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Neutrophil function in patients with cystic fibrosis and chronic pulmonary infection

A thesis submitted for the degree of

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by

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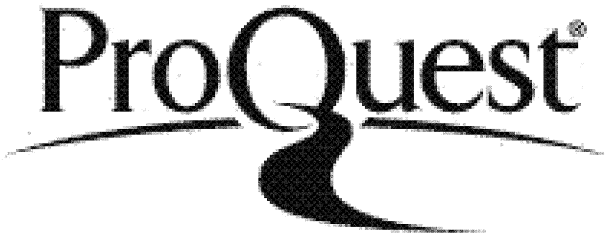

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Neutrophil function in patients with cystic fibrosis and chronic pulmonary infection.

Summary

The thesis investigates reasons for a failure of neutrophils to clear pulmonary bacterial infection in cystic fibrosis (CF). Patients with CF experience bacterial colonization of the lungs associated with progressive lung injury and poor prognosis. Neutrophil responsiveness *in vitro* was determined in patients with CF at different clinical states, and compared to healthy subjects. Neutrophils from the patients were able to phagocytose and kill *Pseudomonas aeruginosa* effectively, but the presence of sputum sol reduced intracellular killing. Superoxide generation and elastase release in response to fMLP were shown to be reduced in neutrophils from patients with an exacerbation of respiratory symptoms. This was not observed when the cells were stimulated with PMA, which acts intracellularly, rather than through cell surface receptors. There was no difference in the down-regulation of cell surface L-selectin and up-regulation of CD11b in response to fMLP suggesting no alteration in number or function of the fMLP receptors. There was increased adherence to nylon columns by neutrophils from patients with CF. Infection resulted in a greater proportion of band neutrophils, and this correlated with the reduced superoxide generation and elastase release, although it was not possible to separate the band forms to prove this conclusively. Alterations in circulating lipid and fatty acid composition in patients could potentially affect neutrophil membrane composition and fluidity and therefore signal processing or release of products. The reason for the observed reduced responsiveness appears to be multifactorial. Band cell number, post receptor signaling and/or receptor desensitization may result in the observed reduced responsiveness, which returns towards healthy subject levels after treatment of an exacerbation, is likely to be the result of chronic infection. The continued effect of reduced killing in the presence of sputum sol may be more related to the impact of CFTR, but more research into the factors responsible is necessary.

Publications containing results included in this thesis

Nixon L.S., Yung B., Bell S.C., Elborn J.S., Shale D.J. 1998 Circulating immunoreactive interleukin-6 (IL-6) in cystic fibrosis. American Review of Respiratory and Critical Care Medicine 157, 1764-69.

Publication of results included in this thesis in abstract form from oral or poster presentations at conferences and meetings.

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Abbreviations

AA	Arachidonic acid
AP	Alkaline phosphatase
ARDS	Acute respiratory distress syndrome
ASF	Airways surface fluid
AZM	Azithromycin
BAL	Broncheoalveolar lavage
BALF	Broncheoalveolar lavage fluid
BCB	Bicarbonate Coating Buffer
BHT	Butylated hydroxytoluene
BMI	Body mass index
BPI	Bactericidal / permeability increasing protein
CAM	Cellular adhesion molecule
cAMP	Cyclic adenosine mono phosphate
CB	Cytochalsin B
CD	Clusters of Differentiation (sequential numbered cell surface markers)
CD16	Fcgamma receptor III
cJUN	Amino-terminal kinase JNK
C5a	Complement factor 5a
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CGD	Chronic granulotomas disease
Cl-	Chloride ion
COPD	Chronic obstructive pulmonary disease
CR3	Complement Receptor Type III
CRP	C Reactive Protein
CSF	Colony stimulating factors
DAG	Diacyl glyceride
DGLA	Di-homo-gamma linolenic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EM	Erythromycin
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-related kinase
ETA	Eicosatrienoic acid
FACS	Fluorescent activated cell sorting
FBC	Full blood count
FEV_1	Forced expiratory volume in 1 second
FFM	Fat free mass
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
fMLP	N-formylmethionyl-leucyl-phenlyalanine
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Graulocyte Macrophage Colony Stimulating Factor
GDP	Guanine di phosphate
GLA	Gamma-linolenic acid
GTP	Guanine triphosphate
HIV	human immunodeficiency virus
hNE	Human neutrophil elastase
hNEAPC	Human neutrophil elastase anti-proteinase complex
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC	High pressure liquid chromatography
HRP	Horse Radish Peroxidase
ICAM	Intercellular cellular adhesion molecule
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IRAP	Interleukin-1 receptor agonist
$[Ca^{++}]_i$	Intracellular calcium ion concentration
JAK	Janus kinases

JNK	c-Jun NH_2-terminal protein kinase
LBP	Lipopolysaccharide-binding protien
LPS	Lipopolysaccharide
LT	Leukotriene (group of mediators including LTB_4)
MAPK	Mitogen-activated protein kinase
MEF	Molecules of equvalent fluorescence
MEP	Mucoid exopolysaccharide
MMP	Metalloproteinases
MPO	Myeloperoxidase
NBF	Nuclar binding fold
NSAID	Non steroidal anti-inflmmatory drug
OD	Optical density
O2-	Superoxide
OH-	Hydroxyl radical
ORCCs	Outwardly rectifying Cl^- channels
PA	Phosphatidic acid
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PDE	Phosphodiesterase
PE	R-phycoererythrin
PECAM	Platelet endothelial cell adhesion marker
PFA	Para formaldehyde
PG	Prostaglandin
PI3K	Phosphatidyl inositol 3-kinase
PIP	Pnostol phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLD	Phospholipase D
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophil

PPI	Polyphosphoinositides
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLPI	Secretory leukoproteinase inhibitor
SOD	Superoxide dismutase
STAT	Signal transducers and activators of trasncription
TIMP	Tissue inhibitors of metalloproteinases
TMB	Tetra methyl benzidine
TNFα	Tumour necrosis factor alpha
TNF RI	Tumour necrosis factor receptor 1
TNF RII	Tumour necrosis factor receptor 2

Chapter 1

Introduction

1.1 The Neutrophil

Neutrophils, also called polymorphonuclear neutrophils (PMN), are the most abundant white blood cells in the circulation accounting for 40 to 80 %. There are 1.2 to 7.6 x 10^9 cells per litre of blood in a healthy person with the numbers rising rapidly at times of infection to over 20 x 10^9/l within 24 hours in diseases such as pneumonia and septicaemia [Braun *et al* 1997, Friedland *et al* 1992]. Mature human neutrophils have a relatively short life span in the circulation and undergo spontaneous or constitutive apoptosis within 24 hours [Kilpatrick *et al* 2002]. In the healthy individual 10^{11} new granulocytes enter the blood stream from the bone marrow every minute [Mollinedo *et al* 1999], and the liver plays a major role in the control of their numbers by removal of apoptotic cells in the circulation [Shi *et al* 2001]. Structurally the neutrophil consists of a granular cytoplasm and a multi lobed nucleus (Figure 1.1).

Neutrophils play an important role in the host defence mechanisms against invading micro-organisms. They ingest invading bacteria and destroy them by the use of chemicals that are delivered either to phagolysosomes or to the extracellular space. The physiological regulation of neutrophil function may vary between body compartments. Activation signals received when in the circulation lead to altered expression of adhesion molecules and the process of rolling, margination and migration into the site of infection or injury [Tandon & Diamond 1998]. The movement of the neutrophil through the tissue compartment may depend on a network of chemotactic and activation signals from other host cells or pathogens at the site of inflammation or infection [Wagner & Roth 2000, Ley 2003]. The array of cytotoxic chemicals used to destroy invading pathogens can be split primarily into products produced *de novo* in the membrane or cytoplasm, such as reactive oxygen species, and products released from pre-formed granules such as elastase. Ideally invading bacteria will first be phagocytosed and cytotoxic agents released into the newly formed phagosomes to reduce their toxic effects on the surrounding tissues [Weiss *et al* 1982, Brown & Donaldson 1988].

The neutrophil must be able to detect a variety of pro- and anti-inflammatory signals in its microenvironment and then respond accordingly. Host derived signals may include, pro-inflammatory cytokines (e.g.IL-1β, TNFα), cell products (e.g. LTB_4, C5a) and plasma exudate [Chertov *et al* 2000]. The response to these signals include a transient rise in intracellular calcium ion concentration ($[Ca^{++}]_i$, up-regulation of receptor number and function, adherence, diapedesis, chemotaxis, particle binding, phagocytosis, degranulation and production of reactive oxygen species. Structural modification such as actin polymerisation, formation of pseudopods and increased movement also occurs [Skoutelis *et al* 2000]. There may also be, where appropriate, the generation of further pro-inflammatory mediators such as eicosanoids and secondary cytokines. Normally, the influx of neutrophils along with their subsequent triggering is carefully regulated and ceases when the initiating signal is destroyed [Savill 1997]. The functioning of the neutrophil is tightly regulated by the immune system [Burg & Pillinger 2001].

Figure 1.1a **Diagram of a neutrophil**

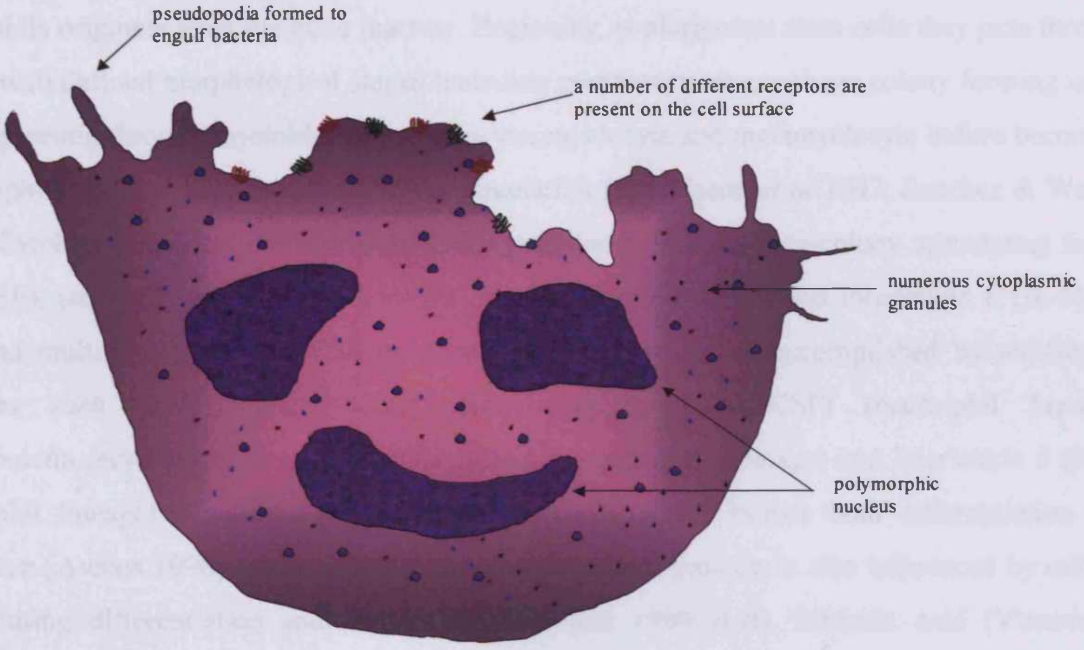
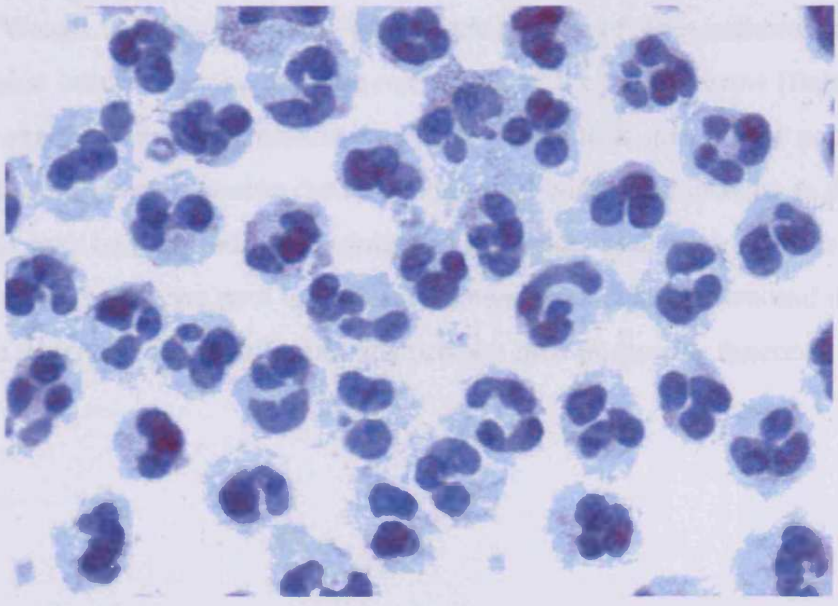


Figure 1.1b **Cytospin of isolated neutrophils stained with Leishmans**



1.1.1 Origin of neutrophils

Neutrophils originate from the bone marrow. Beginning as pluripotent stem cells they pass through several well defined morphological stages including granulocyte-macrophage colony forming units, and progressing through myeloblast, promyelocyte, myelocyte and metamyelocyte before becoming a neutrophil, which is then released into the circulation [Sigurdsson *et al* 1997, Sanchez & Wangh 1999]. Cytokines such as Interleukin 3 (IL-3), granulocyte macrophage-colony stimulating factor (GM-CSF), stem cell factor (SCF) fms-like tyrosine kinase-3 ligand and Interleukin 6 (IL-6) are early and multi-lineage growth factors. Terminal differentiation is accomplished by uni-lineage cytokines such as granulocyte-colony stimulating factor (G-CSF) (neutrophil lineage), erythropoietin (erythrocyte linage), thrombopoietin (megakaryocye linage) and Interleukin 5 (IL-5) (eosinophil lineage) [Caldenhoven 1998] with G-CSF able to induce both differentiation and maturation [Avalos 1996]. The content of neutrophil granule proteins is also influenced by mRNA levels during differentiation and maturation [Cowland 1999 JLB]. Retinoic acid (Vitamin A derivative) plays a role in terminal neutrophil maturation [Lawson & Berliner 1999], and the remodelling of nuclear laminins might underlie the mechanism for nuclear segmentation of neutrophils [Yabuki 1999]. At the metamyelocyte stage condensation of the chromatin and nuclear indentation occurs to form a horseshoe shaped nucleus and the cell is described as a band form, and further segmentation into two, three, four or five lobes connected by fine chromatin strands occurs [Sanchez & Wangh 1997]. It is unclear whether the number of lobes indicates cell age, and whether nuclear division occurs after the cells are released from the bone marrow [Bainton 1992]. Release may be into margination and circulating pools, to give a total granulocyte pool. Neutrophils may travel from the margination pool to the circulating pool without an increase in the total granulocyte pool, and this may be triggered by physiological influences such as exercise or adrenaline [Smith 1997]. This total granulocyte pool is the transit between the bone marrow and the tissues and body secretions, in which the neutrophils normally perform their phagocytic function.

1.1.2 Mature Neutrophils

Neutrophils have the shortest half-life among leukocytes, and are programmed to die within 24 hours of leaving the bone marrow [Ogura 1999]. Bloodstream transit of mature neutrophils is typically complete within 12 hours, and the blood clearance half-life of neutrophils is 6 to 7 hours, at which time the cells migrate into normal tissues or to sites of infection. The transfer of neutrophils from blood to tissue is considered to be 'one way only', with fewer than 5% of the total number of neutrophils in the body normally in the circulation [Dale & Conrad 1998]. The regulation of granulopoiesis is by a homeostatic balance between stimulatory and inhibitory humoral factors, in which colony stimulating factors play a major stimulatory role [Dale *et al* 1995, Molineux 2002].

1.1.3 Apoptosis

Ageing neutrophils spontaneously undergo apoptosis (programmed cell death) and are recognized and phagocytosed by macrophages, which minimizes the risk of tissue injury from release of neutrophil-derived toxic mediators such as proteinases and oxygen free radicals [Squier 1995]. Presence of cytokines or pro-inflammatory agents may delay this removal [Savill *et al* 1989], increasing the life of the neutrophil several fold once it enters infected or inflamed tissue [Akgul *et al* 2001]. Apoptosis is suppressed in the presence of inflammation through the action of cytokines such as TNFα and IL-8 [Dunican *et al* 2000]. *In vitro* neutrophils rapidly die with changes characteristic of cells undergoing apoptosis. Morphologically these cells have a condensed and fragmented nuclear structure, blebbing of the plasma membrane and a decrease in cell size [Oberholzer *et al* 2001]. Neutrophils undergoing apoptosis have decreased oxygen radical production, degranulation and phagocytosis in response to stimuli [Kilpatrick *et al* 2002]. This process represents a mechanism *in vivo* to limit the release of neutrophil content from disintegrated cells that could have the potential to cause tissue injury and amplify inflammation [Haslett 1999].

1.2 Neutrophil products

1.2.1 Plasma membrane products

Neutrophils produce a number of short lived reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide anions (O_2^-), in response to a number of stimuli, which is known as the respiratory burst [Clark 1999]. These agents are among the most important toxic agents that neutrophils produce in response to the presence of micro-organisms. These molecules play an important role in pathogen killing as they are highly bactericidal. They are indiscriminate agents in terms of the targets they attack, with host tissue and structures also affected. Due to this they are a major factor in lung tissue destruction. ROS production occurs rapidly in response to stimuli and is accompanied by a large increase in oxygen consumption. Phosphorylation of numerous proteins occurs as part of the activation process in a tightly controlled process that involves assembly of cytosolic and endogenous membrane components [Barboir *et al* 1973].

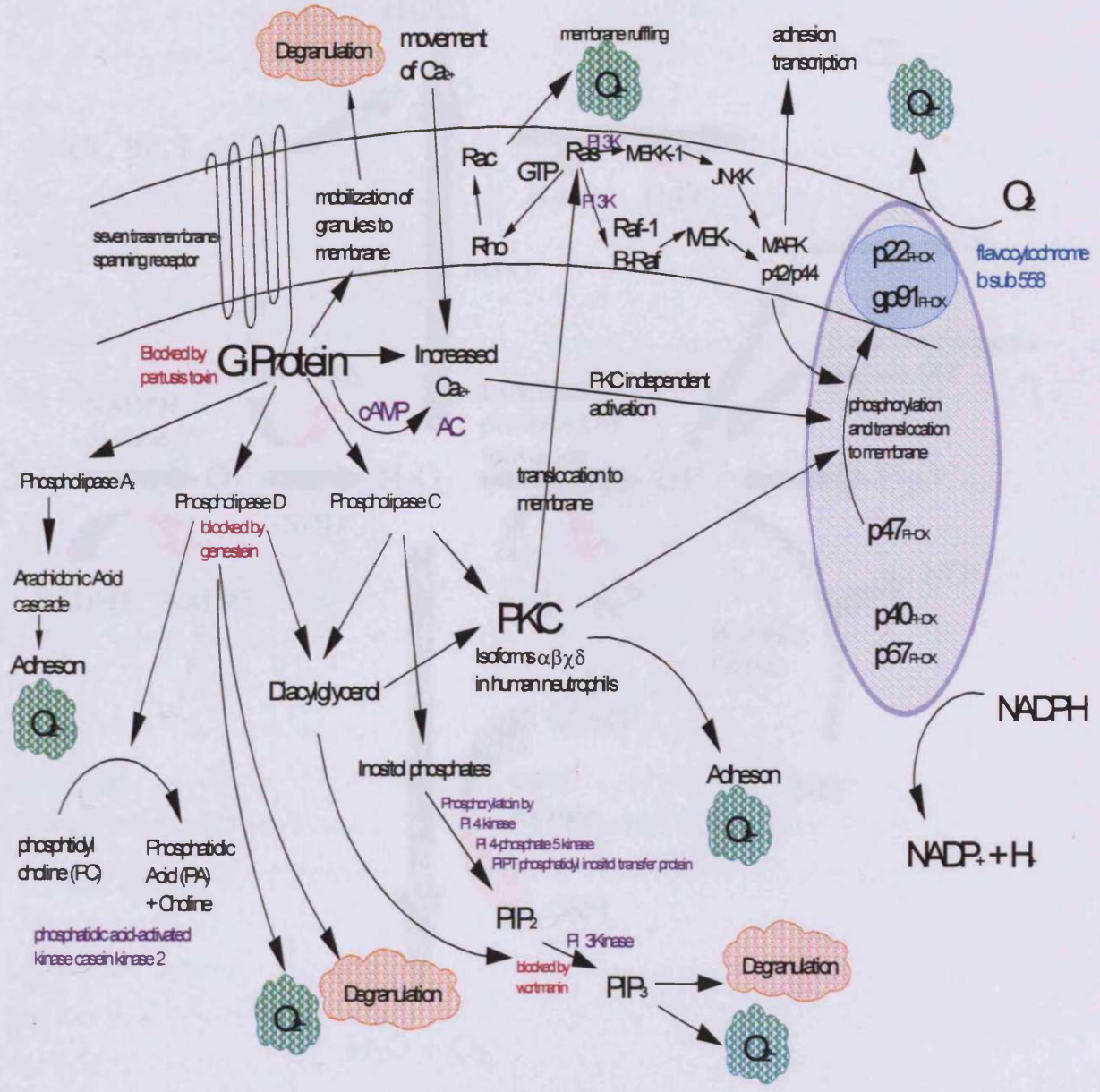
NADPH oxidase is the key enzyme which allows the neutrophil to generate this family of reactive oxidising chemicals. Upon cell activation this forms a functional mulitcomponent electron-transfer system that catalyses the reduction of molecular oxygen at the expense of NADPH [Karlsson & Dahlgren 2002]. The electron transporting component of this NADPH oxidase is a low-potential flavocytochrome b, cytochrome b_{558}, with $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, $p22^{phox}$ and $gp91^{phox}$ units. It is the $p47^{phox}$ which translocates to the membrane forming the active complex on activation, where it binds to the cytosolic side of the plasma membrane [Wientjes *et al* 1997]. NADPH oxidase reduces oxygen (O_2) to O_2^-, and the dismutation of O_2^- produces H_2O_2 catalysed by superoxide dismutase [Test & Weiss 1984]. Myeloperoxidase (MPO) an enzyme contained in azurophil granules, catalyses the H_2O_2-dependent oxidation of chloride (Cl^-) to hypochlorous acid (HOCl) and the reaction of HOCl with H_2O_2 yields O_2^- [Condliffe *et al* 1998]. HOCl can react with amines and ammonium ion to produce toxic chloramines such as monochloramine (NH_2Cl), the so-called long-lived oxidants. A number of other enzymes are also involved in the production of oxygen species (Figures 1.2 & 1.3).

Wientjes *et al* [1997] have shown that the NADPH oxidase components are located bound to the cytoplasmic side of the neutrophil plasma membrane upon the activation of cells. They also noted that there was a wide variation between neutrophils in the amount of flavocytochrome labelled. It has also been shown that both intra- and extracellular Ca^{++} are required for full activation of the respiratory burst of human neutrophils, and the Ca^{++} influx from extracellular space plays an important role either in generation of reactive oxygen metabolites or in activation of protein kinase C [Bei 1998]. This leads to a number of downstream actions including Ca2+ mobilization, modification of phosphoinositide metabolism, and activation of mitogen-activated protein kinase (MAPK). The N-formly-leucyl-methionyl-phenylalanine (fMLP) and Fcγ receptor-mediated neutrophil activation pathways differ not only in the responses they elicit but also in the requirement for elevated $[Ca^{++}]_i$.

The predisposition of patients with chronic granulotomas disease (GCD) to bacterial infections has been associated with decreased oxygen consumption as well as defective microbial killing [Karlsson & Dahlgren 2002]. Other diseases have been linked to free radical damage arising from an imbalance between radical-generating systems and radical-scavenging systems, a condition known as oxidative stress. Reactive oxygen species, in particular OH^- can react with all biological macromolecules (lipids, proteins, nucleic acids and carbohydrates). The host uses a number of oxygen scavengers, or antioxidants such as superoxide dismutase (SOD), GPx and Vitamin E to maintain oxidative homeostasis [Kinnula & Crapo 2003] .

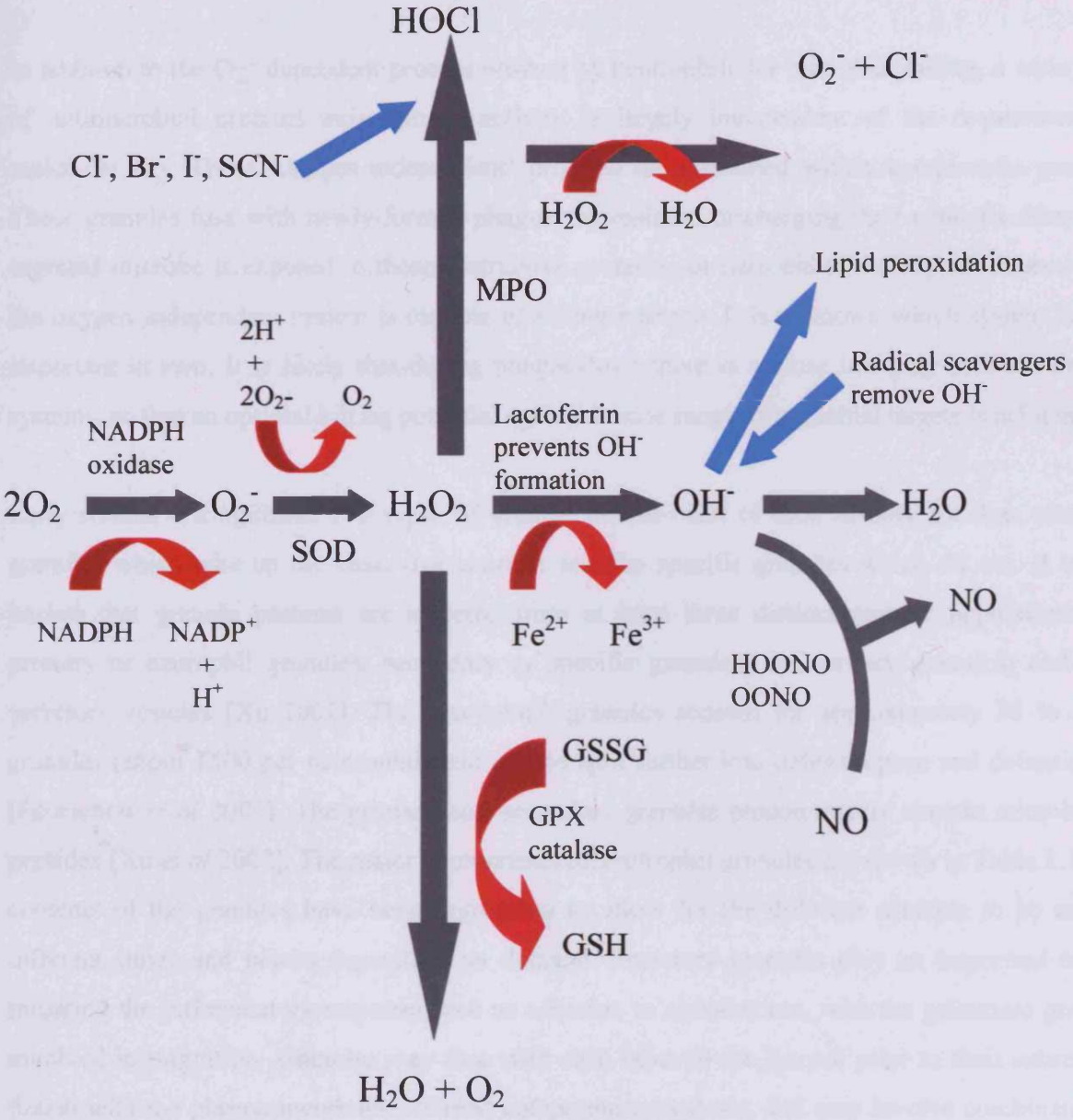
The other main products produced by the neutrophil *de novo* are lipid mediators, which include products of the arachidonic acid (AA) cascade, such as leukotrienes (LT), prostanoids and eicosanoids, and the phospholipid mediator platelet activating factor (PAF). The AA cascade occurs as a result of cellular activation of cytosolic phospholipase A_2, and results in the production of prostaglandins (PG), LTs and thromboxane [Levy *et al* 2000, Haeggstron & Wetterholm 2002].

Figure 1.2 ***General intracellular pathways in neutrophils***



Information collated from; Thompson [1993], Avdi [1996], Kent [1996], Bauldry & Wooten [1997], Gay [1997], Kular [1997], DeLeo *et al* [1998], Dewas [2000], Burg & Pillinger [2001], Huber Lang [2002], Karlosson & Dahlgren [2002], Chen [2003].

Figure 1.3 ***The oxygen free radical production in neutrophils***



MPO Myeloperoxidase
SOD Superoxide dismutase
H_2H_2 Hydrogen peroxide : much of this is consumed by the neutrophil
OH^- Hydroxyl radical : more powerful oxidant than H_2O_2
HOCl Hypochlorous acid

1.2.2 Intracellular granules

In addition to the O_2^- dependent process utilized by neutrophils for microbial killing, a wide range of antimicrobial proteins exist whose activity is largely independent of the requirement for molecular O_2. These 'oxygen-independent' proteins are contained within cytoplasmic granules. These granules fuse with newly-formed phagocytic vesicles, discharging their contents. Hence the ingested microbe is exposed to these destructive proteins. *In vitro* either the oxygen dependent or the oxygen independent system is capable of killing bacteria. It is unknown which system is most important *in vivo*. It is likely that during phagocytosis there is a close interplay between the two systems, so that an optimal killing potential against a wide range of microbial targets is achieved.

Early studies distinguished two types of granule on the basis of their affinity for dye: azurophil granules which take up the basic dye azure A and the specific granules which do not. It is now known that granule proteins are secreted from at least three distinct granule populations (i.e. primary or azurophil granules, secondary or specific granules, and tertiary granules) and from secretory vesicles [Xu 2002]. The azurophilic granules account for approximately 30 % of all granules (about 1500 per neutrophil) and can be split further into defensin poor and defensin rich [Faurschou *et al* 2002]. The primary and secondary granules predominantly contain microbicidal peptides [Xu *et al* 2002]. The major components of neutrophil granules are shown in Table 1.1. The contents of the granules have been segregated to allow for the different contents to be used at different times and places depending on demand. Secretory granules play an important role in initiating the inflammatory response such as adhesion to endothelium, with the gelatinase granules involved in migration. Granules may fuse with each other in the cytosol prior to their subsequent fusion with the plasma membrane, termed compound exocytosis, and may involve combination of five granules, which may be a mechanism for efficient targeting of granule contents [Lollike *et al* 2002].

The primary granules are formed at an early stage of the neutrophil formation in the bone marrow. It has been shown the number of granules per cell is reduced by cell division in the mitotic pool. Therefore a reduction in the number of divisions in the mitotic pool occurs as part of the systemic response to stress [Bainton 1992]. The secondary, or specific granules are twice as abundant. The

gelatinase-containing (tertiary) granule also contains fMLP receptors, and enzymes such as diacylglycerol lipase and β-glucuronidase [Kjeldsen *et al* 1994]. Secretory vesicles are not formed until the final stages of neutrophil development, and therefore are mainly present in segmented neutrophils. Specifically they contain alkaline phosphatase and CD35, not found in the more immature cells [Borregaard & Cowland 1997]. Granules have also been shown to be important reservoirs of membrane proteins that become incorporated into the surface membrane of the neutrophils when these organelles fuse with the plasma membrane and exocytose their content. In this way, granules and secretory vesicles may fundamentally change the ability of the neutrophil to interact with its environment [Borregaard 1996].

The contents of the granules are biologically inert within the cytoplasm because of the impermeability of their surrounding membrane. Their release may be into a phagocytic vesicle, or by secretion extracellularly. Movement and discharge of granules, termed degranulation or exocytosis, can be regulated by separate control mechanisms such that degranulation of specific versus azurophilic granules can occur independently. Granule components may be released extracellularly if vesicles do not adequately seal during phagocytosis, or if the target organism is too large to be fully enclosed.

1.2.2.1 Granular enzymes, specifically elastase

Granules contain different types of proteins with different actions and roles. Proteases, by nature, degrade proteins, and as such are capable of destruction of host tissue. The major protease that has been implicated in the pathogenesis of lung injury is neutrophil elastase, which is expressed and released upon neutrophil activation [Gadek 1992]. Human neutrophil elastase (hNE), also called human leukocyte elastase, is a serine protease, and a cationic glycoprotein which exists in four isoenzymes. The potential substrates of hNE include almost all components of the extracellular matrix, as well as proteins as diverse as clotting factors, complement, immunoglobulins and cytokines [Lee 2001]. It also acts to enhance the activity of other neutrophil antimicrobial proteins, and functions in the degradation of cell walls to kill bacteria such as *E.coli* [Belaaouaj 2000].

It is thought that even before the neutrophil has reached the lungs it may cause damage to lung tissue as elastase is released as part of the adherence and chemotaxis stages of migration into the lung. In addition if it is activated prior to adherence, elastase can cause cleavage of the L-selectin adherence ligand, and adherence will not take place [Gardiner *et al* 2001]. The release of elastase is thought to be via a calcium dependent mechanism [Houston *et al* 1997], although a much reduced degranulation may occur in the presence of a calcium chelator [Seetoo *et al* 1997]. Degranulation also involves Phospholipase D [Tamura *et al* 1998] and Phospholipase A [Tou 2002] and after simulation elastase is expressed on the cell surface [Owen 1995].

1.2.2.2 Anti-proteinase defences

To control possible host tissue destruction by proteases release from neutrophils the host also produces anti-proteases such as α_1-antitrypsin (α_1-proteinase inhibitor), secretory leukoproteinase inhibitor (SLPI), plasminogen activator inhibitor-1, α_2-macroglobulin and $\gamma_2$globulin. As well as being present in the serum, some of these anti-proteases are contained within the granules, and as such α_1-antitrypsin is released at the same time as elastase [Paakko *et al* 1996]. Such protease inhibitors confine the activity of proteolytic enzymes of inflammatory cells, but fail to protect substrates in the immediate pericellular zone, which is controlled by the enzyme inhibitor ratio [Liou 1996]. Triggered neutrophils use chlorinated oxidants to inactivate antiproteinases. Matrix metalloproteinases (MMP) are proteinases that participate in degradation of the extracellular matrix. These enzymes are also precisely regulated under normal physiological conditions, primarily by tissue inhibitors of metalloproteinases (TIMP) [Visse & Nagase 2003]. At high concentrations elastase can hydrolyse and inactivate these TIMP as well as α_1-antichymotrypsin and α_1-antitrypsin, affecting control of such proteases.

1.2.2.3 Other granule constituents

Non-specific host defences contained within the granules include iron-binding proteins such as lactoferrin and transferrin. Iron is an essential requirement for the establishment and maintenance of bacterial infections. Bacteria must possess iron sequestration and transport systems in order to compete for available iron. Proteins in the outer membrane act as specific receptors for iron

siderophore (iron chelators) complexes [Que & Woods 1987]. Bactericidal/permeability increasing protein (BPI) and lipopolysaccharide-binding protein (LBP) bind LPS. The LBP-LPS complex is recognised through CD14 and acts to initiate an inflammatory response through monocytes and T cells, while the BPI-LPS complex is cytotoxic to gram-negative bacteria [Mollinedo *et al* 1999].

Table 1.1

The contents of human neutrophil granules

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Matrix Elastase[1] Cathepsins (including G)[1] Proteinase 3[1] Lysozyme Myeloperoxidase (MPO) Defensins Azurocidin[1] (CAP37, Heparin binding protein) Acid hydrolases N-acetyl-β-glucosamidase β-glucuronidase β-glycerophosphatase α-mannosidase acid-β-glycerophosphatase Sialidase Proteoglycans α_1-antitrypsin Bactricidal permeability increasing protein Ubiquitin-protein acid mucopolysaccharide **Membrane** CD63 CD68 V type H^+-ATPase	**Matrix** Lactoferrin β_2-microglobulin Collagenase Gelatinase Vitamin B_{12} binding protein Lysozyme NGAL SGP28 (Cathelicidin) properdin hCAP-18 heparanase UKPA Sialidase **Membrane** CD15 antigens CD66 CD67 Fibronectin receptor G-protein a-subunit Laminin receptor cytochrome b_{558} CD11b (MAC-1) fMLP receptor NB 1 antigen Rap 1, Rap 2 Thrombospondin receptor TNF receptor Vitronectin receptor VAMP-2	**Matrix** Gelatinase Acetyltransferase Lysozyme β_2-microglobulin **Membrane** CD11b (MAC-1) fMLP receptor Diacylglycerol-deacylating enzyme V type H^+-ATPase cytochrome b_{558} SCAMP UKPA receptor VAMP-2	**Matrix** Plasma proteins, e.g. tetranectin albumin **Membrane** Alkaline Phosphatase cytochrome b_{558} CD11b (Mac-1) UKPA receptor fMLP receptor CD10 CD13 CD14 CD45 CD16 CR1 DAF V type H^+-ATPase SCAMP VAMP-2 C1q receptor

[1] hematopoietic serine proteases

Abbreviations: Urokinase type plasminogen activator (UKPA), Neutrophil gelatinase associated lipocalin (NGAL)

Collated from: Borregaard *et al* [1993], Gullberg *et al* [1997].

1.3.1 Neutrophil activation

Neutrophil activation includes a combination of shape change, adherence, chemotaxis, transient rise in cytosolic free calcium concentration, granule release and respiratory burst. Different types of activation may result in a different combination of these effects through a complex network of intracellular pathways. The majority of agents which activate neutrophils exert their effect via receptors coupled to G proteins. How such a broad variety of chemical compounds activate receptors which share a preserved overall tertiary structure, and are able to result in specific activation of intracellular signalling cascades has been the focus of much research [Gether *et al* 2002].

Unstimulated neutrophils are spherical, but become polarized on activation. The front end of the cell is more sensitive to stimulation than the rear, possibly because of an asymmetrical distribution of receptors [Gallin & Seligmann 1984]. Bound receptors are capped to the tail and are internalized by endocytosis. In order for the neutrophil to be able to sustain chemotaxis along a gradient, there must be continuous re-expression of receptors at the front of the cell. This may occur via recycling of receptors or else via the mobilization of new receptors from internal pools. The majority of re-expressed receptors that appear on the plasma membrane within 4-10 min. after stimulation arise from the mobilization of internal pools - the membranes of specific granules (and possible gelatinase-containing granules, or secretary vesicles). Recycling of internalized, ligand-bound receptors to the plasma membrane may also occur, but only more slowly [Koenig & Edwardson 1997]. The time for this recycling however, does vary a great deal with receptor type.

G Proteins, heterotrimeric GTP-binding proteins, are characterised by a seven-transmembrane configuration, and blocked by pertussis toxin [Bokoch 1995]. There are three groups of G-proteins, A, B and C, split on the basis of sequence similarity. The majority of receptors are in Family A. G-proteins are generally referred to by their a subunits including those which activate adenylate cyclase (G_S) and those that inhibit the enzyme (G_i). At rest, the G-protein is in the low-affinity state. After agonist binding a transient high-affinity complex of agonist, activated receptor and G-protein is formed. GDP is released from the G-protein and replaced by GTP. This leads to dissociation of the G-protein complexes into α subunits and $\beta\gamma$dimers, which both activate several effectors [Pierce

et al 2002]. Of importance in neutrophil activation is the $G\alpha_s$ activation of adenylate cyclase, which leads to an increase in cyclic AMP (cAMP). This increase in cAMP in turn activates protein kinase A (PKA), which is a serine/theonine kinase that phosphorylates many different substrates which are important in intracellular signalling [Pierce *et al* 2002]. The majority of chemo attractants such as fMLP, IL-8, LTB_4 have been cloned and are seven transmembrane-spanning, and are associated with G_{i2} and G^{i3}. C5a couples to $G\alpha_{16}$ which is pertussis-toxin insensitive. G_i dissociates into the GTP bound Ga and the $\beta\gamma$ subunit complex [Bokoch 1995].

Downstream of the G-protein, the Ras/mitogen-activated protein kinase (MAPK) cascade and Phosphatidylinositol 3-Kinase (PI3K) play an important role in neutrophil activation. The MAPK cascade is a major signalling system that is shared by various types of cells. Three MAPK modules have been identified in neutrophils, p38 MAPK, extracellular signal-related kinase (ERK), and c-jun amino-terminal kinase (JNK). The ERK cascade is activated in response to signals from receptor tyrosine kinases, haematopoietic growth factor receptors, or some heterotrimeric G-protein-coupled receptors and appears to mediate signals promoting cell proliferation or differentiation [Suziki *et al* 1999].

Neutrophils must follow both endogenous and bacterial chemoattractant signals out of the vasculature and through the interstitium to arrive at a site of infection. The presence of multiple chemoattractants at different concentrations means the neutrophil must prioritise its response [Heit *et al* 2002]. This is done by an intracellular signalling hierarchy where chemotaxis via p38 MAPK has an inhibitory effect on that of the PI3K/Akt pathway, and when the p38 MAPK pathway was inhibited the PI3K pathway increased in magnitude and duration [Heit *et al* 2002]. In addition the p38 MAPK, activated by Src Family kinases has a central role in eliciting the fMLP-induced release of primary and secondary granules, but not in that of secretory vesicles [Mocsai *et al* 2000]. In septic patients there is a failure of correct neutrophil emigration [Wagner & Roth 1999], and this chemoattractant hierarchy may be involved in this.

1.3.1.1 Activators of neutrophils

Bacterial derived products include endotoxin, LPS and fMLP. Endotoxin is the most general inflammatory stimulus, as it is the shed components of the cell walls of bacteria. The component of endotoxin responsible for its dramatic pro-inflammatory activity is the lipid A fraction of LPS [Aldridge 2002].

fMLP is a peptide of bacterial origin. It is commonly used as an activator to model receptor mediated processes. It can activate many neutrophil functions, including chemotaxis, aggregation, reactive oxidant production, cytoskeletal changes and degranulation. Its effects are enhanced by cytochalasin B (CB). fMLP binds rapidly to specific G protein which results in the broad range of rapid functional responses which can occur in neutrophils. The release of superoxide anion is dependent on the activation of p38 MAPK [Nick 1997], but may also occur via a different mechanism. Tumour Necrosis Factor alpha (TNFα), GM-CSF, fMLP, phagocytosis and FcγR cross-linking stimulate the activity of p38 MAPK and ERK but not JNK in human neutrophils [Ward 2000].

Host derived products include cytokines, C5a, leukotrienes (LT) and platelet activating factor (PAF). Cytokines which activate neutrophils include Interleukin-8 (IL-8), TNFα, and Interleukin 6 (IL-6). IL-8 is extremely chemoattractant for neutrophils and can cause degranulation, reactive oxygen metabolite production, and elevations in $[Ca^{++}]_i$. TNFα is chemotactic for neutrophils, can prime degranulation and reactive oxidant production, enhance phagocytosis and up-regulate the expression of some surface receptors such as CR3. High concentrations of TNFα alone can activate low levels of oxidant production, which is more pronounced if the neutrophils are adhered to surfaces [Decleva *et al* 2002]. The various cytokines are discussed in more detail in 1.6.

The complement fragment, C5a, is a heat-stable chemotactic substance produced as a result of the activation of the complement system, by either the classical or alternative pathway. It is chemotactic, can induce degranulation and stimulate the respiratory burst in human neutrophils. C5a also increases vascular permeability and stimulates histamine release from mast cells.

LTs are bioactive lipids, in a family of paracrine hormones derived from the oxidative metabolism of arachidonic acid (AA). Bone marrow derived cells are the main producers, particularly neutrophils, which predominantly generate LTB_4. LTs possess a wide range of biological activates elected via specific G-protein coupled cell surface receptors [Haeggstrom & Wettterholm 2002]. LTB_4 is a powerful chemoattractant for neutrophils, and can promote adherence of neutrophils to endothelial cells, induce neutrophil aggregation and up-regulate expression of complement receptor 3 (CR3). Higher concentrations can also activate degranulation and NADPH oxidase activity, and CB augments these responses [Bates 1995]. Due to their potent biological activities, several drugs have been developed which block the synthesis and action of leukotrienes [Drazen *et al* 1999]

PAF includes a family of structurally-related lipids that are all acetylated phosphoglycerides, which are mostly synthesised and released by leukocytes, with eosinophils being the richest source [Kroegel *et al* 1992]. PAF acts on neutrophils through a G-protein receptor, to cause chemotaxis, chemokinesis, aggregation, adherence, degranulation and stimulates the respiratory burst. PAF has more marked effects on eosinophils than on neutrophils, and many of its actions on neutrophils may be related to enhancement of arachidonic acid release and LTB_4 synthesis [Krump & Borgeat 1999].

1.3.1.2 Neutrophil priming

Priming refers to a process whereby the response of neutrophils to an activating stimulus is potentiated by prior exposure to a priming agent. This applies to both production of reactive oxygen species and degranulation. Often such agents do not elicit a response on their own, although they may do so when applied at very high concentrations [Condliffe *et al* 1998]. Substances such as GM-CSF, Substance P, L-selectin cross-linking and IL-8 are among the more well known priming agents, of which GM-CSF has been used widely *in vitro* [Dale *et al* 1995]. Mechanisms of priming the respiratory burst have not been well delineated. At this time, it is thought to occur by either partial mobilization of components to the plasma membrane (lipopolysaccharide) or by partial phosphorylation of the p47(phox) component without translocation [Dang *et al* 1999, Quaid *et al* 2001]. Many priming agents have clear biological relevance *in vivo* and are released in response to infection and trauma. For example, TNFα has been linked to poor outcome in septic shock [Pinsky *et al* 1993] and TNFα infusion in healthy subjects results in systemic neutrophil activation [Van der

Poll *et al* 1992, Drost *et al* 1999]. Primed neutrophils have been identified in patients with bacterial infection [Bass *et al* 1986], and TNFα has been shown to prime neutrophils *in vitro* [McLeish *et al* 1998].

1.3.1.3 Inhibitors of neutrophil function

Inhibitors of neutrophil function include anti-inflammatory cytokines such as IL-10 and adenosine. Receptors for adenosine (P1) and (P2) adenine nucleotides belong to completely different families but are often grouped together as purinoceptors. Neutrophils express A_1, A_{2A} and A_{2B} receptors, and it is predominantly through the A_{2A} receptors that adenosine antagonises the superoxide generation induced by fMLP [Fredholm 1997]. Adenosine, acting through A_{2A} receptors is also able to suppress neutrophil functions by suppressing arachidonic acid release and LTB_4 biosynthesis [Krump & Borgeat 1999].

1.3.2 Adhesion and surface markers

The recruitment of neutrophils from the bloodstream into infected tissues depends on them being able to attach to the endothelial walls and then squeeze through the spaces between theses cells to begin their migration up a chemoattractant concentration gradient towards the site of infection. Adhesion occurs as a cascade with three main steps: rolling, activation, and firm adhesion and migration. Rolling is selectin dependent, activation is integrin dependent, and firm adhesion and migration involves the interactions of integrins with intercellular adhesion molecule (ICAM). There are three groups of cellular adhesion molecules (CAM) responsible for these processes; the selectins, the integrins and the immunoglobulin (Ig) superfamily.

1.3.2.1 Sequence of Adhesion

Rolling:

The selectins are key in the rolling process. They are a family of adhesion molecules discovered in 1989 when the cDNA sequences of the three cell surface glycoproteins found on endothelium (E-selectin), platelets (P-selectin) and lymphocytes (L-selectin) were reported [Patel *et al* 2002]. They bind to sialyated Lewis X blood group antigen (SLeX) which is expressed on the neutrophil. The presentation of the SLeX to the E-and P- selectins is mediated through L-selectin. The attachment of neutrophils is initially transient because L-selectin is shed quickly from the leukocyte. This is thought to permit neutrophils to 'let go' during subsequent stages of transmigration [Burg & Pillinger 2001], and these adhesive contacts have high tensile strength to allow rolling under shear stress [Greenburg *et al* 2000]. Neutrophils in the circulation must resist tremendous shear forces in order to stop along the vascular endothelium. The activity of the selectins is controlled in large by their appearance and disappearance from the cell surface. L-selectin is clustered on the most distal aspects of the cell surface, which is thought to enhance the molecule's bio availability during brief collision between rapidly flowing leukocytes and ligand-bearing endothelial cells [Fors *et al* 2001]. Shedding of L-selectin from the surface of leukocytes leaves the carboxyl terminus amino acid residues membrane bound. The remainder of the protein is shed from the leukocytes into the plasma and hence described as soluble L-selectin (sL-selectin). *In vitro* studies have demonstrated that soluble L-selectin retains functional activity and inhibits adhesion of leukocytes to an endothelial

cell monolayer in a static system [Schleiffenbaum *et al* 1992], at concentrations seen in the serum of patients with sepsis [Ferri *et al* 2001]. Anti-L-, P-, or E- selectin antibodies will completely block this rolling process [Marshall & Haskard 2002]. The transition from selectin mediated adhesion to CD18 mediated adhesion occurs rapidly and involves the activation of CD18.

Adhesion: The key ligands in adhesion are the β2 integrins which are comprised of three α/β heterodimer membrane glycoproteins, with a common β subunit designated CD18. The α chain designates CD11a, CD11b or CD11c. α and β subunits have a relatively small cytoplasmic domain which contains regions capable of binding to cytoskeletal elements [Alpin *et al* 1998]. These bind to the ICAMs. Activation with cytokines (e.g. IL-1, TNFα and IFNγ) results in an increase in ICAM-1 expression on vascular endothelium and lymphocytes. The CD18 integrins undergo a conformational change after activation which results in increased affinity for their ligands, ICAM-1 and -2 on the endothelial cells. This increased adhesive capacity occurs through both qualitative (conformational) and quantitative (up-regulation of surface expression) changes in the integrin molecules. Integrin independent adhesion (without involvement of CD18) is also thought to play a role *in vivo*, particularly in sequestration of neutrophils into the lungs [Doerchuk 2000]

Migration: Neutrophils can migrate from vessels into tissues under both normal and pathological circumstances. The mechanisms controlling this process of migration are complex. The movement of neutrophils through the endothelial cell monolayers occurs preferentially at tricellular junctions, and platelet endothelial CAM (PECAM-1, CD31) plays a key role in this passage [Wagner & Roth 2000]. Following this initial movement, subsequent migration is thought to be associated by the release of neutrophil derived proteases, as anti-proteases are able to inhibit the process *in vitro* [Delclaux *et al* 1996], but not when basement membrane matrices are involved [Allport *et al* 1997].

1.3.2.2 Adhesion and cell activation

Activation of neutrophils by exposure to bacterial products (e.g. LPS, fMLP) or host mediators (e.g. IL-8, TNFα, PAF) alters CAMs on the cell surface. The proportional effect on the different CAMs may be dependent on the mediator, and the p38 MAPK and the PKC pathways have been implicated in this mechanism [Cara *et al* 2001, Jung *et al* 1990]. C Reactive Protein (CRP) has been shown to rapidly downregulate the expression of L-selectin on the neutrophil surface [Zouki *et al* 1997], with the hypothesis that CRP, by attenuating neutrophil adhesion to the endothelium and consequently neutrophil traffic into tissues, may be a major mechanism to attenuate or limit the inflammatory response. Other activators of neutrophils such as IL-8 and TNFα are also able to induce shedding of L-selectin from the neutrophil surface. Another negative feedback mechanism is that lactoferrin is able to reduce the LPS binding to L-selectin providing a protective effect which occurs at concentrations of lactoferrin seen in the serum of patients with septic shock. Through this mechanism the superoxide generation is reduced in response to LPS [Baveye *et al* 2000]. In these ways humans may prevent tissue damage following infection.

1.3.2.3 Effect of infection and inflammation on adhesion molecules and their function

Total leukocyte and neutrophil counts and the neutrophil expression of L-selectin was increased in trauma, particularly in patients who developed acute lung injury. However there was a corresponding reduction in the plasma sL-selectin [Rainer *et al* 2000]. Since circulating sL-selectin competes with cell-bound selectin for endothelial ligands and interferes with leukocyte-endothelial adhesion in a dose-dependent manner [Schleiffenbaum *et al* 1992] it is possible that low concentrations of sL-selectin will shift the balance in favour of neutrophil rolling and adhesion. This would be supported by other studies which have also showed a reduction in sL-selectin in trauma [Muller *et al* 1998], inflammation [Haught *et al* 1996] and cardiac arrest [Gando *et al* 1999]. Contrastingly increased sL-selectin levels have been seen in sepsis [Ferri *et al* 2001, systemic inflammatory response syndrome [Ahmed *et al* 1996] and acute injury [Maekawa *et al* 1998]. The raised sL-selectin in acute lymphoblastic leukaemia [Spertini *et al* 1994], and the lack of change of sL-selectin with steroids in multiple sclerosis [Crockard *et al* 1998] suggest a role for lymphocytes in the production of sL-selectin *in vivo*.

In systemic inflammatory response syndrome the neutrophils are highly adhesive, have enhanced expression of CD11b and decreased expression of L-selectin [Brown *et al* 2001]. In asthma no difference was seen in non-stimulated neutrophils, but fMLP stimulation resulted in higher levels of CD11b in the asthmatic children than in the non-asthmatic children [Berends *et al* 1993]. *In vivo* drug treatments are able to modify the CD11b expression on neutrophils with chemotherapy producing an increase [Engervall *et al* 1998] and erythromycin a reduction [Lin *et al* 2000]. In a baboon model of hemorrhagic-traumatic shock, treatment with anti-L-selectin resulted in a decreased mortality and improved survival time [Schlag *et al* 1999] suggesting it plays a crucial role in inflammation.

1.4.1 The role of neutrophils in inflammation

Almost 17 billion granulocytes enter and exit the lung each minute. There is some evidence that normal functions of the lungs may be influenced by granulocytes, and some findings suggest humoral communication between granulocytes and vascular endothelium which affects granulocyte margination and migration [Brigham & Meyrick 1984]. Under normal circumstances the neutrophils entering the lungs do not cause inflammation or damage. Neutrophils are also involved in inflammation in other parts of the body, such as rheumatoid arthritis (RA) which is a systemic inflammatory disease localized preferentially in the synovial joints. Although a result of different initiation, parallels may be drawn between this chronic disease and the inflammation seen in chronic lung disease such as Cystic Fibrosis (CF). Concentrations of IL-6 may be considered as an indicator for local pro-inflammatory cytokines and neutrophil activation in both diseases [Van Leeuwen *et al* 1995].

Inflammation is a characteristic response of the lung to infection and has been established to occur in a number of diseases. The cause of inflammation in the lungs may not always be due to infection, and may occur as an allergic response such as in asthma. Here there are increased numbers of eosinophils in the airways, accompanied by increased eosinophil cationic protein concentrations in the broncheoalveolar lavage fluid (BALF) [Kroegel *et al* 1992]. Zimmerman *et al* [1983] showed that neutrophils in acute respiratory distress syndrome (ARDS) are in a functionally and metabolically activated state in the majority of patients. Their enhanced chemotactic and chemiluminescence response *in vitro* occurred even when no evidence of bacterial infection was shown. Hypoxemia has also been implicated in neutrophil mediated inflammation in the absence of infection [Madjdpour *et al* 2003].

Mucosal pathogens use diverse and highly specific molecular mechanisms to activate mucosal inflammation. It may even be argued that their virulence depends on the inflammatory response that they induce. Some bacteria target epithelial cells and trigger them to produce inflammatory mediators but others cross the mucosa and activate macrophages or dendritic cells. Although systemic release of inflammatory mediators causes many symptoms of infection, local chemokine production leads to the recruitment of inflammatory cells and lymphocytes that participate directly

in the clearance of bacteria from mucosal sites. In this way, mucosal inflammation is a two edged sword responsible for disease associated tissue destruction and crucial for the antimicrobial defence.

It is unclear whether neutrophil mediated inflammation precedes infection where Khan *et al* [1995] and Balough *et al* [1995] have shown that neutrophil influx occurs in the airways and may precede evidence of infection. However, there is also a lot of evidence to link infection and inflammation [Wilmott *et al* 1990, Bonfield *et al* 1995, Armstrong *et al* 1997]. There is a clear link between inflammation and neutrophil mediated lung injury in CF which can be demonstrated both in the circulatory and airways compartment in which levels of complexed or free elastase, and other granule products can be measured [O'Connor *et al* 1993, Koller *et al* 1995, Regelmann *et al* 1995, Sagel *et al* 2002]. There is also evidence of connective degradation products such as desmosine [Bruce *et al* 1985]. BALF has been shown to have very elevated elastolytic activity, due to a serine-protease (assumed to be neutrophil elastase). However the possibility of *Pseudomonas aeruginosa* elastase could not be ruled out. CF respiratory immunoglobulins (except IgM) were significantly raised when compared with healthy subjects, but albumin levels were decreased [Fick *et al* 1984]. In infants with respiratory syncytial virus (RSV) bronciolitis, the majority of elastase in BALF was shown to be active, and associated with increased IL-8 [Abu-Harb *et al* 1999] suggesting that this is does not only occur in CF. Free elastase has also been shown in acute pneumonia, but only in 18% of patients studied [Braun *et al* 1994].

In chronic obstructive pulmonary disease (COPD), a chronic lung disease usually without a clear and directly linked genetic abnormality such as in CF, it seems likely that it is smoking which causes an increase in the number of neutrophils in the lungs, associated with greater concentrations of elastase in BALF [Janoff *et al* 1983]. The chronic infection that follows is associated with high concentrations of elastase and MPO in the airways [Aaron *et al* 2001, Sethi *et al* 2000, Lacoste *et al* 1993]. Management of these patients is often pre-occupied with symptom control rather than primarily aimed at prevention of progression of disease. [Munro 1992]. Radiological progression of disease was shown in patients where the signs and symptoms appeared largely unchanged, suggesting suppression of symptoms is not sufficient to resolve the inflammation.

There are a number of endogenous products which suppress neutrophil function such as prostaglandin E2 and adenosine to counterbalance exaggerated cell activation [Ottenolo *et al* 1999]. Adenosine has been recognised as an important modulator of neutrophil function, and may be protective against neutrophil mediated tissue injury [Bouma *et al* 1997] and against reperfusion damage [Newby 1991]. Adenosine increases cAMP by activation of adenylate cyclase, and its effects are mimicked by phosphodiesterase IV (PDE-IV) inhibitors which act to increase the intracellular cAMP. It has been shown to reduce the fMLP induced release of elastase in TNFα primed cells and this was reversed by the addition of adenosine deaminase [Ottenolo *et al* 1999]. CGS21680 acting at the A2a adenosine receptor subtype, suppressed the activity of both elastase and superoxide generation [Visser 2000]. CC16, a recently characterized immunosuppressive agent, has wide range of actions which include inhibition of fMLP induced chemotaxis and phagocytosis of neutrophils. Higher concentrations of CC16 were observed in BALF fluids of ARDS patients who survived compared to those that die, pointing to a protective role in neutrophil mediated lung injury [Geerts *et al* 2001]. It is unclear whether the continued inflammation seen in chronic conditions is linked to a failure of these endogenous products in the resolution of inflammation.

1.4.2 Modulation of neutrophil responsiveness in disease

Burnett *et al* [1987] showed that neutrophils isolated from the peripheral blood of patients with emphysema have an increased chemotactic response to fMLP compared to neutrophils isolated from healthy subjects. Neutrophil isolated from patients with adult respiratory distress syndrome also show increased oxidative products compared to healthy subjects [Zimmerman *et al* 1983]. Neutrophils from patients with human immunodeficiency virus (HIV) had increased expression of adhesion receptors (CD11b) and increased H_2O_2 production [Elbim *et al* 1994]. Hypoxemia was shown to prime neutrophils for enhanced superoxide generation and elastase release [Tamura *et al* 2002]. However when circulating neutrophils were compared with those from bronchial lavage neutrophils the superoxide generation was significantly impaired [Martin *et al* 1991].

Chronic cigarette smoking produces a 20-25% increase in the peripheral blood leukocyte count compared with non smokers, and this chronic increase correlates with a decrease in lung function over time. This provides one link to the role of neutrophils in the pathogenesis of both chronic airways obstruction and emphysema associated with cigarette smoking [van Eeden & Hogg 2000]. In the more acute setting of ARDS there is much evidence to suggest that the neutrophil is a key cell in causing pulmonary damage. The multiple effector mechanism combine to result in the associated lung and tissue injury [Aldridge 2002].

There is evidence that there is impaired neutrophil oxidative burst and reduced phagocytic capacity in type 1 and type 2 diabetes [Marhoffer *et al* 1993, Wykretowicz *et al* 1993]. Infectious complications in type 1 diabetes mellitus are generally recognized to be a major cause of morbidity and mortality. Marhoffer *et al* suggest inhibitory effects of elevated glucose concentration on PMN functions which might be of clinical importance concerning impaired host defence. Neutrophils from patients with glycogen storage disease type 1b have been shown to have reduced respiratory burst activity, which included superoxide generation. This was associated with attenuated calcium mobilization. However degranulation was not affected [Kilpatrick *et al* 1990].

Macrolide antibiotics, in particular erythromycin (EM) and azithromycin (AZM) have been shown to be clinically effective (particularly in the treatment of diffuse panbronchiolitis) at concentrations

lower than their antimicrobial actions [Sugihara 1997]. *In vivo* inhibitory effects of EM and AZM on active oxygen generation and chemotaxis of neutrophils demonstrated that this may be responsible for their therapeutic efficacy. However, the concentrations necessary for inhibition of neutrophils was again higher that the effective therapeutic dose. Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to inhibit fMLP stimulated neutrophil chemotaxis but not superoxide generation. Glucocorticoids have also been reported to inhibit neutrophil functions, as well as inhibit apoptosis and produce neutrophillia [Barton *et al* 2000].

More specific to cystic fibrosis is the control of Chloride ions (Cl^-). Neutrophils use part of their energy to pump Cl^- from the extracellular environment into the intracellular compartment, thereby accumulating a considerable amount of this anion [Simchowitz & De Weer 1986]. The resting neutrophil has an unusually high (80-100 mM) Cl^- concentration that is 4- to 5-fold higher than predicted on the basis of the Nernst equation. A Cl^- efflux seems to be one of the early neutrophil responses to several soluble agonists, of which TNFα is most powerful [Menegazzi *et al* 2000]. This also has the potential to affect the intracellular pH, which has been shown to be altered in patients with CF [Coakley *et al* 2000].

Destruction of lung epithelium accompanied by an increase in epithelial permeability is a frequent finding in most types of lung disease. Neutrophils are able to induce tissue injury through their ability to release cytotoxic compounds, including reactive oxygen metabolites and granule proteins. In vitro the proteinases elastase and cathepsin G have been shown to cause degradation of the extracellular matrix, injure both endothelial and epithelial cells and affect ciliary function. *In vitro* both elastase and cathepsin G cause detachment of alveolar type II cells [Van Wetering *et al* 1997] however defensins induced cell lysis instead, but were able to reduce elastase and cathepsin G induced cell detachment, with an additive increase when the two enzymes were mixed.

1.5 Cystic Fibrosis

1.5.1 The genetic basis of Cystic Fibrosis

Cystic Fibrosis (CF) is a multisystem, autosomal recessive monogenic disorder which predominantly affects secretory tissues including the lungs and results in recurrent or chronic infection, initially with *Staphylococcus aureus* and *Haemophilus influenza* and later with *Pseudomonas aeruginosa* [Hutchinson & Govan 1999]. The latter organism is associated with copious quantities of purulent sputum which is extremely difficult to expectorate and is at least partly through up-regulation of human muc2 and muc5 genes and the production of alginate by the bacterium [Hutchinson & Govan 1999]. *P.aeruginosa* is a cofactor in the progressive lung damage which is eventually fatal in these patients [Elborn 1991]. Repeated courses of antibiotics are prescribed and although they have improved quality and length of life, infections caused by *P.aeruginosa* are difficult to treat and require repeated hospitalisation and administration of parental antibiotics [Davies 2002]. Despite improved treatment, the major cause of morbidity and mortality in CF results from chronic progressive broncho-pulmonary infection leading to bronchiectasis and eventually to respiratory insufficiency. However, the life expectancy of these patients has risen from 14 years in 1969 to 32 in 2000 [Ratjen & Doring 2003].

Cystic Fibrosis is the most common autosomal recessive disorder amongst northern European populations. The affected gene is on chromosome 7, and the mRNA codes for a protein of 1480 amino acids, called the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The CFTR is a phosphorylation-dependent epithelial Cl^- channel. It is located primarily in the apical membrane, where it provides a pathway for Cl^- movement across epithelia and regulates the rate of Cl^- flow [Sheppard & Welsh 1999, Reddy & Quinton 2003]. CRTR occurs at various sites in the cells where it is expressed. Thus CFTR is central in determining transepithelial salt transport, fluid flow, and ion concentrations. The most common mutation in the U.K. is the delta F508 mutation (70%), with another 1000 mutations having been reported [Goldberg & Pier 2000, Hart *et al* 2002]. The different mutations have different effects on the functioning of the CFTR and therefore is one factor in severity of disease. CFTR genotypes can be divided into five classes according to their molecular consequences. Their effects, including examples, are shown in Table 1.1. These genes

may be associated with a secondary gene on chromosome 7 to further the severity of the disease [Pilewski & Frizzell 1999]. The manifestations and severity of the disease are very variable between individuals and a number of modifier genes separate from the CFTR locus significantly influence severity [Merlo & Boyle 2003].

Table 1.2

The CFTR genotypes and their effects

	Consequence	Effect	Examples
I	severe	No synthesis	G542X
II	severe	No maturation	ΔF508
		Block in processing	
III	severe	Defective regulation	G551D
		Block in regulation	
IV	mild	Decreased conductance	R117H, R347P
V	mild	Decreased abundance	A455E
		Reduced synthesis	

P.M Quinton discovered that secretory epithelia derived from CF patients have a low Cl^- conductance [Quinton 1983]. Since then identification, cloning, and a number of other investigations have been carried out. The CFTR consists of two halves, each containing six transmembrane spanning segments and linked to a consensus sequence for a nucleotide binding fold (NBF). The two halves are joined to a large polar R domain, containing multiple sites for phosphorylation by protein kinase A and protein kinase C. CFTR is a member of a superfamily of ATP dependent transport proteins [Sheppard & Welsh 1999]. The CFTR functions to regulate both Cl^- and Na^+ conductive pathways; however the cellular mechanisms whereby CFTR acts as a conductance regulator are unknown. CFTR and outwardly rectifying Cl^- channels (ORCCs) are distinct channels but are linked functionally via an unknown regulatory mechanism. One theory is

that CFTR functions to regulate other Cl- secretary pathways in addition to itself conducting Cl^-, and that purinergic (P_{2U}) receptors are involved [Schwlebert *et al* 1995]. Huang *et al* [2001] have shown that the signalling elements include adenosine A_{2B} receptors, G proteins, adenylate cyclase and protein kinase A.

Why defective electrolyte transport by affected epithelia leads to the wide variety of clinical manifestations is not clear. A number of theories have been proposed to explain the overwhelming susceptibility of patients with CF to *P.aeruginosa* lung infections. These include changes in airway liquid composition and viscosity [Smith *et al* 1996, Krouse 2001], enhanced bacterial binding to mucin and epithelial cell receptors [de Bentzmann *et al* 1996, Bals *et al* 2001, Lillehoj *et al* 2001], increased innate inflammation owing to altered cytokine production [Kammouni *et al* 1997] disruptions in lipid metabolism [Bhura-Bandali *et al* 2000] and a signalling role for the CFTR protein in bacterial ingestion and clearance [Schroeder *et al* 2002]. The increased mucus leads to obstruction of small airways, and results in decreased clearance of airways at both micro and macro levels.

There are several indications that airway inflammation is a contributing factor to the pathology of lung disease in CF even before lung infection is established. Balough *et al* [1995] showed increased infiltration of airways with inflammatory cells such as neutrophils and markers such as IL-8 in very young infants before infection had occurred. Inflammation was indicated by raised neutrophil and macrophage numbers, and increased IL1β and IL-8 concentrations in BALF. There was also an inverse correlation observed between the IL-8 and oxygenation as measured by pulse oximeter, even at the very early stage of lung disease. In contrast, Armstrong *et al* [1997] showed that these parameters were raised above healthy subject levels in those patients with CF who had evidence of infection. Production of IL-10, a cytokine that decreases inflammatory responses and T-cell stimulation, was reduced in epithelial cells from CF patients suggesting that there may be a defect with the down-regulation of inflammation [Bonfield *et al* 1995]. A four year placebo controlled study [Konstan *et al* 1995] showed that high dose ibuprofen significantly slowed the progression of lung disease in a group of patients with CF and mild lung disease, and without serious adverse effects. Further analysis of the data suggested that the effect was mainly in children with CF below 13 years. Since ibuprofen inhibits the migration, adherence, swelling, and aggregation of

neutrophils as well as the release of lysosomal enzymes, the results of this study put further emphasis on the neutrophils role in the damage to lungs in patients with CF.

Since the discovery of CFTR, and the link that this faulty channel was the underlying cause of CF, the potential of gene therapy to correct this has been recognised. Gene therapy can be achieved either using recombinant viruses carrying the therapeutic cDNA within the genome, or synthetic vectors complexed to plasmid DNA (lipoplexes and polyplexes) [Ferrari *et al* 2003]. Early on many *in vitro* studies showed that gene transfer of the normal CFTR gene to CF cells could correct abnormalities in ion transport [Chinet 1994]. However *in vivo*, studies have failed to produce more than a transient expression of the corrected CFTR at a low level [Harvey *et al* 1999]. A recently published Phase II trial showed safety, but could not prove efficacy, of a viral vector/gene construct [Wagner *et al* 2002].

1.5.2 Infection in Cystic Fibrosis

Patients with CF seem unable to eradicate infection with *P.aeruginosa* even with intensive antibiotic treatment. There is a marked antibody response, but the bacteria are not eradicated after the infection is established, and this is partly due to the versatility of the *P.aeruginosa* bacteria [Tummier & Kiewitz 1999]. The infection is contained within the small airways, and there is little evidence of invasion of the host, although there is evidence of inflammatory response occurring in several anatomical compartments. This infection remains localised in the lungs and very rarely causes sepsis or other complications. Several studies have linked these clinical parameters of a respiratory exacerbation with increased levels of inflammatory mediators such as CRP and neutrophil elastase anti-proteinase complex (hNEAPC) in the blood [Suter *et al* 1989, Norman *et al* 1991, Elborn *et al* 1993PP, Bell *et al* 2000, Ionescu *et al* 2000]. Studies have also linked levels of such markers to bronchoalveolar lavage and sputum levels, although such links are not as clear cut. Neither serum, BALF or sputum are an exact representation of what is occurring in the lung, and studies continue to compare and link clinical and biochemical measurements to the disease state.

Clinical management of cystic fibrosis is at present mostly dependent on clinical symptoms, with lung function looked at as a way of determining severity of disease, and a reduction signifying the necessity to treat with *i.v.* antibiotics. Sputum production, weight loss, and how the patient feels are also taken into consideration [Shale 2003]. Raised CRP levels may also be used in some instances. There is much debate as to whether this is the best management of the disease. The Danish guidelines have recommended that *i.v.* antibiotic treatment be given every 3 months from time of bacterial colonisation of the lungs, and have shown reduced clinical decline in lung function [Hoiby 2000]. However other studies have not been able to demonstrate an advantage of elective over symptomatic treatment [Elborn *et al* 2000]. More recently the impact of infection on the whole body has been considered more important including its impact on body composition [Ionescu *et al* 2002].

No primary immune deficiency appears to be associated with CF and yet unlike subjects with normal pulmonary function, CF patients almost universally fail to eradicate *P.aeruginosa* from their lungs. Lung Injury is thought to occur from the presence of proteases, which may have originated from the host (neutrophils, macrophages, monocytes, fibroblasts and eosinophils) or

bacteria. During infection neutrophils are thought to be highly activated and release products which cause lung damage [Weiss 1989]. Thus, the neutrophil has been implicated as the major injury producer in its release of antibacterial factors, but agents released by the *P.aeruginosa* are also implicated. The anti host factors produced by *P.aeruginosa*, and its evasive mechanisms are discussed in further in chapter 3. The central pathogenic problem of CF airways has been described as a vicious cycle of inflammation, mucus secretion or accumulation of infection.

Abnormal mucocilliary transport and tenacious sputum in CF will delay clearance of mediators and inflammatory cells from the respiratory tract, possibly prolonging their biological effects. A single infection might then contribute to a vicious circle of persistent inflammation with increased mucus production, persistent cellular chemotaxis and activation, and further mediator production. The impaired mucocilliary transport described in CF patients appears to be secondary to infection, or linked to the thick secretions present in CF airways. The CFTR plays a role in ion transport and therefore is probably related to the tenacity and viscosity of lung fluids. How much this is linked to the development of infection form a non diseased state is unclear.

There is no evidence to support a specific defect in neutrophil function in patients with cystic fibrosis. There is unpublished evidence that CF plasma does not enhance the proteolytic capacity of neutrophils *in vitro* and that CF neutrophils are no more active than non-CF neutrophils in this context. In addition there was no suggestion of priming of these cells which suggests that the protective, but potentially injurious functions of the neutrophil only become evident in the close vicinity of infection as the cells transit from the capillary to the airways.

1.5.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa (originally named pyocyanea) is a gram-negative rod bacteria. It is both a plant pathogen and an opportunistic pathogen, and is notorious for its ability to survive in the environment, being a dangerous contaminant, since it has the ability to cause serious infections in immunocompromised patients. *P.aeruginosa* can contaminate pharmaceutical products such as disinfectants, antibiotic eye ointment or drops and intravenous fluids. The organism only needs traces of organic substances present in water, hence can be found in drains, sinks, taps, and cleaning equipment which is stored wet.

Environmental factors affect antimicrobial sensitivity by influencing the phenotypic expression of micro-organisms. In the natural environment growth is controlled by the restricted availability of one or more nutrients. Growth will cease when all of a restricted nutrient is consumed. The cells are termed 'nutrient-depleted'. The important consequences of nutrient-depletion is that the cells display properties which are strongly dependent upon the nature of the particular depleted nutrient. e.g. *P.aeruginosa* grown in a magnesium depleted state is highly resistant to the quaternary ammonium compound (QAC), benzalkonimum chloride, whereas the same strain grown under carbon depletion is extremely sensitive. The antibiotic sensitivity and resistance of *P.aeruginosa* is also affected by its tendency to grow in a biofilm in the environment, which occurs in the lungs as well. Alginate production is particularly prevalent in strains of *P.aeruginosa* isolated from patients who have experienced chronic infection.

P.aeruginosa has a natural resistance to antimicrobials, and an extensive repertoire of phenotypic variations. The basis of the greater resistance of *P.aeruginosa* compared with other gram-negative bacteria is not at all clear. The answer may lie in the properties of the envelope because when this is removed, the resulting spheroplasts are just as sensitive as those of other organisms. The outer membrane is not different from that of other organisms in terms of overall composition. The same components (LPS, proteins, phospholipid, peptidoglycan) are present. One difference is the number of phosphate groups present in the lipid A region of the LPS. This is significantly greater in *P.aeruginosa* than in members of the Enterobacteriaceae and might account for the unusual sensitivity of the organism to ethylenediaminetertra acetic acid (EDTA). The high phosphate

content means that the outer membrane is unusually dependent upon divalent metal ions for stability; their removal by EDTA therefore has a dramatic effect upon cell integrity. Magnesium-depleted cells of *P.aeruginosa* are extremely resistant to EDTA. Presumably the lower magnesium content of the cell envelope reflects a decreased phosphorylation of lipid A.

1.5.4 Pseudomonas aeruginosa in lung disease and cystic fibrosis

P.aeruginosa produces a number of exoproducts which have been implicated in lung injury including exotoxin A, proteases, phospholipase C and exoenzyme S. A chronic rat lung infection model has been used by a number of investigators to assess the role of *P.aeruginosa* exoproducts in lung disease due to this organism. Similarities between the rat model and the lung infections seen in patients with CF include similar virulence factors expressed by *P.aeruginosa*, histological examination of lung tissue at autopsy, high titres of antibody to *P.aeruginosa* antigens, development of immune complexes, and a failure to clear the organism from the lungs [Que & Woods 1987]. Intratracheal administration of *P.aeruginosa* exoproducts has resulted in histopathological changes that closely resembled those seen in the lungs of infected rats [Cash *et al* 1982, Gray & Kreger 1979]. Leukocidin, another *P.aeruginosa* product, has been shown to be cytotoxic to neutrophils.

The proteases produced by *P.aeruginosa* have also been shown to degrade a number of cytokines, including IL-2, TNFα and interferon gamma (IFNγ). These cytokines are important in an intricate multicellular co-operation which affects T cells, B cells, neutrophils, macrophages and epithelial cells as the host response. Although not fully understood degradation of part of this complex network has the potential to have far reaching effects.

P.aeruginosa has a number of siderophore systems, of which pyochelin is the most well-characterized. This is an example of *P.aeruginosa's* ability to overcome the host's ability to withhold iron as a non-specific defence mechanism [Que & Woods 1987]. Yields of exotoxin A, elastase and alkaline protease are inversely proportional to the iron concentration of the medium. The type and amount of exoproducts produced varies depending on the site of isolation between different clinical sources. The anatomical pathology varies with the clinical situation, such as the differences between 'necrotizing pneumonia' and community-acquired pneumonia. These are both

different to the pulmonary histopathology of *P.aeruginosa* infection in cystic fibrosis which is dominated by bronchial changes.

P.aeruginosa has emerged as the principal cause of morbidity and mortality in patients with CF. Chronic colonisation of the CF respiratory tract with *P.aeruginosa* is characterised by a gradual transit of the pathogen from a non mucoid to a mucoid phenotype [Buret & Cripss 1993]. It has been reported that the capacity of *P.aeruginosa* to convert to mucoid form is present in all strains, and it is the environmental influence of the CF lung which causes such a conversion [Deretic *et al* 1994]. Histological changes include epithelial metaplasia with loss of cilia, predominance of mucous versus serous cells in hyperplastric bronchial glands, goblet cell hyperplasia, acute and chronic inflammatory infiltrates, bronchiectasis and mucopurulent plugging of the airways.

1.5.5 Immune response to P.aeruginosa

The characteristic susceptibility of CF patients to chronic *P.aeruginosa* lung infection may be indicative of a colonisation or growth advantage in the CF environment for this bacterium, or of exaggeration of CF abnormalities in the lung, or immune cells caused by certain *P.aeruginosa* components or exoproducts. Insipiated mucus, as seen in CF patients, is also present in non-CF patients with chronic bronchitis and COPD and does not predispose these patients to *P.aeruginosa* infection.

Colonisation of the lungs with *P.aeruginosa* is associated with a marked antibody response, with high IgG titres inversely correlated with pulmonary function [Cowan & Winnie 1993] . It induces a strong antibody response in serum, saliva and pulmonary secretions in both healthy subjects and patients with CF [Buret & Cripss 1993, Hendry *et al* 2000]. Many patients with CF have antibodies to *P.aeruginosa* prior to colonisation, however this does not appear to prevent colonisation. Once the chronic phase of the infection is established the production of large amounts of non-opsonic antibodies is accelerated. This suggests that the quantity and quality of antibodies is a secondary response to the degree or duration of antigenic stimulus in any given patient, and that there may be an intrinsic immunologic characteristic that determines the ability to resist chronic infection [Tosi *et al* 1995].

Protection against chronic pulmonary infection with *P.aeruginosa* in vaccinated patients has not yet been achieved despite positive serum antibody responses. Measurement of serum opsonophagocytic killing antibodies specific to the mucoid exopolysaccharide (MEP) antigen in patients with CF has shown that the levels of these antibodies were significantly raised in older, non-colonized, and relatively healthy patients [Pier *et al* 1991]. A specific *P.aeruginosa* antibody response does not suffice in providing protective immunity against this pathogen within the CF lung. Action may depend on opsonic activity of serum. [Peir *et al* 1993]. Use of such vaccines has achieved antibody response but limited protection against *P.aeruginosa* infection [Cryz *et al* 1997]. However, there remains a lack of suitable controlled trials to assess such vaccines [Keogan & Johansen 2000]. Several studies have demonstrated that IgG antibodies in the bronchial secretions from patients with CF were fragmented into Fab and Fc pieces, and that this proteolytic activity is from elastase and alkaline protease synthesized and released by *P.aeruginosa.* These enzymes have also been shown to cause proteolysis of the C3b opsonic molecule and its receptor, hence affecting phagocytosis. *P.aeruginosa* also inhibits opsonin recognition and therefore phagocytosis by a protective layer of alginate which is produced by mucoid strains, and is an adaptation to colonisation of the lungs of patients with CF [Drenkard & Ausubel 2002].

Other pathogenic bacteria in the lungs of adults with CF include *Burkholderia cepacia* which is also an opportunistic pathogen and was originally classed as a *Pseudomonas spp.* Strains of this organism seem to produce a greater inflammatory response in patients and many are more virulent leading to outbreaks of the same strain within a clinic leading to segregation of patients. A sub group of these patients exhibit the 'cepacia syndrome' *i.e.* rapid clinical deterioration and death within one year. *B. cepacia* are highly resistant to antibiotics and to neutrophil-mediated non-oxidative killing, and nine strains have been grouped together as the *B.cepacia* complex [Speert 2002].

Many patients with CF also suffer from asthmatic bronchoconstriction. Forteza *et al* [1994] showed that supernatants from cultures of *P.aeruginosa* caused bronchoconstriction in sheep, suggesting the presence of proinflammatory metabolites. Another theory is that damage to the bronchial epithelium makes the airway more susceptible to effects by chemicals. *Aspergillus fumigatus* is also a common

pathogen in patients with CF, although often causing a significant IgE as well as IgG response [Greenberger 2002].

1.6 Inflammatory markers

A simple definition of an inflammatory marker would be 'a chemical that is altered in infection or inflammation compared to healthy subjects, and changes with effective treatment of the condition'. An inflammatory marker is generally one that is raised with infection, and falls with treatment. By association they are therefore often mediators of inflammation as well. A number of systemic markers of inflammation have been measured in patients with CF. The levels of most proinflammatory markers, such as CRP, hNEAPC, IL-1and IL-8, are raised in patients compared to healthy subjects, especially at times of infective exacerbation, and are reduced with antibiotic treatment [Norman *et al* 1991, Dean *et al* 1993, Kronborg 1993, Bell *et al* 2000]. However, treatment does not always reduce markers to that of the healthy subjects [McGraph *et al* 1999, Ionescu *et al* 2000]. Increases in inflammatory markers also occur in other diseases such as COPD [Eid *et al* 2001], sepsis [Friedland *et al* 2000] and fever [Engervall *et al* 1995].

Inflammatory markers include acute phase proteins, cytokines, and neutrophil granular products. Of the acute phase proteins, CRP is the most commonly referred to. It is produced by the liver in response to infection. The major degradative protease produced by the neutrophil is elastase which is widely considered to cause degradation of lung tissue [Brown & Donaldson 1988, Gadek 1992]. In the circulation, its enzymatic activity is prevented by the presence of anti-protease molecules present in the blood stream, in particular α_1-anti-trypsin, and circulating levels are measured as a complex; hNEAPC. A number of cytokines are considered to be pro-inflammatory and form part of a complex network involving a balance with those cytokines considered to be anti-inflammatory. They are regulated by a complex pattern of pro- and anti-inflammatory effects produced by stimulation of cells often mediated through other cytokines. There is a subset of leukocyte chemotactic cytokines referred to as chemokines of which IL-8 is most associated with neutrophil infiltration into the lungs during inflammation and infection [Luster 1998].

Cytokines produce their effect by binding to cell-surface receptors. These receptors commonly have four cysteine residues with an amino and a carboxy terminal end. The receptors in the cytokine family share common signal transducing receptor subunits [Taga & Kishimoto 1995]. Their signalling pathways include activation of cytoplasmic tyrosine kinases of the JAK family and

cytoplasmic transcription factors or activators (STATs). The JAK-STAT system also plays a role in cytokine specific gene expression [Sato & Miyajima 1994]. These receptors are often found in the circulation in their soluble form, and may act as inflammatory markers in their own right.

IL-1 and TNFα are often considered similar as they have many overlapping functions including induction of fever, induction of the acute phase response, anorexia, cachexia and neutrophillia. IL-1 is a 'multifunctional' cytokine, with effects on nearly every cell type. IL-1 is highly inflammatory and agents that reduce the production and/or activity of IL-1 are likely to have an impact on clinical medicine [Dinarello 1998]. IL-1 has two forms, α and β, which have similar biological activities [Dinarello 1988]. The production and activity of IL-1 is tightly regulated by gene expression, synthesis and secretion. This also applies to the IL-1 receptor antagonist (IRAP) which competitively blocks *in vitro* and *in vivo* effects of IL-1α and β, and has been shown to increase during the course of infection [Kronborg 1993]. Clinical trials of the synthetic IL-1 receptor antagonist, anakinra, has shown significant improvement of inflammation and pain in rheumatoid arthritis [Kary & Burmester 2003].

TNFα has also been called cachectin from its early association with catabolic weight loss in patients [Beutler & Cerami 1987]. TNFβ has similar biological activities, but has been less well studied. The effects of TNFα are mediated by two receptors: TNFRI (55kDa) and TNFRII (75kDa) [Vandenabeele *et al* 1995]. The level of expression of the two receptors can be regulated independently, both receptors can be shed, and their soluble form measured in serum. TNFα is able to stimulate the production of other pro- and anti-inflammatory cytokines, as well as priming and activating superoxide generation and granule release in neutrophils [Rumalla 2002]. TNFα will activate signalling pathways for cell survival or programmed cell death. TNFα is an incomplete secretagogue in neutrophils and requires input from both ligated integrins and TNFα receptors to trigger superoxide generation, degranulation and activation of enzymes such as PI3K. The adherence of neutrophils to matrix proteins such as fibronectin provides signalling from β-integrins, a requirement for neutrophil responsiveness to TNFα [Kilpatrick *et al* 2002].

IL-6 has a range of overlapping functions with TNFα and acts in an endocrine fashion to mediate the synthesis and release of acute phase proteins such as CRP from the liver [Gauldie *et al* 1992]. IL-6 also stimulates T cells, and has effects on osteoblasts, and elevated levels of IL-6 have been reported to be associated with a variety of diseases such as rheumatoid arthritis [Cohick *et al* 1994], myelomas, lymphomas and malignancies [Bauer & Herrmann 1991]. The IL-6 receptor has two different membrane glycoproteins; an 80-kD protein (CD126), the ligand-binding protein, and a 130kD protein, the signal transducing protein. The latter can bind to cells not bearing the IL-6 receptor and therefore extends the range of sites at which IL-6 can act [Roth 2000]. The 50kd portion of the receptor is shed as a soluble form. Both IL-6 and the IL-6/sIL-6 complex appear to be biologically active [De Benedetti 1994]. It has been demonstrated that CRP activates soluble IL-6 receptor production and release by human neutrophils [Jones *et al* 1999]. Although predominately considered a pro-inflammatory cytokine, some reports indicate that it may have anti-inflammatory activity, particularly relating to its effect on T cells and the immune response [Tafuto 1994].

The chemokines are homologous 8- to 10-kd proteins that are subdivided into families on the basis of the relative position of the cystein residues in the mature protein. The α-chemokines are also referred to as CXC and are chemotactic for neutrophils and the β-chemokines, also referred to as CC, act on lymphocytes [Luster 1998]. IL-8 is a CXC chemokine which activates neutrophils in addition to its chemotactic activity and has recently been renamed CXCCL8 according to new nomenclature [Adams *et al* 2001]. IL-8 is inversely related to lung function in children with cystic fibrosis [Sagel *et al* 2002], and positively related to BALF neutrophil number [Balough *et al* 1995]. High levels of IL-8 in BALF relating to severity of disease in patients with CF has been reported by other groups [Dean *et al* 1993, Bonfield *et al* 1995] and in COPD [Wilkinson *et al* 2003]. IL-8 has been shown to be key in the chemotactic activity if sputum [Beeh *et al* 2003]. IL-8 is also raised in the BALF of babies with brochiolitis [Abu-Harb *et al* 1999], and patients with interstitial pneumonia [Fujimori *et al* 2003] where it is associated with increased elastase and neutrophil density.

Colony stimulating factors (CSF) may also be indicators of infection and inflammation. G-CSF is a pleiotropic cytokine best known for its specific effects on the proliferation, differentiation, and activation of haematopoietic cells of the neutrophilic granulocyte lineage. G-CSF not only supports

the proliferation and neutrophilic differentiation of normal haematopoietic progenitor cells *in vivo* but also their survival [Avalos 1996]. G-CSF also appears to have the potential to activate functions of mature neutrophils, influencing recruitment to sites of inflammation and tissue injury through inducing adherence to intercellular adhesion molecules-1(ICAM-1) [Chakraborty *et al* 2003]. G-CSF primes neutrophils for enhanced superoxide generation and antibody dependent cellular cytotoxicity as well as stimulating the release of AA from neutrophils and the production of leukocyte alkaline phosphatase and MPO. The production of pro-inflammatory cytokines, TNF and IL-6 by neutrophils was reduced by G-CSF treatment [Valente *et al* 2002] suggesting that G-CSF might simultaneously enhance the host defence whilst reducing the risk of developing uncontrolled systemic inflammation. CSFs stimulate glucose uptake in target cells, presumably to provide increased metabolic fuel for heightened cellular activity [Vera 1998]. Granulocyte Macrophage CSF (GM-CSF) is also a pleiotropic cytokine with many similar functions to G-CSF. It is better known for its ability to prime neutrophils [Dale *et al* 1995]. In mice infected with Listeria the degree of elevation of CSFs depended on the infecting dose, and the reduction in bacterial numbers followed the peak in bone-marrow progenitors [Cheers *et al* 1988], providing evidence for their role in fighting infection through specific cell-medicated immunity.

Neutrophil granule proteins are released upon activation of neutrophils, and their presence in the circulation can indicate infection and/or inflammation. Elastase, MPO and lactoferrin are the most commonly measured indicators of neutrophil activity. MPO has been shown to be released from neutrophils on adherence, in particular to fibronectin, as well as on stimulation to both fMLP and PMA [Xu 2002]. Both basal and stimulated release of lactoferrin and MPO was reduced in patients with pneumonia compared to healthy subjects [Zimmerman 1999ICM]. Elastase, MPO and Cathepsin B were raised in patients with COPD producing purulent green sputum, and elastase and MPO were related to each other [Stockley *et al* 2000T, Ren 2002]. There are a number of reports in patients with CF of raised elastase concentrations in the ariways [Sagel *et al* 2001] and as a complex in serum [Suter *et al* 1989, Meyer *et al* 1991, Rayner *et al* 1991].

Neutrophils utilize the lipoxygenase pathway to generate leukotrienes (LT), the majority of which are pro-inflammatory. LTs are 5-lipoxygenase metabolites of arachidonic acid, produced primarily by inflammatory leukocytes, and arachidonic acid (AA) itself is generated largely via the activity of

phospholipase A_2 on membrane polyunsaturated fatty acids. Neutrophils have been shown to release free AA in response to phagocytic stimuli including opsonized zymosan and PAF. This free AA may then be further be metabolised via the lipoxygenase pathway into biologically active metabolites such as 5-hydroxy-eicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4). *In vivo* LTs are able to cause increased mucus production, bronchoconstriction, leukocyte chemotaxis, and increased vascular permeability [Bates 1995, Crooks & Stockely 1998]. BALF from patients with cystic fibrosis patients has been reported to contain sufficient amounts of LTB_4 both to recruit additional neutrophils into the airways and to stimulate neutrophils to release granular products. Levels of LTB_4 were significantly higher in CF patients than healthy subjects [Konstan *et al* 1993]. An abnormality in the regulation of arachidonic acid release from lymphocyte membrane phospholipids of CF subjects has been demonstrated [Carlstedt-Duke *et al* 1986]. Pathological regulation of arachidonic acid release in CF could potentially lead to an increased generation of LTs [Saak *et al* 1990].

Platelet Activating Factor (PAF) is involved in the stimulation of acute and chronic inflammation, but probably also plays a role in the regulation of normal tissue processes including heart, liver and kidney function. Its effects during inflammation are to induce hypotension, cause bronchoconstriction and increase vascular permeability. Many of the biological activities of PAF are modulated by interactions with eicosanoids, and it is likely that PAF formation and arachidonic acid metabolism are intimately linked during cell activation. Release of PAF only occurs after a critical intracellular concentration in reached, and some PAF remains cell associated. This may play a role in cell-cell communications. Increases in intracellular Ca^{2+} may allow PAF synthesis, but not release, whereas extracellular Ca^{2+} increases both synthesis and release in a dose-dependent manner.

There are a large number of other inflammatory markers, with well over 100 cytokines currently identified which could potentially be measured in various diseases and have the potential to be inflammatory markers. This section gives only a very brief outline of those most commonly used, and most relevant to CF and it's associated lung disease and infection.

Table 1.3 ***Summary of key cytokines involved in inflammation and neutrophil function***

Cytokine	Functions
C-CSF	Proliferation and differentiation of granulocyte-macrophage population.
GM-CSF	Survival, proliferation and differentiation of granulocyte-macrophage populations.
GRO	CXC chemokine functionally related to IL-8.
Interferon α, β, γ	Anti-viral and anti-parasitic, proliferate effects on tumour cells.
IL-1 α, β	Important in the inflammatory response, and acts as pyrogen by inducing the production of prostaglandins and activates the hypothalamus.
IL-1 receptor antagonist (IL-1RA)	Inhibits both forms of IL-1, implicated in the regulating the severity of the inflammatory responses.
IL-2	Induction of proliferation of T_H and T_C cells and the stimulation of T cells to produce other cytokines.
IL-3	Stimulates colony formation of megakaryocytes, neutrophils and granulocytes from bone marrow. Involved in regulation of T cell growth, macrophage proliferation and differentiation and IgG secretion.
IL-4	Numerous actions, many as a result of indirect action whereby IL-4 stimulates various cell types to produce other cytokines.
IL-5	Role in production, activation and localisation of eosinophils. Implicated in a range of allergic conditions.
IL-6	Multifunctional, influences antigen-specific immune reactions and inflammatory reactions. Induces B cell differentiation and immunoglobulin secretion, T cell growth and cytotoxic T cell differentiation. Induces the production of hepatic acute phase proteins during the inflammatory response.
IL-8	CXC chemokine, potent chemoattractant for neutrophils, T cells and basophils. Inhibits adhesion of leukocytes to endothelial cells.
IL-10	Inhibiton of pro-inflammatory cytokines from activated monocytes and macrophages. Promotion of B cell proliferation and immunoglobulin secretion.
IL-15	Stimulation of growth and differentiation of B and T cells. Role in NK cell proliferation, maturation and cytokine production and the induction of mast cell proliferation.
IL-18	induces IFNγ production from TH1 NK cells. Activates NFκB and induces the production of CC and CXC chemokines.
Interferon inducible protein-10	CXC chemokine, potent chemoattractant for activated T lymphocytes.
Leukaemic Inhibitory Factor (LIF)	member of IL-6 family. Inhibits migration of neutrophils
Lymphotactin	Chemotactic for lymphocytes
monocyte chemotactic protein (MCP) 1 to 5	Overlapping functions; chemotactic for monocytes, lymphocytes, basophils. Regulation of adhesion molecule expression and cytokine production in monocytes.
Macrophage inflammatory Proteins (MIP)(1α, 1β, 3α, 3β, 4)	CC chemokines, 1α is capable of neutrophil activation, 1α and 1β are HIV suppressive factors, 4 is chemotactic for T lymphocytes.
Neutrophil Activating Peptide 2 (NAP 2)	CXC chemokine less potent chemoattractant of neutrophils than IL-8. Causes neutrophil uptake of Calcium, neutrophil degranulation and respiratory burst.
RANTES	Activation of basophils, NK cells and eosinophils
Stem Cell Factor (SCF)	Haematopoietic growth factor which acts synergistically with CSF.
Transforming Growth Factor (TGF) α, β	Induces the production of acute phase proteins, stimulates the expression of MHC antigens, and the expression of various cytokines and CSF. β form has similar functions but less potent.

1.7 Lung structure and function and the role of Airways fluid

The lung structure consists of two bronchi which branch repeatedly into bronchioles and then capillaries, each only 0.2 to 0.6 μm, whick make up the alveoli where oxygen transfer takes place. A thin layer of a mucous secretion covers the surface of the healthy large airways and participates in the removal of foreign particles through mucocilliary transport. Mucus glycoproteins (mucins) and proteoglycans are prominent macromolecular components of this [Thornton *et al* 1991].

The airway surface fluid is present as a bylayer, with a superficial gel or mucous layer and a layer of periciliary fluid, referred to as the sol, interposed between the mucous layer and the epithelium. The mucins of the sol are smaller than those of the gel, and may be proteolytic fragments of the larger species in the gel [Thornton *et al* 1991]. A thin layer of surfactant separates the mucous and pericilliary fluid layers [Rubin 2002]. The airways surface fluid (ASF) is produced by airway submucosal glands located throughout the bronchioles and movement of the ASF is very dependent on its consistency. In CF the malfunction of the CFTR leads to a thicker more tenacious consistency to the ASF, the result being the cilia can no longer effectively clear the ASF of any foreign matter including microorganisms [Verkman *et al* 2003]. Physiotherapy is a well established procedure to remove these secretions and has helped to improve life expectancy through increased clearance, but its lack of effectiveness in clearing all the airways particularly the most distal parts is accepted.

Abnormal muciliary transport and tenacious sputum in CF will delay clearance of mediators and inflammatory cells from the respiratory tract, prolonging their biological effects. A single infection might then contribute to a vicious circle of persistent inflammation with increased mucus production, persistent cellular chemotaxis and activation, and further mediator production. Cytokines are thought to be present in the lung. A few have been measured in sputum recently [Kronborg 1993], but not at different times during an exacerbation.

Surfactant is a mixture of phospholipids and protein (surfactant protein (SP) A, B, C and D), with the primary function of reducing surface tension at the air-liquid interface. Surfactant components are synthesized and secreted by type II pneumocytes within the alveolar spaces. The secretory mechanism is counterbalanced by clearance from the alveolar spaces, surfactant components being

degraded and recycled primarily by type II pneumocytes and, to a lesser extent, by alveolar macrophages. Total protein was not significantly greater than in control BALF, but levels of Albumin were lower, counteracted by the fact that levels of IgG, IgA, were higher [Fick *et al* 1984].

Connective tissue of the lung is largely made up of collagen, elastin and fibronectin, all of which are susceptible of the action of proteolytic enzymes produced by neutrophils. Once the integrity of the overlying airways mucosa is breached exposing connective tissue, the elastase activity which is likely to be present in the epithelial lining fluid and sol phase of sputum, can act unopposed on the substrates. In addition, alterations in the integrity of the surface epithelium will enhance the capacity of bacteria to remain within the lung and stimulate more host mediated response.

Matrix components fall into 3 general categories: collagens, proteoglycans, and glycoproteins. Collagens are the major structural elements in tissues. Proteoglycans are highly charged molecules regulating fibre size, hydration and tissue permeability. The glycoproteins link matrix components and cells. Each matrix such as fibrous tissue, basement membrane, or cartilage has a distinct combination of matrix proteins that determines its characteristic form, tensile strength, and its interactions with various cells [Martin *et al* 1985]. Ten collagen proteins have been identified to date, and it is the type III collagen which is particularly abundant in the matrix of blood vessels and in internal organs, and type IV is present only in basement membranes [Martin *et al* 1985], and therefore the ones which are degraded in lung inflammation.

It is essential that both elastase and collagenase, which are required for host defence, be carefully regulated to prevent them from damaging the tissues that they are trying to protect. One way that the body accomplishes this regulation by means of anti-proteinases. An appropriate equilibrium between proteases and antiproteases is essential to maintaining the health and integrity of the lung [Gadek 1992].

1.8 Experimental Factors

Neutrophils are sensitive to a number of agents and can be activated by trace amounts of endotoxin [Haslett *et al* 1985] or by 'rough' handling. They have a short lifespan and therefore must be isolated from venous blood immediately prior to study. Attempts at culture have been most successful with HL60 cells although these cells do not have exactly the same properties as neutrophils. Neutrophils are considered fragile cells, easily damaged by improper handling. A review by Glasser & Fiederlein [1990] reported that the method of erythrocyte sedimentation and density gradient separation gives a relatively pure preparation, without background superoxide generation or granular release, and no adverse effects on biological phagocytosis or killing. It also confirmed that room temperature was best for the preparation.

ELISAs provide both quantitative and qualitative measurement of proteins with good sensitivity and specificity. We therefore used ELISAs for the majority of measurements of marker protein in both circulation, sputum and neutrophil supernatant cultures.

CRP is routinely measured by Medical biochemistry in hospitals, but only with a minimum detection of 1mg/l. To allow detection in all healthy subjects as well as patients we used an in house ELISA able to detect down to 200ng/ml in serum (200pg/l). The ELISA for neutrophil elastase was developed for use with sputum and the supernatant of cell functional studies, where much of the elastase is not complexed. It does detect complexed elastase.

The degree of destructiveness exhibited by neutrophils is an integrated response to both excitatory and inhibitory signals regulating normal functions, such as phagocytosis, degranulation and intracellular killing of bacteria. Many of the signals to such functions will be found in the sol-phase of sputum. Because neutrophils have both an anti-infectious and proinflammatory role in CF, the investigation of neutrophil activity is of crucial importance.

1.9 Why do neutrophils fail to clear the infection from the lungs in CF?

From previous research it is established that:

- The primary role of the neutrophil is to kill and remove invading bacteria.
- Bacteria, particularly *P.aeruginosa,* colonise the lungs of patients with CF
- Chronic colonisation of the lungs of patients with CF leads to progressive deterioration of lung function.
- Neutrophils do not clear the chronic lung infection in CF.

We attempted to find out the possible reasons that neutrophils fail to clear lung infections. This has been addressed by a number of research groups and has included investigations into the salt concentration [Quinton 1983, Smith *et al* 1996, Krouse 2001, Coakely *et al* 2000], preferential binding of *P.aeruginosa* to mucin, epithelial cells and pilli [Saiman & Prince 1993, de Bentzmann *et al* 1996, Goldburg & Pier 2000, Bals *et al* 2001], resistance and immunoevasive actions of *P.aeruginosa* [Hoiby *et al* 2001, Drenkard & Ausubel 2002], metabolic effects [Freedman *et al* 2000, Ionescu *et al* 2002], and alterations in adhesive properties of neutrophils which could affect recruitment to the lungs [Mackerel *et al* 2001].

Based on neutrophils isolated from other diseases we might expect neutrophils to show enhanced activation such as was observed in patients with COPD and emphysema [Burnett *et al* 1987]. This may only occur in a sub population such as has been seen with ARDS [Cholett-Martin et al 1992]. There may be reduced responsiveness as has been seen in patients with diabetes [Marhoffer *et al* 1993, Wykretowicz *et al* 1993]. Alteration of cell surface receptor number by acute phase proteins [Elbim *et al* 1994, Zouki *et al* 1997], or desensitisation of previously activated receptors [Shaked *et al* 1994, Lee *et al* 1989], or after migration [Martin *et al* 1991] may also occur. Enhanced reactivity by *in vivo* priming by cytokines [Van Leeuwen *et al* 1995, Zimmermann *et al* 1983] or hypoxia may also occur [Tamura *et al* 2002, Madjdpour *et al* 2003].

We therefore approached this from a different perspective to determine whether the neutrophils themselves were functioning incorrectly and participating in the overall *in vivo* situation. This thesis compares the neutrophils from patients with CF with those from healthy subjects for a number of

different aspects of functioning including phagocytosis and intracellular killing of *P.aeruginosa*, superoxide generation, elastase release, cell surface receptor expression, adhesion and morphology. We have also studied neutrophils from patients with CF at different clinical states to see whether functioning is altered at different clinical states and therefore affected by the level of infection and inflammation. Addition of sputum sol provided a crude determination of how neutrophil functioning may be influenced *in vivo* in the lungs. For completeness the level of some key inflammatory markers were determined in both the sputum sol and circulation to provide information on the substances that the neutrophil may be in contact with which could influence functioning.

Chapter 2

Methods and Development of Assays

This chapter comprises a full description of all methods and their development and validation where appropriate. Details of materials, buffers, equipment and ELISA kit and other protocols can be found in Appendices 1, 2 and 3. The details of the patient groups for the different studies are detailed here clarifying where there is overlap of patient results between chapters.

2.1 Development and modification of Assays

2.1.1 Enzyme Linked Immunosorbent Assay (ELISA) - Introduction

Enzyme immunoassays are based on the discriminatory power of antibodies, and their combination with specific enzymes with a high catalytic power. The observation that antigens or antibodies could be immobilized on solid phases to develop methods for the quantitative detection of immunoreactants was made by Engvall and Perlmann [1971]. Further developments including the labelling of an antibody with an enzyme without seriously affecting its activity [Rubenstein *et al* 1972] and the ability to produce monoclonal antibodies [Köhler and Milstein 1975] has allowed an extremely wide range of enzyme immunoassays to be developed. Sandwich ELISAs where an antigen is detected by the use of two antibodies, one to capture the antigen and a second to detect the captured antigen were used throughout this project. At each step unbound and unwanted proteins and reagents are removed by washing. All in house assays use Immulon IV plates (Thermo Labsystems, Ashford, Middx. UK; formerly Dynex technologies) which are the most absorbent in the range. The basic steps of an ELISA are shown in Figure 2.1

Bovine serum albumin (BSA) is the most commonly used blocking protein at a concentration of 0.1 to 1%. Most antibody-antigen binding occurs at physiological pH (e.g .7.4). Gently shaking enhances antibody-antigen binding rates by both increasing the kinetic energy, and the likelihood of the antigen and antibody coming into contact with each other. Therefore, for all in house assays the plates underwent gentle shaking at all incubation steps except coating. Antibody-antigen

interactions occur faster at physiological temperatures, i.e. 37°C. In practice all reagents, and ideally procedures, must be at the same temperature otherwise consistency between wells is lost, particularly edge wells which cool more quickly than those in the middle. Since it is impractical to carry out ELISAs at 37°C, room temperature has been used throughout except where otherwise stated. Coating is routinely carried out at 4°C overnight after which the assay plate and all reagents are left to equilibrate to room temperature before starting the assay. Samples, particularly serum, contain a number of other proteins, which may affect the binding of antibodies, and this is referred to as matrix effect. Dilution or adding chemicals to the sample are done to minimise this matrix effect.

The secondary antibody may be conjugated directly to an enzyme, to biotin where an avidin-enzyme conjugate is used, or an anti-IgG–enzyme conjugate (against the species which the secondary antibody was raised in) can be used. The advantage with biotin-avidin system is that it may amplify detection however non-specific binding may be increased, and the assay length is increased. For some antibodies where the binding strength is of lower efficacy, the extra washing may actually remove some of the antibody complex, causing a reduction in sensitivity of the assay. The two enzymes most commonly used are Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (AP) which both alter the colour of the specific substrate.

Following substrate colour development the absorbance at a specific wavelength is measured using a plate reader (a specially adapted spectrophotometer), which reads each well vertically through the bottom of the plate. Here all ELISA readings were made using the BioRad model 3550 plate reader. This was linked to a computer and operated with specific software, which also calculated the results according to entered format and standard values. This colour intensity is measured as optical density (OD) which develops in a direct relationship to bound enzyme activity. This in turn is directly related to the concentration of antigen bound. A standard curve is plotted and used to calculate the concentration in the test samples. A standard curve must be carried out on every assay plate.

Figure 2.1

Layout of an ELISA

A Primary antibody is immobilized, best in an alkaline bicarbonate buffer

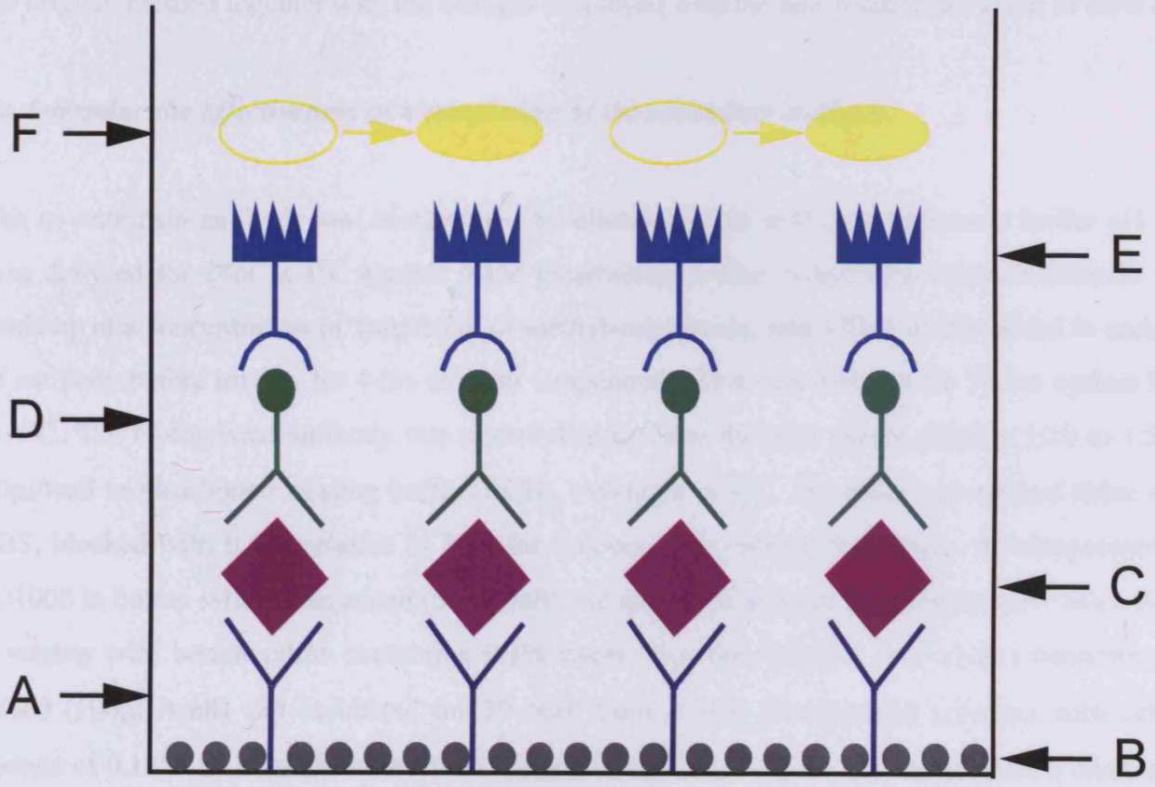
B Blocking of surface area not covered by the coating antibody with a non-reactive protein

C Antigen (standard / sample) binds to the primary antibody

D Secondary antibody binds to the antigen

E Conjugate binds to the secondary antibody (where secondary antibody is not conjugated to an enzyme)

F Substrate changes colour when antigen is present. Intensity is proportional to concentration.



2.1.2 Neutrophil Elastase α_1 anti-trypsin complex (hNEAPC)

This assay was adapted from a previously reported ELISA (Rayner 1991). This assay used a sheep anti human elastase (hNE) capture antibody, hNE - α_1-antitrypsin (α_1AT) complex as the standard, and a biotinylated sheep anti human-α_1AT detection antibody. Binding of the latter was detected with an avidin-peroxidase conjugate and 5-amino salicylic acid as substrate. The assay used both Phosphate Buffered Saline (PBS) and borate saline as buffers, and gelatine as the blocking protein and in the assay buffer (Appendix 1). Using the original method a standard curve could not be obtained. To investigate this problem each assay step was separately studied. However, since the method for biotinylation of the α_1-AT antibody contained insufficient practical information for accurate replication, it seemed likely this had been carried out differently. Insufficient biotinylation of the antibody was likely to be the reason for lack of colour change of the substrate. An outline of the original method together with the changes employed with the new method are given in table 2.5.

To determine the effectiveness of biotinylation of the secondary antibody:

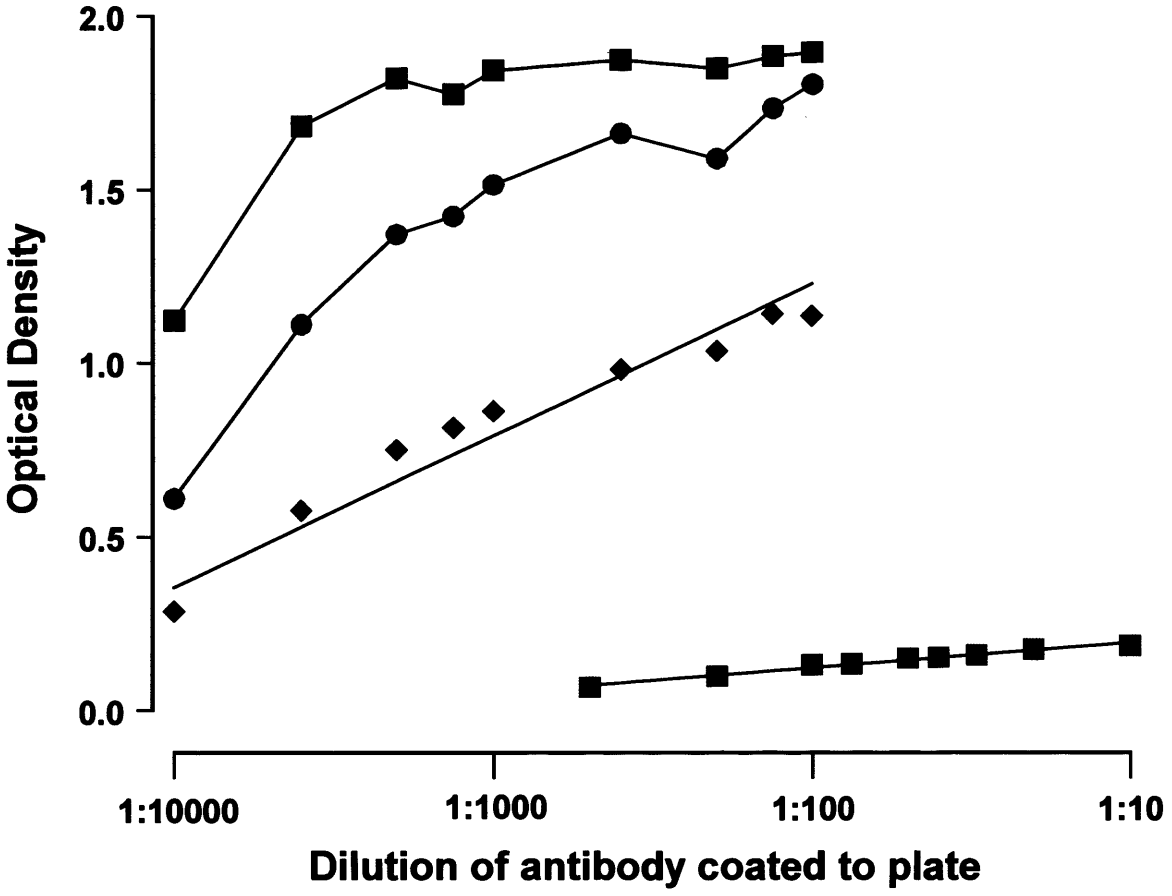
The α_1-antrypsin antibody was biotinylated by dilution (1:10) in 0.1M bicarbonate buffer pH 9.0, then dialysed for 24hr at 4°C against 0.1M bicarbonate buffer. N-hydroxy-succinimidobiotin was made up at a concentration of 1mg/ml in di-methyl-sulphoxide, and 120ml of this added to each ml of antibody before mixing for 4 hrs at room temperature. This was dialysed for 24 hrs against PBS at 4°C. The biotinylated antibody was coated directly onto the plate (serial dilution 1:10 to 1:500) 50µl/well in bicarbonate coating buffer (BCB), overnight at 4°C. The plate was washed twice with PBS, blocked with 0.5% gelatine in PBS for 2 hours, then washed four times. Avidin-peroxidase (1:1000 in borate saline) was added (50µl/well) and incubated at room temperature for 1 hour. After 4 washes with borate saline containing 0.1% tween, 5-amino salicylic acid (ASA) substrate was added (100µl /well) and incubated for 30 min. Little colour development occurred with only a change of 0.185 OD at λ455 for the 1:10 dilution of antibody (Figure 2.2). Although the dilution of biotinylated antibody was related to the optical density it was clear the antibody had not been effectively biotinylated by the method used here.

Use of an alternative secondary antibody:

An improved result was obtained by replacing the biotinylated α_1-AT antibody with a goat anti-human α_1-AT peroxidase conjugate as the second antibody and omitting the now redundant avidin peroxidase step. This improvement can be seen in Figure 2.2. After only 3 minutes substrate incubation, there was a clear concentration-related colour development with a good correlation between antibody dilution and OD ($r=0.983$, $p<0.001$).

Figure 2.2

Comparison of α_1-antitrypsin detection by anti α_1-antitrypsin conjugated with biotin in house, and commercially available peroxidase-conjugated antibody.



Commercially conjugated antibody: ■ 30 min. incubation,
● 10 min. incubation,
◆ 3 min. incubation

Biotinylated abtibody ■ 30 min. incubation

Optimisation of buffers and incubation times:

The original assay used borate and PBS buffers for the sample and antibody incubation phases. The method was changed so that all steps were carried out in PBS. Buffers containing 0.1% gelatine ofr0.1% BSA were compared and lower blank values were found with the latter. In addition, a more comparable sample value for spiked (100ng/ml hNEAPC) plasma was obtained and consequently 0.1% BSA was substituted for 0.1% gelatine in all subsequent assays (Table 2.1).

Sample incubation times of 1hr, 2hr and 3hr were compared, showing little difference between blanks, and only slightly more standard binding at the longer incubation times (Table 2.2). The 2hr incubation was chosen, as this was a compromise between the greater amount of standard binding and the lesser amount of non-specific plasma binding.

Table 2.1

Comparison of Gelatine and Bovine Serum Albumin in sample buffer for the hNEAPC ELISA

	Gelatine in sample buffer	BSA in sample buffer
Blank	0.551 OD	0.206 OD
1ng/ml hNE	0.648 OD	0.293 OD
10ng/ml	0.821 OD	0.526 OD
100ng/ml	1.425 OD	0.766 OD
1000ng/ml	1.645 OD	1.005 OD
Plasma 1:5 dilution	6.7 ng/ml	35 ng/ml
Spiked plasma (100ng)	15 ng/ml	118 ng/ml

Table 2.2

Effect of different standard/sample incubation times on the optical density

	Blank	50ng/ml	500ng/ml	1:2 plasma NSB	1:2 plasma
1 hour	0.126	0.693	0.918	0.203	0.540
2 hour	0.131	0.852	1.056	0.261	0.583
3 hour	0.141	0.668	1.077	0.351	0.638

Changes to the blocking phase of the assay

Blocking of the microtiter plates with gelatine (1%) was replaced by BSA (1%) as this was found to reduce the non-specific binding without reducing the binding of the standard. Blank O.D. values were 0.206 for gelatine and 0.167 for BSA. In comparison 100ng/ml hNE gave total OD of 0.487 for gelatine and 0.489 for BSA, giving a change in absorbance of 0.281 and 0.322 respectively. The best concentration of BSA for blocking was determined to be 1%, as this gave the lowest non-specific binding whilst maintaining a good maximum OD. This buffer (PBS, 1% BSA) is referred to as blocking buffer.

Washing

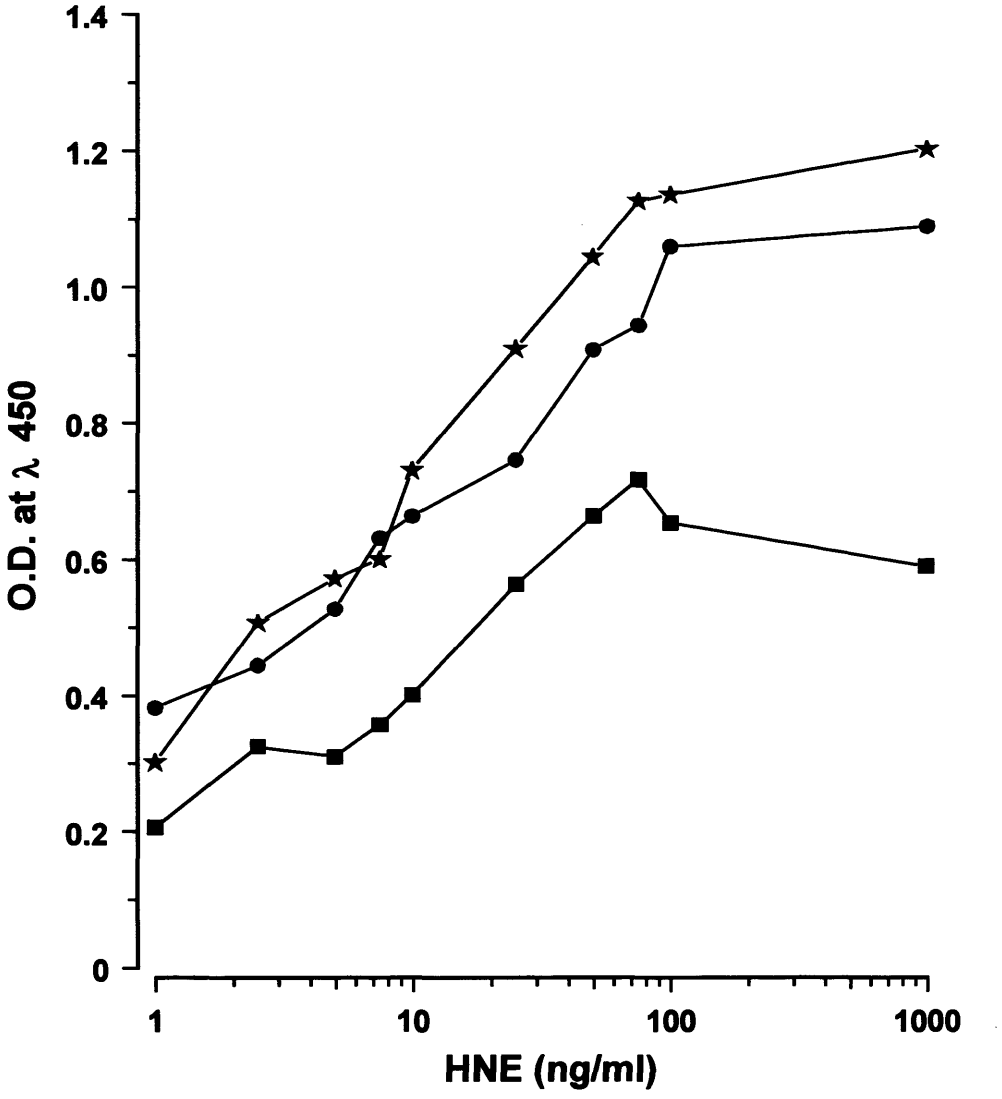
Washing was standardised to use wash buffer (PBS, 0.1% BSA, 0.1% tween 20) throughout.

hNEAPC standard

The standard hNE was combined with α_1-anti-trypsin (α_1-AT) at a molar ratio of 1:3 based on published values [Fujita et al 1992]. The MW of hNE is 29500 and MW of α_1-AT is 52000 therefore 5.2μg α_1-AT must be added to 1μg of hNE for the α_1-AT to be in (1:3) excess. The standard was made up in this way and aliquoted, prior to dilution in buffer for each assay. However this gave a lower maximal response than was obtained with plasma samples, or standard made up in buffer containing plasma (Figure 2.3). There is a possibility that this may have been due to dissociation of the complex during the sample incubation, although the binding of α_1-AT to hNE is reported to be nearly irreversible [Britigan 1999]. Since α_1-AT is inactivated by hNE there is the possibility of functionally inactive α_1-AT being present, and therefore not binding to the hNE. The typical bell shaped curve of the standard indicated a limiting step. This problem was resolved by including α_1-AT in the sample buffer at 5 μg/ml (Figure 2.3).

Figure 2.3

Measurement of hNEAPC in different buffer systems: Effect of additional α_1-antitrypsin



■ PBS, 0.1% BSA ● PBS, 10% plasma ★ PBS, 0.1% BSA, 5μg/ml α_1-AT

Changes in substrate

The peroxidase substrate of 5-amino salicylic acid used in the original assay was changed to 3,3',5,5'-Tetramethylbenzidine (TMB) for a more consistent colour development. The problem became apparent when curves were very poor, culminating in the blank O.D of 0.095, with a top standard O.D. of 0.144 after 60 min. colour incubation. A new batch of anti-α_1-AT antibody showed some improvement. Changing the substrate had a much greater effect (Table 2.3). This could have been due to either the substrate having degraded, the H_2O_2 having degraded, or a problem with the activated charcoal. The TMB was found to react much quicker, and colour development was not limited.

Conditions for Table 2.3 were using the second half of the hNEAPC ELISA (i.e. coating with α_1-AT and then detecting with the anti-α_1-AT antibody). Here the results for TMB were read at λ490, further tests showed that the optimal wavelength was λ450, which gave three times greater O.D. for equivalent colour (all Ref λ655).

Assay performance characteristics after modification

Intra- and inter-assay variation was 12 and 5.0% respectively. This is similar to the published values for the 'old' assay, which were 6.7 and 12 % for intra- and inter-assay variation [Norman *et al* 1991]. Serial dilutions of plasma were shown to give equivalent values when multiplied by the dilution factor down to a 1:10 dilution (Table 2.4). A 1:20 dilution was chosen, as both healthy subject and patient sample values fell on the linear part of the standard curve. A sample was tested after being thawed and frozen, with values of a 1:50 dilution being 135.5ng/ml in the sample frozen once, and 137.2ng/ml in the thawed and re-frozen one.

Table 2.3

Comparison of TMB and ASA substrate

α_1-AT coating concentration	TMB substrate 10 min colour development	ASA substrate 60 min colour development
Blank	0.137	0.099
1 μg/ml	0.219	0.106
5	0.202	0.108
10	0.290	0.139
50	0.405	0.208
100	0.821	0.815
500	0.963	1.254
1000	1.023	1.284

Table 2.4

Values for dilutions of samples showing linearity of values.

	S1	S2	S3	S4	S5	S6
1:5	19.3	47.9	61.9	25.5	32.3	55.6
1:10	25.1	72.5	80.9	35.9	39.7	83.8
1:20	24.3	100.6	114.3	37.4	50.5	106.7
1:40		112.6	108.4	39.9	48.8	110.7

Values are ng/ml hNE after multiplication by dilution factor.

Subsequent further improvements to this assay

Change of secondary antibody source from sheep to rabbit

Following the observation that some control figures were 10-100 fold higher that expected, it was concluded that some sera contained antibody to sheep immunoglobulins. Replacement of the sheep anti-human α_1-AT HRP conjugate with rabbit anti-human α_1-AT HRP conjugate eliminated these false positive results.

Change of detecting enzyme from peroxidase to alkaline phosphatase

The rabbit anti-human α_1-AT HRP became available as an alkaline phosphatase conjugate (AP). The substrate used with this enzyme was p-nitrophenol obtained from Sigma (104-0). An improvement both in the percent of change and rate of colour development was seen compared to the peroxidase conjugate. For example two assays carried out on the same day, with 30 min. substrate incubation, using the HRP conjugate gave an O.D. of 0.178 for blank, and 0.912 for 500ng/ml NE, whilst the AP conjugate gave an O.D. of 0.055 for the blank and 1.154 for 500ng/ml NE. When the value of the blank is expressed as a percentage, this gives 20% and 5% respectively. When equivalent AP and HRP antibodies have been tested in parallel, with p-nitrophenol and TMB substrate respectively, faster and greater percentage colour change is seen with the AP conjugate (data not shown). This is thought to be due to the AP enzyme being able to convert more substrate molecules in a set time than the HRP.

Table 2.5

Comparison of ELISAs to measure neutrophil elastase α_1-antitrypsin complex

	Original Assay	'New' modified Assay
Coating Antibody	Sheep anti human elastase 1:1000 in BCB, overnight 4°C	
Wash	x2 PBS	x2 PBS, 0.1%BSA, 0.1 %tween
Block	0.5% gelatine in PBS, 200µl/well, 2 hours room temp gently shaking	1% BSA in PBS, 200µl/well, 1 hour room temp gently shaking
Wash	x4 PBS, 1% gelatine, 0.1% tween	x2 PBS, 0.1% BSA, 0.1% tween
Standard / Sample	hNEAPC complex (PBS, 0.1% gelatine), 50µl/well, Standard curve 1 to 50 ng/ml, samples 1:20 dilution. 2 hours room temp gently shaking	hNEAPC complex (PBS, 0.1% BSA) 100µl/well, Standard curve 1 to 50 ng/ml, samples 1:20 dilution. 2 hours room temp gently shaking
Wash	x4 PBS, 1% gelatine, 0.1% tween	X4 PBS, 0.1% BSA, 0.1% tween
Detection Antibody	1:100 of goat anit-α_1-antitrypsin (biotinylated) in PBS 0.1% gelatine 1 hour room temp gently shaking.	1:2000 goat anti-human α_1-antitrypsin HRP conjugate OR 1:2000 Rabbit anti α_1-antitrypsin AP conjugate PBS, 0.1% BSA 2 hours room temp. gently shaking
Wash	X4 PBS, 0.1% gelatine, 0.1% tween	
Conjugate	1:1000 borate saline 50µl/well 1 hour room temp. gently shaking	
Wash	X4 PBS, 1% gelatine, 0.1% tween	X4 PBS, 0.1% BSA, 0.1% tween
Substrate	5-amino salicylic acid, 100µl/well 30 min. room temp. gently shaking Stop with 50µl 3M NaOH, Read λ455	TMB for the HRP conjugate OR Sigma 104 for the AP conjugate

2.1.3 Human Neutrophil Elastase (hNE) ELISA

An ELISA to measure free elastase un-complexed with α_1-antitrypsin was developed for two applications in this project. One was the measurement of hNE in sputum, where the majority is free, and the other for the measurement of hNE in the supernatants from neutrophil stimulation experiments. Although possible to measure hNE in such samples using the hNEAPC assay, the hNE is only detected when complexed to $\alpha_1$antitrypsin. Hence, this would have involved the addition of large amounts of α_1-AT to the assay samples, and it would then have been necessary to prove that all the elastase had been complexed.

Setting up the assay

The polyclonal goat anti-elastase used in the hNEAPC assay was used with a monoclonal mouse anti-hNE antibody (The supplier had changed the anti-hNE antibody from sheep to goat, but this makes no difference to the ELISA as the two animals are immunologically similar leading to equal cross reactivity i.e. anti-goat IgG will recognise antibodies raised in sheep or goat equally).

Determination of the optimal primary and secondary antibodies.

Initially a plate was coated half with the mouse anti-hNE, and half with the goat anti-hNE (both at 1:1000 dilution in BCB, 100µl/well) incubated overnight at 4°C. After two washes and blocking (200µl/well) (Appendix 2), and two further washes, standard hNE (1 to 100ng/ml) was added, and incubated for 1½ hours. After washing the 'other' anti-hNE antibody was added (goat where the coating was mouse, and mouse where the coating was goat) 1:1000 dilution in assay buffer, and incubated for 1½ hours. After washing the appropriate anti IgG-AP conjugate was added at 1:500 in assay buffer, and incubated for 30 min. After washing substrate (Sigma 104) was added and read at λ405 (ref λ490).

Initially it appeared that coating with goat was better, as this gave a greater colour change with the hNE standard, and this was not affected by the presence of α_1-AT in the sample buffer (Table 2.6). However, both the linearity of dilutions and the recovery of a spike from sputum sol was found to

be poor, and variable between experiments e.g. 0.5ng/ml hNE gave recovery figures of 0 to 80% in different sputum sol samples and experiments. The addition of Tween 20 at 1% to the sample buffer appeared to improve measurement of hNE in sputum sol particularly at lower dilutions (Table 2.7). Coating with the mouse anti-hNE was again looked at to determine whether improved measurements of hNE could be obtained in sputum. Recovery was considerably better, with a mean recovery of 29.36ng/ml (n=5) of a 25 ng/ml hNE spike (1:1000 dilution of sputum). Concentrations of the mouse anti-hNE and goat anti-hNE could both be reduced to 1:2000 without altering the sensitivity of colour development. The anti-goat IgG-AP conjugate was being used at 1:1000 dilution, and gave a colour change of 1.7 in 15 minutes. If this antibody concentration was reduced to 1:5000, there was still a colour change of 1.0 in 15 minutes, and the blank was lower. The change made no difference to measurements in samples.

Analysis of samples

Sputum-sol: Most samples had to be diluted considerably due to their high hNE content, and testing sputum-sol at 1:100, 1:1000 and 1:10000 ensured that one of the 3 dilutions used fell on the straight part of the standard curve.

Neutrophil incubation supernatant: No matrix effect from the HBSS buffer (method 2.2.8) (containing Cytochrome C) used for the neutrophil incubation studies occurred, and samples were tested at a 1:5 dilution for the elastase release to be determined in the absence of sputum sol, and at the same concentration as the sputum sol when this was present.

Comparison of values: The two assays were compared by running test sputum-sol samples in both the hNEAPC ELISA, which involved the addition of excess α_1-antitrypsin in the sample buffer, and the hNE ELISA (Figure 2.4). The values seen for the same sample in each of the assays is the same.

Table 2.6

The effect of the order of antibodies on detection of hNE by ELISA

Coating Ab	Mouse	Mouse	Goat	Goat
Secondary Ab	Goat	Goat	Mouse	Mouse
Standard	hNE	hNE + α_1-AT	hNE	hNE + α_1-AT
100	0.389	0.259	0.891	1.641
50	0.090	0.043	0.309	0.461
10	0.049	0.025	0.217	0.262
5	0.007	0.003	0.049	0.041
1	0.002	0	0.025	0.018

(O.D. background subtracted)

Table 2.7

Effect of Tween 20 on measurement of hNE in sputum sol.

Spu sol dilution	µg/ml hNE 0.1% tween	x dil factor	µg/ml hNE 0.5% tween	x dil factor
10	.185	1.85	0.386	3.86
20	.118	2.36	0.326	6.52
40	0.136	5.44	0.277	11.08
80	0.226	18.08	0.225	18.00
160	0.150	24.00	0.168	26.88
320	0.087	27.84	0.078	24.96
640	0.053	33.92	0.069	44.16

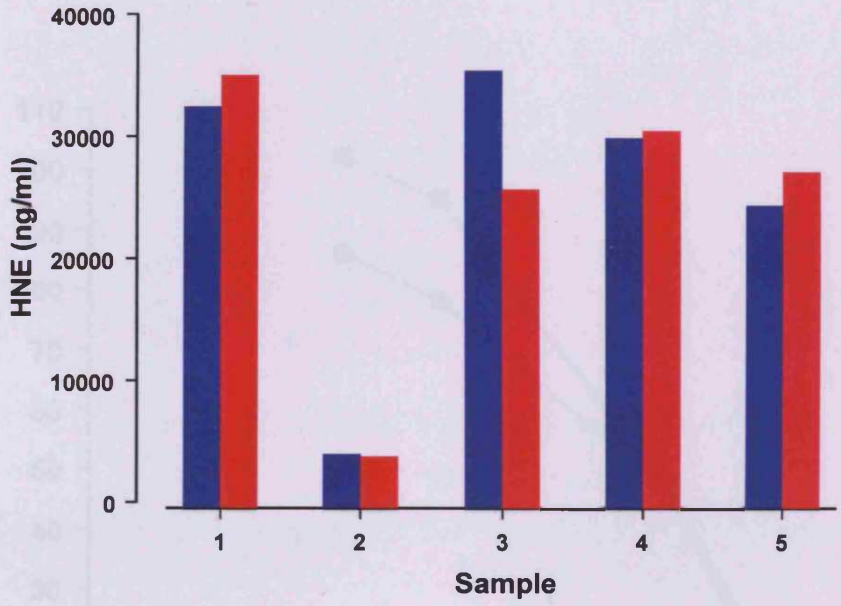
Goat anti-hNE as primary and mouse anti-hNE as secondary antibodies.

The effect of α_1AT on this assay was investigated, as samples may often contain this protein. α_1AT was found to have no effect on hNE measurements in this assay at concentrations below 1μg/ml. However, concentrations of α_1AT greater than 1mg/ml do affect the measurement of hNE by this ELISA (Figure 2.5). This would affect results where total hNE was quoted and there was a significant amount of α_1AT. The measured hNE was also affected by some hNE inhibitors.

We compared the activity of hNE, and the quantity of hNE determined by ELISA in patient samples. Activity of hNE was determined using the chromogenic substrate n-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide. There was a significant correlation between the two measures in patients with cystic fibrosis ($r=0.87$, $p<0.0001$) (Figure 2.6a) [Okeahialam 1995] and in infants with bronciolitis (Figure 2.6b) [Abu-Harb *et al* 1999].

Figure 2.4

Comparison of the two different elastase ELISAs; results (after allowing for dilution)



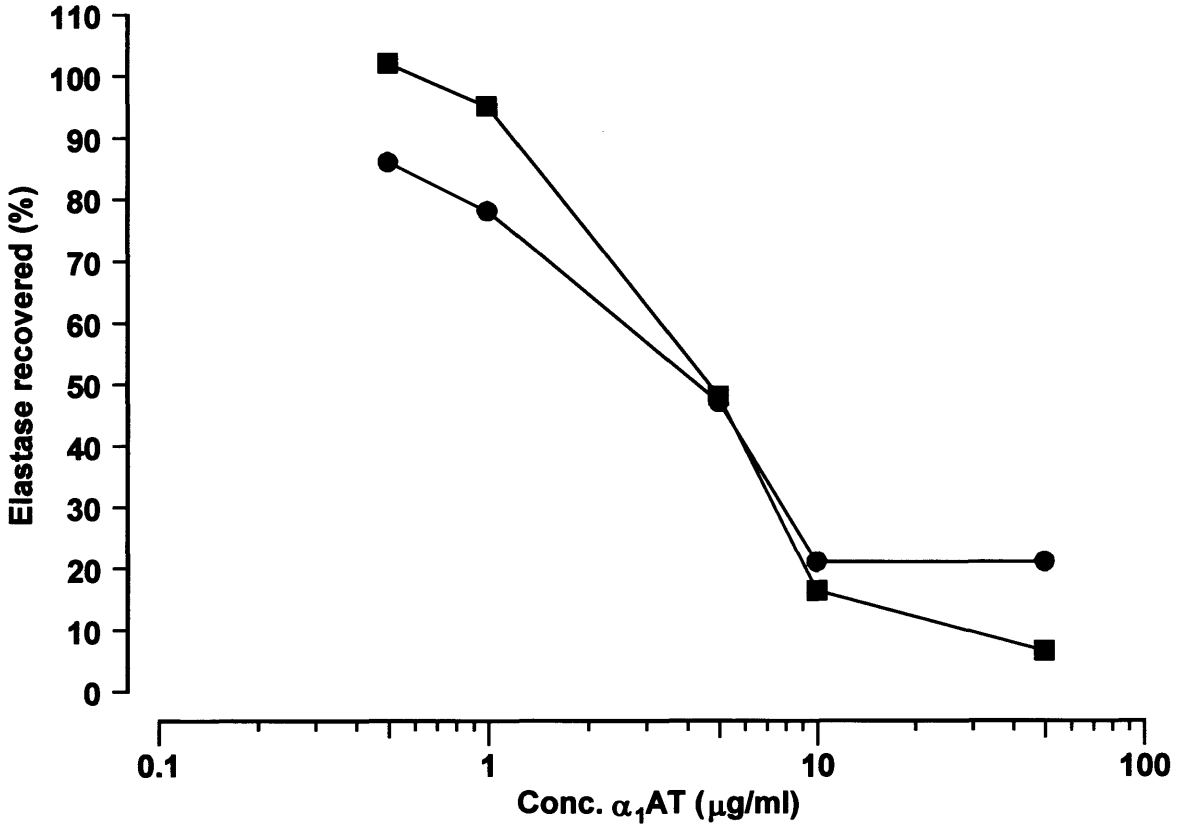
■ hNE content measure by hNEAPC ELISA,

■ hNE content measured by hNE ELISA

p=0.737 by paired t test

Figure 2.5

The effect of the presence of α_1 antitrypsin on elastase measured by ELISA

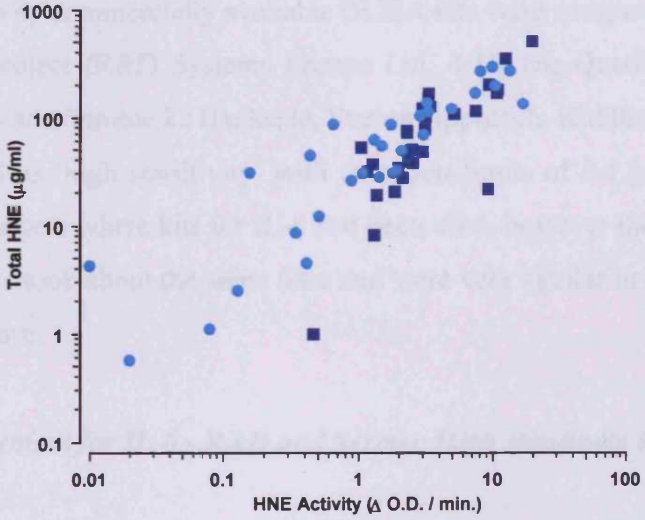


■ Elastase at 500 ng/ml

● Elastase at 50 ng/ml

Figure 2.6a

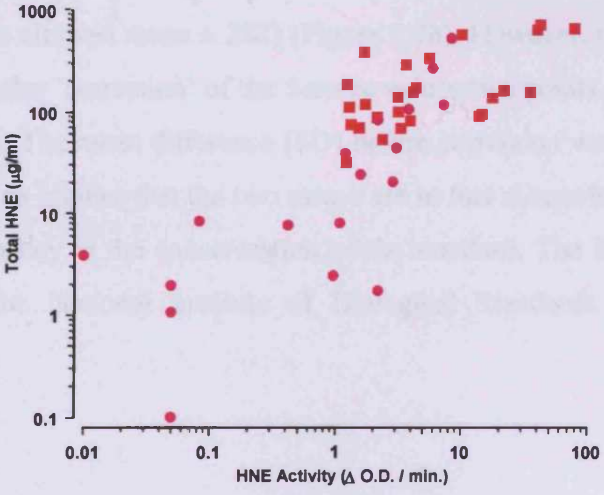
Comparison of hNE determined by chromogenic substrate and hNE determined by ELISA in sputum sol from patients with Cystic Fibrosis.



■ Patients with CF at a time of respiratory exacerbation ● Patients with CF after treatment

Figure 2.6b

Comparison of hNE determined by chromogenic substrate and hNE determined by ELISA in BALF from infants with bronchiolitis.



● Age matched healthy subjects ■ Infants with bronchiolitis

2.1.4 Comparison of IL-6 Cytokine Kits and validation to be used for sputum

IL-6

Two high sensitivity commercially available ELISA kits were compared to determine their potential for use in this project (R&D Systems Europe Ltd. 4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS and Serotec 22 Bankside, Station Approach, Kiddlington, Oxford OX5 1JE). Both kits were classed as 'high sensitivity' with detection limits of 0.1 pg/ml. The R&D kit had been quoted in many papers where kits for IL-6 had been used, however the Serotec assay was about half the cost. Both kits took about the same time and were very similar in the supply of reagents and the operating procedure.

Analysis of agreement for IL-6 - R&D and Serotec High sensitivity ELISA kits

A Spearman correlation of the results obtained from the two kits gave $r= 0.9256$ ($p<0.0001$) using a total of 48 samples (24 patients, 24 healthy subjects) (Figure 2.7a). Values for IL-6 tended to be lower for the Serotec kit than the R&D kit, and a linear regression indicated a slope of 0.3409. 'Correcting' the Serotec values by dividing them by 0.3409, gave the same values (Figure 2.7c). To determine whether the two assays were performing similarly the mean difference was calculated by the method of Bland & Altman [1986]. Plotting the mean value against the difference gave only two points outside the allowed mean ± 2SD (Figure 2.7b). However, the graph shows clearly that there is a difference. After 'correction' of the Serotec values two points still remained outside the mean ± SD (Figure 2.7d). The mean difference (SD) before correction was 3.52 (8.82) and after correction 0.0059 (3.25). This implies that the two assays are in fact measuring the same protein, but that there may be a discrepancy in the concentration of the standard. The R&D assay was validated using a standard from the National Institute of Biological Standards (South Mimms, Herts) by the manufacturers.

Figure 2.7a

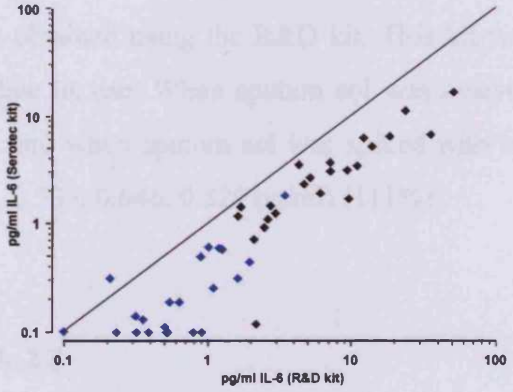


Figure 2.7b

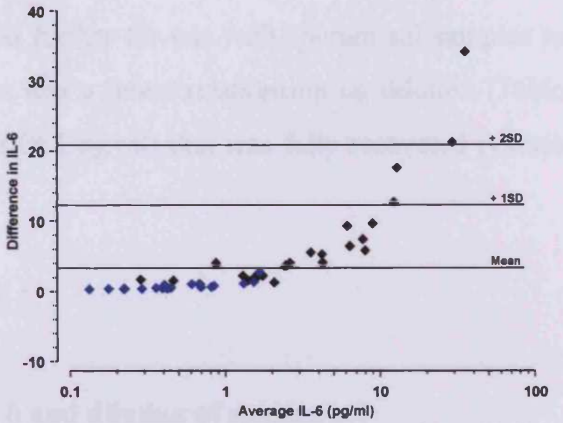


Figure 2.7c

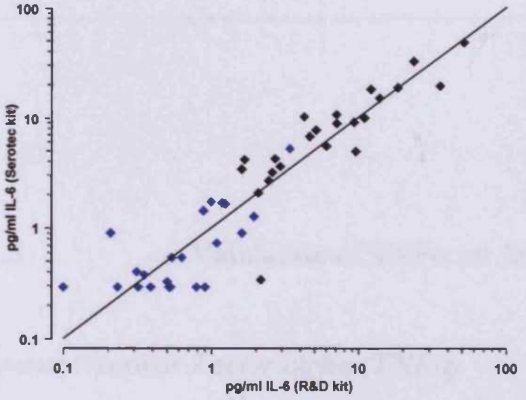
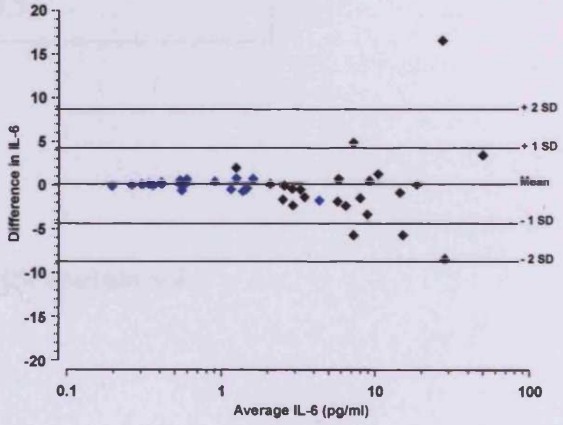


Figure 2.7d



◆ Patients with CF, ◆ Healthy subjects

Analysis of sputum sol

When sputum sol samples were assayed, no values were obtained for the Serotec assay, but values were obtained using the R&D kit. This kit was tested further for use with sputum sol samples to validate its use. When sputum sol was assayed there was a linear relationship on dilution (Table 2.8), and when sputum sol was spiked with standard (0.5 pg/ml) this was fully recovered (values 0.51, 0.535, 0.646, 0.529 pg/ml) (111%).

Table 2.8

Relationship between measured IL-6 and dilution of sputum sol

Spu sol	1:10 dilution	1:100 dilution
A	90.7	84.3
B	23.7	29.5
C	23.7	29.5

2.1.5 Validation of TNFα kit for use with sputum sol

Tumour Necrosis Factor alpha (TNFα)

On the basis of the results from the IL-6 assay, the R&D high sensitivity ELISA kit was purchased to measure TNFα. This assay was validated for use with sputum sol, and its use with plasma confirmed. When sputum-sol was spiked with standard (1.6pg/ml) this was fully recovered (values 2.128, 1.648, 1.692 pg/ml) (112%).

2.1.6 Additional In house cytokine ELISAs

ELISAs for sL-selectin and IL-8 were developed using antibodies and standards from R&D systems Europe and Autogen Bioclear. These were sandwich ELISAs which followed the basic layout of the hNE assay. Problems were experienced with the sL-selectin standard which could not be resolved even with advice from R&D. A 'control' serum sample was used in chapter 9 to compare subjects, but the results should be interpreted with caution.

Soluble L-selectin ELISA

A 96 well microtitre plate was coated with goat polyclonal anti-sL-selectin (N18)(AutogenBioclear) which recognized the amino terminus of sL-selectin at a 1:1000 dilution in bicarbonate coating buffer, 100µl/well and incubated overnight at 4°C. The plate was washed (x2) with PBS gelatine wash buffer (0.1% gelatine, 0.1% BSA), and the plate blocked with 1% gelatine in PBS for 1 hour at room temperature. The plate was washed (x2) before addition of standard and samples, 100µl/well. The plate was incubated overnight at 4°C, and all further incubations were carried out at this temperature. The wash buffer was also stored and used at 4°C. After sample incubation the plate was washed 4 times, before addition of monoclonal mouse anti-sL-selectin (R&D systems), 1:1000 dilution in gelatine wash buffer, 100µl/well. After 4 hours incubation, the plate was washed 4 times, before addition of anti-mouse IgG-Alkaline phosphatase conjugate (Sigma), 1:1000 dilution, 100µl/well. This was incubated for 1 hour, before again washing 4 times. Phosphatase substrate (Sigma 104 in 10% diethanolamine pH9.8) was added at room temperature, and the absorbance read after 90 min. A full description of the assay development has been reported [Ali 1999].

The recombinant standard obtained from R&D was found not to be recognised by the goat polyclonal antibody, and a plasma sample from a healthy subject was given an arbitrary concentration of 1.0. Serial dilutions of this 'standard' were used at a range of 1:5 to 1:320. Without alternative suppliers of either standard, or a polyclonal antibody (not mouse), it was not possible to fully quantify the results.

IL-8 ELISA

A 96 well microtitre plate was coated with mouse monoclonal anti-IL-8 (R&D Systems) at a 1:1000 dilution in bicarbonate coating buffer, 100µl/well and incubated overnight at 4°C. The plate was washed (x2) with wash buffer (PBS, 0.1% BSA, 0.1% Tween 20), and the plate blocked with 1% BSA in PBS for 1 hour at room temperature. The plate was washed (x2) before addition of standard (0.5 to 1000 pg/ml (R&D Systems)) and samples (1:5 dilution) in sample buffer (PBS, 0.1% BSA, 1% Tween 20, 20mM EDTA), 100µl/well. After sample incubation for 2 hours gently shaking at room temperature, the plate was washed 4 times, before addition of polyclonal goat anti-human IL-8 (R&D Systems), 1:2000 dilution in wash buffer, 100µl/well. After 1 1/2 hours incubation, gently shaking at room temperature, the plate was washed 4 times, before addition of anti-goat IgG-Alkaline phosphatase conjugate (Sigma), 1:5000 dilution, 100µl/well. This was incubated for 30 min., before again washing 4 times. Phosphatase substrate 100µl/well (Sigma 104 in 10% diethanolamine pH9.8) was added at room temperature, and the absorbance read after 60 min.

2.1.7 Development of assay to measure protein in sputum-sol

Total protein was measured in sputum-sol, as unrefined sputum is a very heterogeneous mixture of host and bacterial products in CF. Contamination by saliva, particularly where smaller volumes are produced, may have effects through dilution. Total protein was used as a denominator to reduce the effect of this variation in the inflammatory response. BioRad and Sigma P5656 protein assays were compared. The Sigma assay used Peterson's modification of a micro Lowry assay, with Bovine Serum Albumin (Fraction V) as standard. Inclusion of sodium dodecyl sulfate (SDS) in the method with solubilisation allowed recovery of membrane proteins to be included in the results. The protocol gave an optional protein precipitation step, but this was found not to be beneficial and was not used. The BioRad assay was based on a colour change of coomassie brilliant blue G250 dye in response to various concentrations of protein. The standard for the assay was γ globulin. A number of test sputum samples, and both γ globulin and albumin standards were run. The sigma assay was seen to be more effective at determining BSA, γ globulin and gelatine (Figure 2.8a) than the BioRad assay (Figure 2.8b), and was chosen as being the most representative of total protein. Both assays correlated well with absorbance at λ280; Sigma $r=0.720$ ($p=0.006$), BioRad $r=0.780$ ($p=0.002$), and with each other $r=0.940$ ($p<0.001$) Table 2.9. Separate 'aliquots' of sputum from the same sample, processed separately were found to give consistent results for protein (Table 2.10). The coefficient of variation was only 11 % for the Sigma assay for the 5 aliquots tested.

Figure 2.8a

Protein standard curves in the Sigma P5656 assay

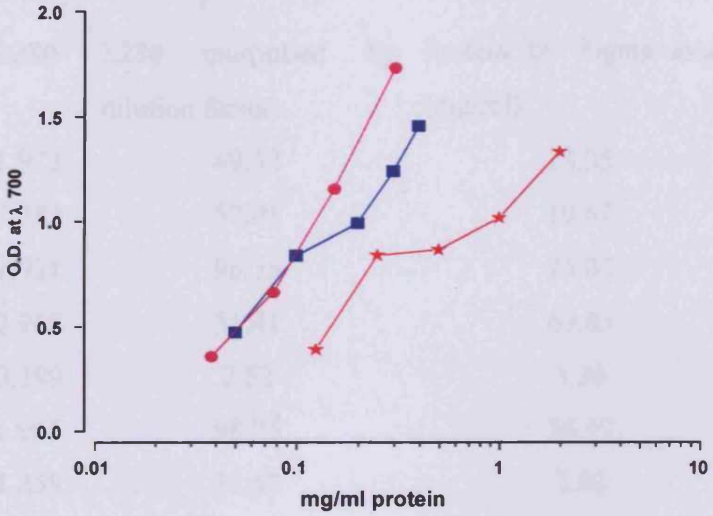
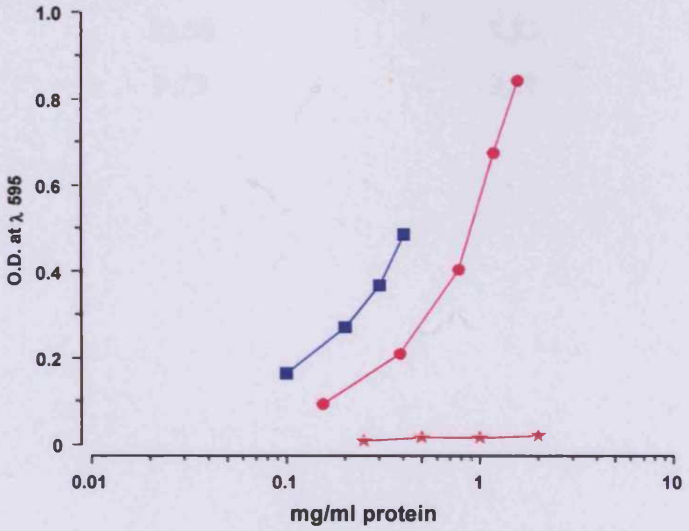


Figure 2.8b

Protein standard curves in the BioRad coomassie blue assay



BSA Fraction V γ Globulin Gelatine

Table 2.9

Protein measurements in sputum sol samples

Sample	λ280	λ280 multiplied by dilution factor	Protein by Sigma assay (mg/ml)	Protein by BioRad assay (mg/ml)
1	1.973	49.33	13.35	16.85
2	2.282	57.05	19.67	18.23
3	1.931	96.55	25.83	39.84
4	2.960	31.41	69.65	45.03
5	0.399	2.52	3.36	2.17
6	1.965	98.25	36.17	25.70
7	1.259	31.47	7.50	13.22
8	0.626	15.65	3.60	2.21
9	0.991	24.78	7.70	9.00
10	2.538	63.45	97.75	33.22
11	0.500	12.50	5.33	4.06
12	0.389	9.73	3.71	2.63

Table 2.10

Sputum sample split prior to processing to show variation

Sample	λ280	Protein by Sigma assay (mg/ml)	Protein by BioRad assay (mg/ml)
A	0.122	15.47	12.76
B	0.224	14.29	10.61
C	0.102	12.11	12.88
D	0.097	13.20	13.65
E	0.164	11.99	9.22
Mean	0.1398	13.41	11.82
SD	0.054	1.48	1.84
%CV	38	11	15

2.1.8 Development of Phagocytosis and Intracellular killing assays

These assays were based on a published method by Marodi *et al* [1983] where the two parts of bacterial clearance by neutrophils are measured separately. Marodi used *Staphylococcus aureus* for quantification of phagocytosis and killing. *P. aeruginosa* was chosen for this study because it is the major infecting organism in the group of patients with CF who were studied. The original assay was changed to allow for the different nature of this bacterium.

Definitions

Phagocytosis was determined as the number of *P.aeruginosa* removed from the incubation suspension by the neutrophils, and expressed as the percentage reduction in colony forming units (cfu).
Intracellular killing was determined as the number of *P.aeruginosa*, which had already been phagocytosed, which were killed, i.e. no longer able to form a colony.

Quantification and storage of P.aeruginosa

An effective means of quantification of *P. aeruginosa* was developed, to ensure that a known number of *P.aeruginosa* were added to each incubation mixture. A concentration of $5x10^7$ cfu/ml was found to be ideal for storage and use in the phagocytosis and intracellular killing experiments (Table 2.11). *P. aeruginosa* gave small round colonies of about 2-5mm in diameter after 24 hours incubation on nutrient agar at 37°C. The colour of *P. aeruginosa* colonies varied from yellow/brown to green. The colonies were visible by eye and counting them was made easier by placing the agar plate on a dark background. The optimal volume for spreading on agar plates was determined to be 100µl. Smaller volumes did not spread evenly over the plate, and with larger volumes colonies tended to 'run' into one another making counting difficult and inaccurate. The counts from each plate could be used to calculate the number of cfu in the original suspension, by calculation to allow for the dilution factors. *P.aeruginosa* could be stored at 4°C in HBSS or distilled water, with the colony number remaining constant for periods of greater than one month.

Table 2.11

The effect of storage of *P.aeruginosa*

Day	0.00	1.00	4.00	8.00	16.00	28.00	46.00
cfu ($x10^7$)	3.10	2.90	3.70	4.50	4.10	5.00	5.40

Development of Phagocytosis assay

P.aeruginosa and neutrophils were mixed together, incubated to allow phagocytosis to occur, and then the neutrophils were separated from the *P.aeruginosa.* The un-phagocytosed *P. aeruginosa* was determined by dilution then plating of the assay mixture onto agar and recording the colony forming units (cfu) after incubation at 37°C for 24 hours.

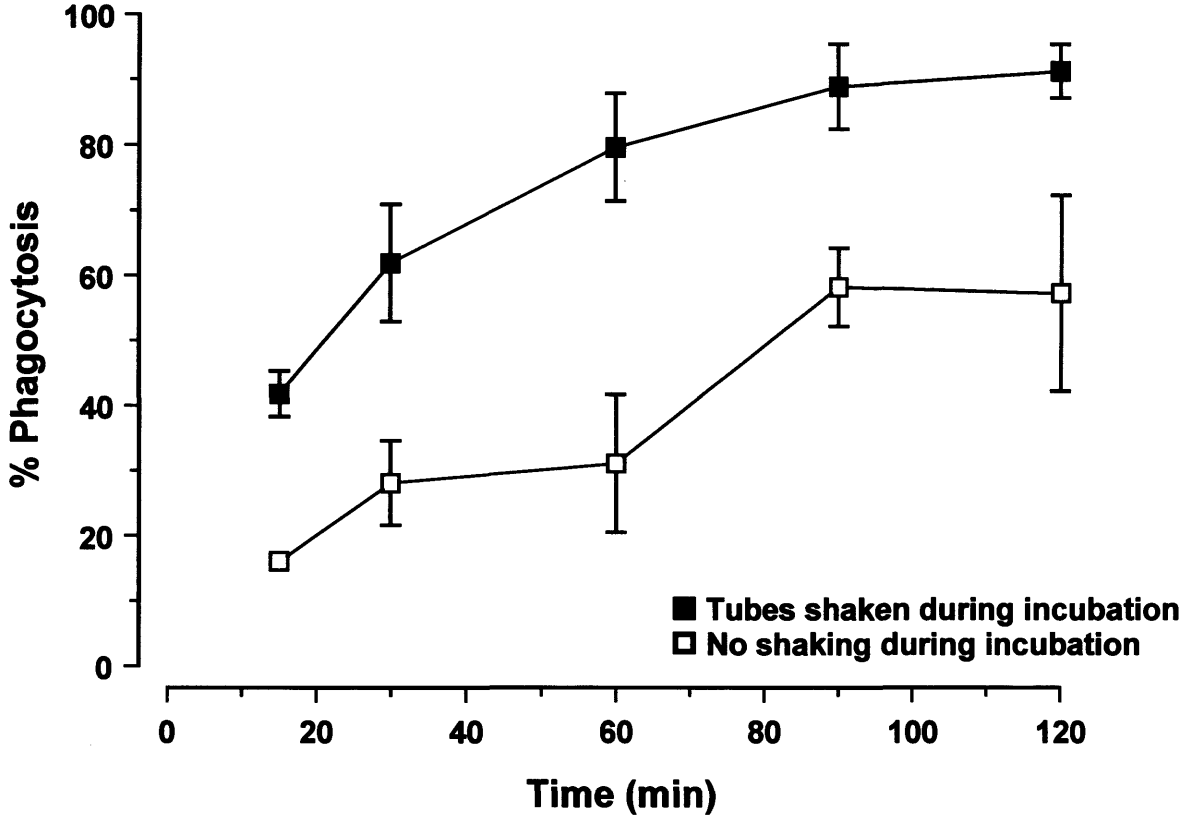
Neutrophils are much larger than *P.aeruginosa* cells, so separation by centrifugation was investigated. Centrifugation at 400g for 6 min reduced a suspension of *P.aeruginosa* from 3.9 x 10^7 cfu/ml to 0.7 x 10^7 cfu/ml, while centrifugation at 100g for 6 min. only reduced the count to 3.5 x 10^7 cfu/ml. It was concluded that centrifugation at 100g would leave the *P. aeruginosa* in suspension whilst removing the neutrophils.

For phagocytosis to occur the *P. aeruginosa* must first be opsonized. *P. aeruginosa* was incubated with autologous serum to prevent any donor cross-reaction.

In the initial experiments incubation was carried out under static conditions, but it was noticed that the cells settled during this period. This was prevented by gentle shaking, sufficient to keep the cells in suspension. Shaking increased phagocytosis compared to static conditions at all time points (Figure 2.9). An incubation time of 60 min. was selected to give a sub maximal amount of phagocytosis, and allow either an increase or decrease of phagocytosis to be determined.

Figure 2.9

The effect of shaking on the rate of phagocytosis of *P.aeruginosa* by neutrophils



The effect of different ratios of *P.aeruginosa* to neutrophils was also addressed. Where there were below 10 *P.aeruginosa* cells per neutrophil similar amounts of phagocytosis occurred. For ratios greater than this the % *P.aeruginosa* phagocytosed for a given time was reduced. It was decided to use a ratio of 5:1 *P.aeruginosa* to neutrophils, to allow for some experimental variation without affecting phagocytosis rate.

Development of Intracellular Killing assay

Initial experiments were carried out to determine the time course, and experimental conditions specific for *P.aeruginosa*.

A number of issues had to be addressed.

i) The removal of non-phagocytosed bacteria prior to the incubation to determine intracellular killing.

ii) A time to give sub maximal intracellular killing, for the effect of sputum to be investigated, as it was possible that it could increase or decrease the killing.

Development

Once the neutrophil has phagocytosed the bacterium, it forms an intra-cytoplasmic phagolysosome where killing occurs. It is necessary to determine the optimum time for phagocytosis, but with minimal killing, so that after separation of non-phagocytosed *P.aeruginosa* the killing is determined in a separate incubation. Also, it was not known how the presence of more than one phagolysosome within the neutrophil would affect the rate of killing.

Effect of serum

In the absence of serum, no *P.aeruginosa* were killed by neutrophils, however in the presence of serum with otherwise identical conditions, killing occurred (Table 2.12).

Table 2.12

The effect of the presence of serum on phagocytosis

		Before incubation	After 1 hour	After 2 hours
Neutrophils	without serum	100 100	116 124	108 139
Absence of neutrophil	without serum	100	124	139
Neutrophils	with serum	100	63	18

A killing effectiveness of 100% would be expected in principle, and therefore the value of 18 in Table 2.12 should be 0. As a result, 3 steps were included into the assay. The *P.aeruginosa* was opsonized as in the phagocytosis method. The opsonized *P.aeruginosa* was mixed with the granulocytes for 10 min at 37°C to allow for phagocytosis of the *P.aeruginosa*. The neutrophils were washed to remove un-phagocytosed *P.aeruginosa*.

A knowledge of how easily *P.aeruginosa* are sedimented by centrifugation came from the development of the phagocytosis assay. The neutrophil/*P.aeruginosa* suspension was mixed with cold HBSS (3x volume), and centrifuged at 200g for 4 min. The supernatant was discarded and the cell pellet resuspended in HBSS. The mixture was again centrifuged, and the pellet resuspended, this time in HBSS 10% autologous serum. The wash step 'improved' the killing by about two fold, indicating that free *P.aeruginosa* had been present. Serum was added to the final incubation mixture after it was noted during the first experiment, that intracellular killing plateaued at about 80%. This effect was not subsequently seen. To check for free *P.aeruginosa*, a sample of the final neutrophil suspension with phagocytosed *P.aeruginosa*, HBSS and serum was plated onto nutrient agar to give the following results:

e.g. 100µl gave 57cfu (=570 cfu/ml).

From time 0 killing determination 200µl mixed with 10ml H_2O, then 100µl onto agar gave 111cfu. This represents [111 x 10 (per ml) x10 (in 10ml) /2 (per ml)] 5550 cfu in an equivalent amount to that taken from the cell suspension.

57 free cfu in a total of 5550 remain = 57/(5550-57)

= 1%

(iii) A determination of the time course of killing was carried out. (Table 2.13). It was decided to use an incubation time of 30 min. as this was sub maximal and hence would show changes in both increase and decrease of intracellular killing.

Table 2.13

Time course of intracellular killing - Results

Time (min.)	10.00	20.00	30.00	60.00	90.00	120.00
Mean (%)	55.50	65.50	65.30	71.30	70.00	71.50
S.E.M.	7.50	7.60	8.90	5.40	3.00	7.50
n=	2.00	2.00	4.00	5.00	3.00	4.00

Addition of Sputum

The effect of sputum on neutrophils had to be determined. Isolated neutrophils were mixed with varying amounts of sputum sol at different concentrations (Range 1:4 to 1:1000 dilutions of sputum-sol). Incubated for 30 min. at 37°C. Trypan blue was added, and the mixture incubated for a further 10 min. at 37°C. The cells were washed, and assessed for exclusion of trypan blue. The lowest viability was 98%, However, there were less cells visible in some of the 'sputum containing' preparations. Clumps of cells were noted. This did not appear to be related to the concentration of the sputum.

Preliminary studies for the effect of sputum sol on phagocytosis and intracellular killing are shown in Table 2.14

Table 2.14

Preliminary results showing the effect of sputum on phagocytosis and intracellular killing

Phagocytosis

Incubation time (min.)		Sputum Concentration							
	Pre-incubation	0		1:100		1:50 or 1:40		1:10 or 1:8		1:4	
		Cfu	%	Cfu	%	Cfu	%	Cfu	%	Cfu	%
90	238 0	12.5	95	54 33	77 86						
90	314 0	24	92	52 38	83 88						
60	267 0	26	90	49	82	55	79	37	86		
60	522 0	386	74			159	68	201	60	259	33
Mean % reduction in phagocytosis by sputum				9.4		8.5		9.0		41	

Killing

Incubation time (min.)		Sputum Concentration							
	Control (time 0)	0		1:100		1:50 or 1:40		1:10 or 1:8		1:2	
		Cfu	%	Cfu	%	Cfu	%	Cfu	%	Cfu	%
30	501	56	89	60	88	124	75	138	72		
30	340	41	88	30	91	53	84	58	82		
30	467	43	91			46	90	81	83	94	80
Mean % killed			89		90		83		79		80
Mean % effect of Sputum				-1		6		10		9	

Black values represent colonies counted, blue values represent the % bacteria phagocytosed / killed.

2.1.9 Development of Flow Cytometry method

Fluorescent Activated Cell Sorting (FACS) is used to determine the fluorescence of cells through a sorter. In simple terms cells pass through a flow cell one at a time, and light is passed across this flow. The forward and side scatter of the cells is measured, and this can be used to determine the type of cell. This technique forms the basis of most haematological counters. In addition, FACS will use light beams of a known wavelength, and when this beam is passed across a fluorescent dye, the dye will emit light at a different frequency, and this is detected by the flow cytometer.

In flow cytometry, cells are labelled with antibodies bound to fluorescent dyes. When cells labelled with these dyes are passed through the FACS, the forward scatter, side scatter, and fluorescence for the specified wavelength is measured for each individual cell. This can be done for several thousand cells per minute. Software is then used to determine the mean fluorescence for each cell type. Dual staining allows the binding of two different antibodies (each labelled with a different dye) to be assessed simultaneously. Flow cytometry has been used extensively in the typing of lymphocytes, and is commonly used in diagnosis of various diseases including leukaemia and AIDS. There are fewer studies using neutrophils, however the literature is growing at a rapid rate in this area.

Methods recommended by the manufactures of the various reagents and antibodies were compared with methods published in the literature [Roos *et al* 1993, Elbim *et al* 1994, Prussin & Metcalfe 1995, Plusa *et al* 1996, Rapport *et al* 1996] and advice from Dr. Terry Hoy (Haematology, UWCM). Some recommended using whole blood, and others washed blood, and some cell suspensions. All red cells had to by lysed, or interference would be seen on the FACS. FACS brand Lysing Solution (Becton Dickinson, 2350 Qume Drive, San Jose, CA, USA) was used as it is best for preserving forward and side scatter patterns (Dr Terry Hoy, advice).

Comparison of red cell lysis on whole and washed blood.

Existing methods seem to use whole blood, diluted blood and 'washed' blood. We compared whole blood, and dilutions of blood in buffer (PBS, 0.5% BSA)(1:2 to 1:40). The forward/side scatter plot showed a lot of 'debris' i.e. counts in areas which were not representative of cells. This debris was not seen with the diluted blood. The signal from antibody binding was better in all cases except with the antiTNFR. Red cell lysis was more effective before the blood was washed, and the 'debris' seen with washed blood may be a reflection of this. Secondary antibodies were used for TNF R and IL-6 R, as they had no fluorescent conjugate. We therefore carried out the lysis step after the first incubation, and before adding the anti IgG FITC conjugate. This resulted in a marked increase in mean fluorescence, and many more cells available for analysis.

FACS lysing solution (Becton Dickinson, 2350 Qume Drive, San Jose CA, USA) was used which was supplied as 10x concentrate, which was diluted in sterile water prior to use. This reagent contains formaldehyde and diethylene glycol in a mixture designed to lyse erythrocytes whilst leaving leucocytes intact. Debris from haemolysis does not interfere with white cell enumeration when using the recommended protocol. In addition to lysis of the red blood cells there is partial fixation of the white cells. White cell recovery, as quoted by the manufacturer, was 92%, and no red cells were present after lysis.

Antibody details

Antibodies were either conjugated to Fluorescein Isothiocyanate (FITC) a green colour, or R-phycoererythrin (PE), a red colour. The FACScan was able to detect both colours at the same time, and therefore neutrophils could be dual stained with antibodies with different conjugates. Details of reagent and antibody suppliers are given in Appendix 1a.

The L-selectin antibody was a mouse monoclonal IgG1 (clone 4GB), and the CD11b antibody monoclonal mouse (Clone 2LPM19c). Their different conjugation meant that neutrophils were stained for both receptor types. The CD11b antibody labelled peripheral blood granulocytes, approximately 80% of monocytes, and a subpopulation of "null cell" peripheral lymphocytes (CD2-positive, CD3-negative) containing most of the circulating natural killer cells, as well as macrophages in many tissues. The antibody to TNF receptor was a monoclonal to p75 (clone MR2-

1), reacting with the extra-cellular part of the TNF-receptor p75. Both the IL6R and TNFR were unlabelled, and therefore a secondary anti mouse IgG FITC conjugate was used to detect their presence on the cell surface.

Determination of optimal antibody concentration

Threshold levels of antibody were obtained in order to ensure that all cells were saturated with antibody, but not excess antibody, as this was both costly and increased the likelihood of non-specific binding. The neutrophils/μl antibody was calculated for comparison between blood samples and dilutions (Table 2.15). From this data we determined the volume of antibody to be used based on an assumed 10^6 neutrophil/ml (considered to be the maximum likely to occur based on previous studies). The unstimulated CD11b data appeared initially that it might be limiting, however, such a large increase was seen upon stimulation with fMLP that this was obviously not the case.

Table 2.15

Determination of antibody threshold concentration for FACS using anti-Lselectin as an example

Antibody	neutrophils/μl antibody	Fluorescence	Threshold ratio
L-selectin FITC	300000	9882	150000
(R&D Systems Europe)	150000	17522	
	150000	20123	
	3750	17778	
	1500	16820	
	375	14324	

Effect of storage

Leaving the blood on the bench for greater than 1 hour before processing resulted in a scatter pattern which was not as clear, fluorescence peaks were not as sharp, and receptor numbers for CD11b and L-selectin were altered. It was therefore decided that blood should be processed within 30 min. of being taken.

Due to limited availability of the FACScan, analysis could not be carried out immediately after the experiment. We determined the effect of storage at 4°C (in the dark) on the fluorescence. It had been shown that there was no loss of fluorescence with this method used during the first week, but a slow reduction in fluorescence intensity was seen with longer periods. FACS analysis was therefore always carried out within one week of staining.

Effect of stimulation with fMLP

FMLP stimulation caused a reduction in mean fluorescence for L-selectin and an increase in mean fluorescence for CD11b, indicating a change in receptor number. Initial results indicated that there was a modest reduction in intracellular MPO after stimulation with fMLP, however later experiments were not able to verify this. It is apparent that only a small percentage of the MPO contained in the cells is released upon stimulation.

Intracellular determination

The 'Fix and Perm' cell permeabilization kit was obtained from Caltag laboratories. Cells are fixed in suspension with reagent A and then permeabilized with reagent B. This procedure gives antibodies access to intracellular structures and leaves the morphological scatter characteristics of the cells intact. No fluorescence was obtained with anti MPO FITC when the cells were not treated with 'Fix and Perm' prior to staining.

To control for inter assay variation

DAKO FluoroSpheres are calibration beads for daily monitoring of the flow cytometer. The beads have a diameter of 3.2μm, and constitute a mixture of 5 bead populations having different fluorescence intensities, and one non-fluorescent bead population. Entrapped in the fluorescent beads is a combination of flurochromes that enables the beads to be excited by light of any

wavelength from 365 to 650nm. With assigned Molecules of Equivalent Fluorochrome (MEF) values for the fluorescent bead populations, their use enables the transformation of arbitrary units of mean fluorescence intensity into absolute units for quality assurance and reporting of flow cytometry data.

Cellular micro spheres are synthetic micro spheres of uniform size with goat anti-mouse IgG covalently bound to the surface of the beads. They can be treated as cell surrogates and stained with conjugated monoclonal antibodies.

2.2.1 Treatment of samples

Plasma and serum

Venous blood collected into EDTA tubes was centrifuged at 2000g, 4°C for 5 min., within 15 min., of collection. The plasma was aliquoted and stored at -70°C. For serum venous blood was allowed to coagulate at room temperature for 60 min. before centrifugation at 2000g for 10 min. and was then aliquoted and frozen at -70°C.

Sputum

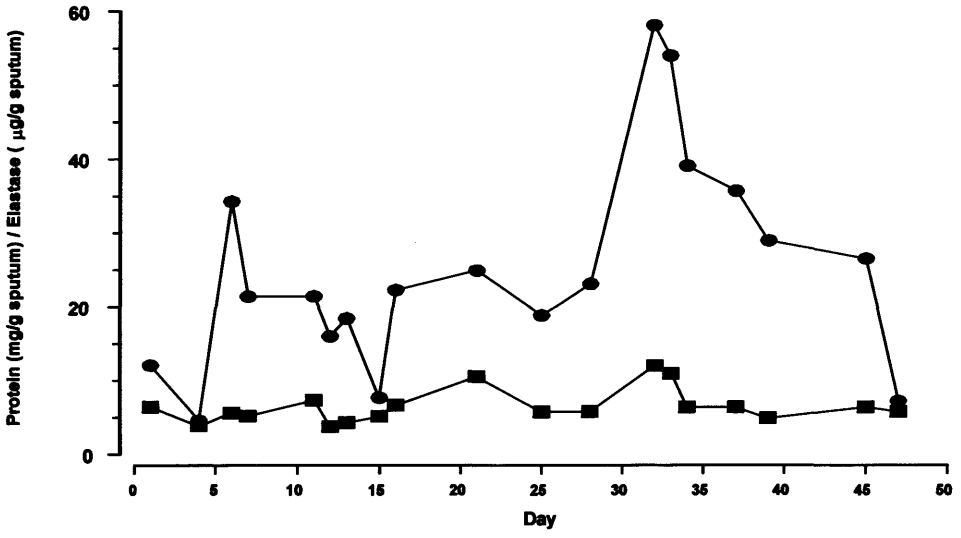
Sputum was collected from the patient and processed within 30 min. Processing involved weighing the sputum. For samples that were large enough (greater that 5g) the sputum was split for 1:2 and 1:5 dilutions, for smaller samples only a 1:5 dilution was carried out (dilutions were weight to weight in light of the viscous nature of sputum). Sputum for the 1:2 dilution was mixed with an equal weight of saline. Sputum for the 1:5 dilution was mixed with 4 times weight of saline. These were mixed for 30 min on a rotary mixer, when an even consistency was observed and then centrifuged at 10000g for 30 min. at 4°C. The upper clear sol layer was removed and divided into the amount needed for the functional experiments, and the remainder aliquoted and stored at -70°C for subsequent assay of inflammatory markers. The supernatant (sputum-sol) from the 1:5 dilution was used to obtain the other dilutions (1:10, 1:20 and 1:40).

Sequential sputum samples were collected from two patients and show the consistency of measurements in sputum sol (Figure 2.10). The large peak for patient B coincides with a pulmonary exacerbation.

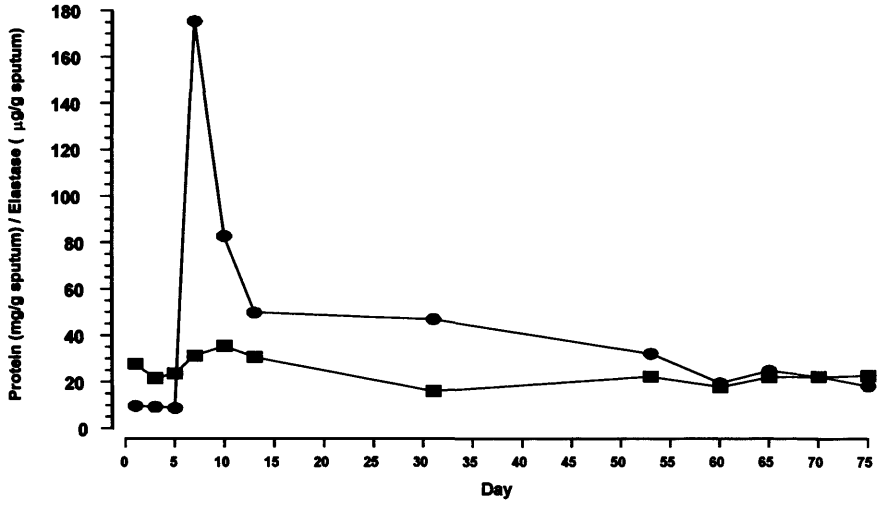
Figure 2.10

Protein and hNE content of sequential sputum samples from two patients with cystic fibrosis

Patient A



Patient B



Key

● Elastase (mg/g sputum)

■ Protein (mg/g sputum)

Patient B had treatment for a pulmonary exacerbation between day 7 and 21.

2.2.2 Protein Determination in Sputum sol

Protein was determined using Sigma protein assay kit P5656 following the standard protocol (Appendix 2). Briefly, the samples, diluted 1:20 in distilled water, and standard (BSA fraction V) 50 to 400 μg/ml were mixed with an equal volume of Lowry reagent and left to stand at room temperature for 20 min. Folin and Ciocalteu's phenol reagent was then added to all tubes and mixed well. After allowing the colour to develop for 30 min. the absorbance was measured at λ700. The protein concentration of the samples was determined from the calibration curve.

2.2.3 C-Reactive Protein ELISA

Serum levels of C-Reactive Protein (CRP) were measured using a double sandwich ELISA. This assay for CRP was adapted by Miss J. Richards, from a previously published method [Rayner 1991].

Goat anti-human CRP (Dako) was coated onto 96-well microtitre plates by incubation (100μl per well of a 1:1000 dilution BCB) overnight at 4°C. Plates were washed twice with wash buffer, blocked with blocking buffer 200μl/ml for 1 hour, and then washed twice more. Standard or sample (50μl/well) diluted in wash buffer was added and incubated for 30 min. The standard curve ranged from 0.45 to 1000ng/ml which gave a sigmoid log-logit curve. Serum samples were assayed at 1:100 1:1000 and 1:3000 dilutions. After four washes rabbit anti-human CRP (Dako) was added at 1:2000 dilution in wash buffer (50μl/well) and the plate incubated for a further 30 min. The plate was washed four times before the addition of anti-rabbit IgG (Sigma), 1:5000 dilution in wash buffer (50μl/well), and incubated for 30 min. The plate was washed four times, this time with a 20 second soak at each wash. Substrate (p-nitrophenol phosphate) was added (100μl/well) and colour allowed to develop for 30 min. or until the highest concentration standard reached O.D. of 1.0. Plates were read at λ405 Ref. λ490.

Intra-assay coefficient of variation (CV) for low and high concentration control sample was 6.4 and 20 % respectively, and the inter-assay variation for the same samples was 12.5 and 8.6%. The CV for the high sample was probably affected by the dilution of the sample 1:5000

2.2.4 Human Neutrophil Elastase – $\alpha_1$antitrypsin complex ELISA

Sheep anti-human neutrophil elastase was bound to 96-well microtitre plates by incubation (100µl per well) of a 1:1000 dilution in BCB buffer overnight at 4°C. The plate was washed twice using wash buffer, blocked for one hour with blocking buffer, before being washed twice again. Standard, human neutrophil elastase-α_1-antitrypsin complex (hNEAPC), and samples were diluted using sample buffer containing 1µg/ml α_1-antitrypsin (100µl/well). The standard range was 0.1 to 100 ng/ml, and samples were assayed at 1:20 dilution, and in triplicate. After 2 hours incubation the plate was washed four times. HRP labelled sheep anti-human α_1-antitrypsin 1:2000 dilution in sample buffer was added (200µl/well) and incubated for 2 hours. The plate was washed four times with a 20 second soak with each wash, and substrate (TMB) 200µl per well was added. The reaction was allowed to proceed at room temperature for 20 min. before being stopped with 1M H_2SO_4 50µl/well and read at λ450nm (Reference λ655nm). This method applies to data described in chapter 3. A rabbit anti a1-antitrypsin AP conjugate with Sigma 104 as the substrate was used for data described in chapters 7 and 8.

2.2.5 Human Neutrophil Elastase ELISA

Mouse anti-human neutrophil elastase was coated onto 96-well microtitre plates (1:1000 dilution in BCB) incubated at 4°C overnight. Plates were washed twice with wash buffer blocked with blocking buffer 200µl/ml for 1 hour, and again washed twice. Samples and standards were diluted in wash buffer and added 100µl/well. Before incubation for 90 min. Standards were in the range 2 to 1000ng/ml. Supernatants from neutrophil incubation studies were assayed at 1:5, 1:25, 1:125 dilutions in study A and 1:5 in later studies where there had not been addition of sputum-sol to the neutrophil incubation. Sputum sol was diluted 1:100, 1:400, 1:1600, and possibly greater for sputum-sol with very high levels of elastase.

Plates were washed four times, and goat anti-human neutrophil elastase 1:2000 dilution in wash buffer was added (100µl/well). Plates were incubated for 90 min. then washed four times. Anti-goat IgG-AP conjugate was added 1:5000 dilution in wash buffer (100µl/well) and incubated for 30 min. After washing four times with a 20 second soak with each wash substrate (p-nitrophenyl phosphate) was added (100µl/well). The plate was read at λ405 (Reference λ490) after 30 min.

2.2.6 Cytokine Kits

Interleukin-6 (IL-6)

IL-6 was assayed using the high sensitivity ELISA kit from R&D according to the protocol supplied with the kit (Appendix 3a) Briefly, a monoclonal antibody specific for IL-6 coated onto an ELISA plate was provided. Samples or standard was added. The standard curved ranged from 0.156 pg/ml to 10 pg/ml in serum diluent. After incubation and washing (by hand), a polyclonal antibody to IL-6 conjugated to alkaline phosphatase was added. Further incubation then washing was carried out before the substrate, then colour amplifier was added. Intra and inter-assay coefficient of variation was quoted as 3.7 and 7.8% respectively. In our laboratory results measured were 6.8 and 7.8% respectively.

Tumour Necrosis Factor alpha (TNFα)

TNFα was assayed using a high sensitivity ELISA kit from R&D using the protocol supplied with the kit (Appendix 3b). Briefly a monoclonal antibody specific for TNFα coated onto an ELISA plate was provided. Samples or standard, 0.5pg/ml to 16pg/ml in serum diluent, were added. After incubation and washing (by hand), a polyclonal antibody to TNFα conjugated to alkaline phosphatase was added A further incubation was carried out, and the plate washed before addition of the substrate, followed by the colour amplifier. The 'shaker' protocol, although recommended, gave a higher than expected CV between sample duplicates, and the bench top protocol was found to be better. Quoted intra and inter-assay variation was 6.0 and 7.5% respectively, but in our laboratory were 17.8 and 25 %, which may reflect low levels of TNFα in plasma or factors that effect assay performance.

Soluble L-selectin (sL-selectin)

Soluble L-selectin was measured using an ELISA kit from R&D, using the protocol supplied with the kit (Appendix 3c). Briefly an ELISA plate had been pre-coated with murine monoclonal antibody to human L-selectin. Plasma samples were diluted 1:100 in the diluent supplied, and added to the plate as well as standards. After 1 hour incubation at room temperature, anti-L-selectin HRP conjugate was added, and the plate incubated for a further 30 min. The plate was then washed 6 times before addition of substrate (tetramethylbenzidine). Intra- and inter-assay coefficient of variation was quoted as 4.7 and 7.6 % respectively.

Granulocyte - Colony Stimulating Factor (G-CSF) and Granulocyte Macrophage - Colony Stimulating Factor (GM-CSF)

G-CSF and GM-CSF were both measured using ELISA kits from R&D, according to the protocol supplied with the kit (Appendix 3d&e). Briefly, ELISA plates had been pre-coated with murine monoclonal antibody to G-CSF or GM-CSF. Plasma samples or standard was added. The standard curve ranged from 80 to 1.25pg/ml for G-CSF and 64 to 1pg/ml for GM-CSF. After incubation and washing, a polyclonal antibody conjugated to HRP to G-CSF or GM-CSF was added. Further washing and incubation was then carried out before the substrate, followed by amplifier, was added.

Table 2.16a

Inter and Intra assay variation for ELISAs

	Low Intra	High Intra	Low Inter	High Inter
hNEAPC	26.1 ng/ml CV=4.4 (n=8)	95.9 ng/ml CV=4.7 (n=8)	26.1 ng/ml CV=14.6 (n=8)	95.9 ng/ml CV=6.7 (n=8)
hNE	2.25ng/ml CV=5.6 (n=4)	6.62ng/ml CV=3.4 (n=4)	2.25ng/ml CV=2.94 (n=4)	6.62ng/ml CV=3.16 (n=4)
CRP	CV=6.4	CV=20	CV=12.5	CV=8.6
IL-8	CV = 5.6		CV=6.5	
IL-6 HS (n=20)	0.47 pg/ml CV=8.5	7.47pg/ml CV=3.2	0.46pg/ml CV=8.7	7.39 pg/ml CV=3.
TNFα HS (n=20)	2.32pg/ml CV=8.2	29.9 pg/ml CV=5.7	2.40 pg/ml CV=10.4	32.34 pg/ml CV=7.8
G-CSF HS (n=20)	2.90 pg/ml CV=8.3	52.11pg/ml CV=4.6	3.66 pg/ml CV=10.9	58.60 CV=7.1
GM-CSF HS (n=20)	3.72 pg/ml CV=6.7	39.6 pg/ml CV=6.7	2.21 pg/ml CV=9.5	28.9 pg/ml CV=4.9
SL-selectin	250ng/ml n=10 CV=2.6	3183ng/ml n=10 CV=3.1	238ng/ml n=40 CV=7.6	2968 n=40 CV=6.3

Intra-assay precision (precision within assay). Inter-assay precision (precision between assays) Quoted CV is % in all cases. Values for IL-6, TNFα, G-CSF, GM-CSF and sL-selectin are those quoted by the manufacturer of the ELISA kit.

Table 2.16b

Recovery and Linearity

	Recovery		Linearity		
	%	Range	1:2	1:4	1:8
hNEAPC			1:4 102 (94-111)		
hNE	103	99-111	1:100[$] 85 (78-90)	1:200[$] 104 (100-106)	1:800[$] 107 (101-111)
CRP	115	111-120	1:50* 89	1:200* 108	1:400* 87
IL-8	91	77-106	1:2=76	1:10=108	1:20=116
IL-6 HS	110	100 - 123	102 (93 – 104)	104 (98 – 108)	111 (103 – 127)
TNFα HS	96	86 - 105	106	102	107
G-CSF HS	98	90 - 110	97 (93 – 100)	95 (91 – 99)	91 (87 – 94)
GM-CSF HS	99	85 - 114	101 (83 – 111)	101 (86 – 110)	99 (81 – 113)
SL-selectin	103	86 - 105	98.1 (96 – 101)	97.7 (94 – 102)	98.4 (96 – 100)

Recovery is where serum is spiked to different levels throughout the range of the assay.

Linearity was assessed by diluting a sample and determining the mean recovery.

[$] For hNE a 1:400 dilution of the sample was on the middle of the standard curve and used for determination of the concentration. Therefore dilutions above and below were quoted to show linearity.

Dilution of 1:100 is the minimum used for the CRP assay, and therefore dilutions are stated as a percentage of the value for the 1:100.

Dilution of 1:5 is used as 100% for IL-8 and 1:20 for hNEAPC.

2.2.7 Separation of Neutrophils

Venous blood was collected into citrate buffer (0.15 x blood volume). A volume of 40ml blood sample typically gave 20 ml of a $4x10^6$/ml granulocyte preparation. This was kept for a maximum of 30 min. at room temperature before separation was carried. All medium and buffers were at room temperature, which has been shown to be best for cell separation (Glasser 1990).

The citrated blood was overlayed onto a mixture of methyl cellulose (1%) and sodium diatrizoate (34%) in 10:16 ratio (2x blood volume). Erythrocyte sedimentation occurred over 30 to 40 min. The supernatant was aspirated into a centrifuge tube and underlayed with Ficoll 400 (0.5 x supernatant volume) and centrifuged at 400g for 20 min. at 20°C. The cells at the interface (the mononuclear layer) were discarded before removal of the supernatant. The remaining granulocyte pellet was gently resuspended in HBSS -Ca^{++}-Mg^{++} (Figure 2.11). The cells were washed by centrifugation at 400g for 4 min. the supernatant discarded and the cell pellet gently resuspended in HBSS- (without Ca^{++} and Mg^{++}). The cells were then washed in HBSS (with Ca^{++} and Mg^{++}) before resuspension in the final volume of buffer for the experiment.

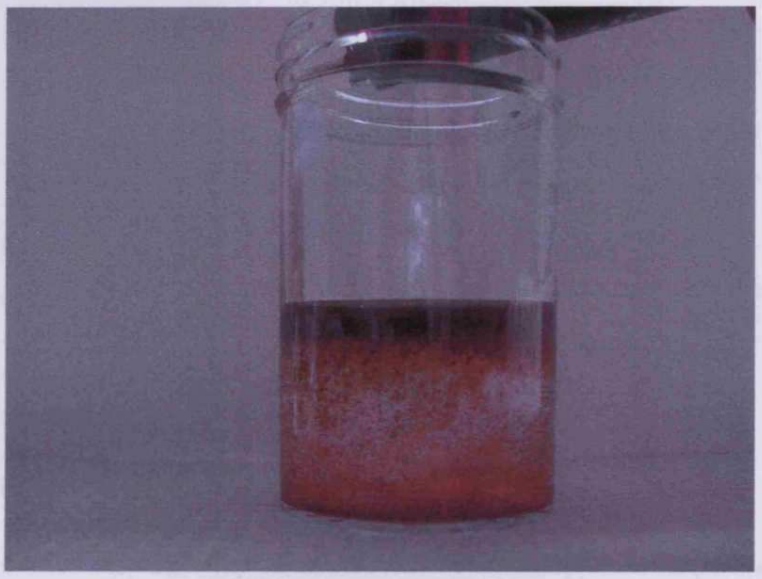
Total cell counts were carried out using a Neubaur chamber. A differential count was carried out on a Leishman's stained cytospin preparation.

Mean	% neutrophil	% Granulocytes (includes eosinophils)
Patients	95.4 SD 2.3	97.3 SD 1.9
Controls	94.7 SD 3.4	96.7 SD 1.9

Viability of the cells was assessed by incubation with Trypan blue for 10 min., washing, then microscopic examination. Most preparation gave 100% viable cells, the lowest (98%) was observed on two occasions (out of 72).

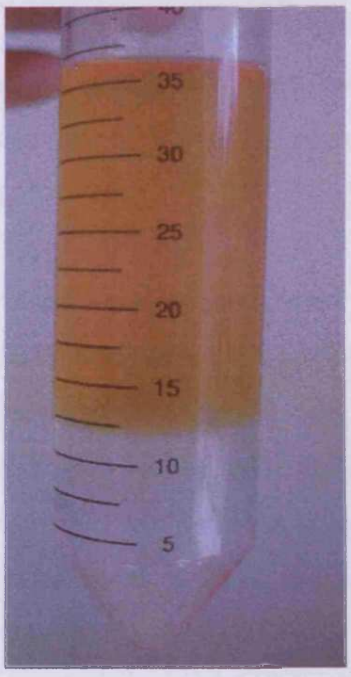
Figure 2.11 Neutrophil separation

Layering of blood on Methyl Cellulose / Sodium Diatriozate mixture, with start of erythrocyte sedimentation



Red cell sedimentation after 45 min. with white cell and plasma layer on top

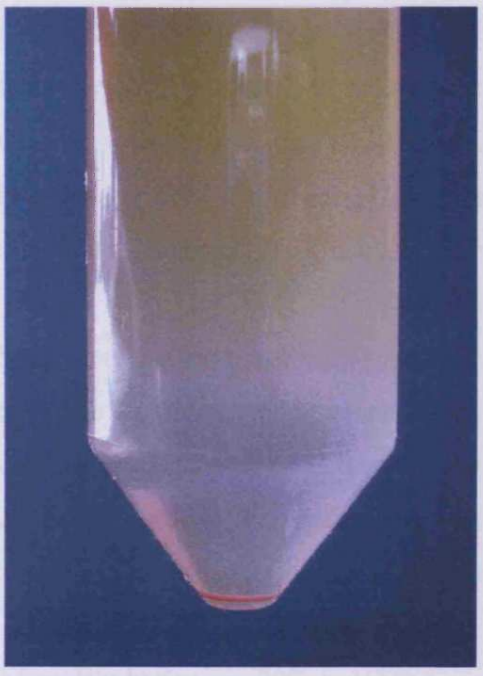
White cell and plasma layer underlayed with ficoll



Aspirated layer of plasma and white cells

Ficoll underlayed beneath

Cell types separated through density gradient (centrifugation for 30 min. at 400g)



Monocyte layer

Ficoll layer

Granulocyte layer

(with some red cell contamination)

2.2.8 Superoxide Generation

Separated neutrophils were resuspended in HBSS containing Cytochalasin B (5mg/ml) and Cytochrome C (0.2mM) to approx. 4 million / ml. (13ml was required for the standard sputum sol experiment).

Blank tubes and sputum control tubes contained HBSS+ without neutrophils. 250µl of neutrophils in HBSS+ was added to each incubation tube, which contained 50µl of buffer of sputum sol, then incubated at 37°C for 30 min. at this point 200µl of HBSS+ some with fMLP at the stated concentration was added to the incubation tubes. and a further incubation at 37C for 20 min. was carried out. Tubes were carried out in triplicate. Tubes were centrifuged for 6 min. at 400g. 300µl of supernatant was aspirated and placed in a microtitre plated for measurement of optical density at λ550nm (Ref. λ490nm).

2.2.9 Phagocytosis of *Pseudomonas aeruginosa* by human neutrophils

Neutrophils were suspended to 10^6/ml in HBSS. An aliquot of *Pseudomonas aeruginosa* was taken from the stock solution (stored at 4°C in HBBS), at 5 x 10^7 colony forming units (cfu)/ml. Bacteria were opsonized by mixing equal volumes (200µl) of *P.aeruginosa* and with autologous serum then incubated at 37°C for 10 min. Opsonized bacteria were mixed with neutrophil suspension at a ratio of 1:4, and incubated at 37°C for 60 min. whilst being shaken gently.

Sputum or buffer was added in an equal volume to this mixture. Incubations were carried out in triplicate in the presence of neutrophils, and duplicate in the absence of neutrophils, for each treatment (absence (HBSS) or presence of sputum at 1:5, 1:10, and 1:20 dilution in HBSS). Where there was insufficient sputum for a 1:5 dilution, a 1:40 was included to replace the 1:5. Final concentrations of sputum for the incubation were ½ of the sputum dilution used.

After incubation, a 200µl volume was removed and mixed with 800µl of HBSS. This was centrifuged at 100*g* for 6 min. to remove the neutrophils, but allowing the *P.aeruginosa* to remain

in suspension. A 1:100 dilution of the supernatant into sterile distilled water was carried out, and this used to spread onto nutrient agar plates, in addition to a further 1:10 dilution. A volume of 100µl was found to be the best for spreading onto the agar plates, as larger volumes tended to form joined colonies, and smaller volumes did not spread evenly. Seeded agar plates were then incubated overnight at 37°C before counting of colonies. *P.aeruginosa* colonies were about 2mm in diameter with a characteristic well in the centre, and green in colour. From the mean colony counts the % phagocytosis could be determined.

2.2.10 Intracellular killing of *Pseudomonas aeruginosa* by human neutrophils

Neutrophils and opsonized bacteria were prepared as in the phagocytosis assay. Neutrophil suspension and opsonized P.aeruginosa were mixed in a ratio of 5:1 and incubated for 5 min. at 37°C to allow for ingestion of the *P.aeruginosa*, with minimal effect of killing. The volume was increased 5 fold with HBSS, and then centrifuged 200*g* for 2 min. at 4°C. The supernatant (containing non phagocytosed *P.aeruginosa*) was removed, and the cell pellet resuspended in HBSS. The above was repeated, and the cell pellet resuspended to 4ml in HBSS (10% autologous serum). This mixture was shown to contain no free *P.aeruginosa*, by plating directly for a couple of test cases. The suspension containing neutrophils with ingested *P.aeruginosa* was aliquoted (250µl) and mixed with an equal volume of buffer of sputum prior to incubation for 30 min at 37°C. A time 0 was taken for every experiment, and this was taken as 0% killing. Triplicate tubes for buffer, and each of 3 concentrations of sputum (3 highest of 1:2, 1:5, 1:10, 1:20, 1:140 depending on size of sputum sample) were carried out. Two aliquots were taken for each reading. Each aliquot was placed into 10ml sterile distilled water at the stated time. This was to lyse the neutrophils, and release any intracellular *P.aeruginosa*. This was then spread onto nutrient agar plates (100µl/plate) and incubated overnight at 37°C, and the colonies counted.

2.2.11 Flow Cytometry

Whole blood was collected into EDTA, and processed within 30 min. of collection. Analysis was carried out in duplicate, and all incubations using fluorescent labelled antibodies were carried out in the dark.

Cell surface markers

To each 50µl of whole blood diluted 1:5 in sterile PBS (0.5%BSA) one of the following was added:

Anti-hu L-selectin-FITC (10µl) + Anti hu CD11b-PE (5µl)

Anti hu IL8RA-FITC (10µl) + Anti hu IL-8 RB-PE (10µl)

Anti-IL-6 (10µl)

Anti-TNFαR (10µl)

Anti-ms IgG-FITC (10µl)

PBS (10µl)

Samples were then incubated for 30 min. at 4°C before the addition of 2.0ml of FACS lysing solution. After a further incubation at room temp for 10 min. tubes were centrifuged at 300g for 5 min. (RT). For the anti-TNFαR and anit-IL-6R determinations there was an additional step where they were washed twice in PBS before addition of anti-ms IgG-FITC (10µl), and a further incubation of 30 min. at 4°C. Unbound antibody was removed by washing twice in PBS, before cells were resuspended in 0.5ml 1% paraformaldehyde (PFA) in PBS. They were then stored at 4°C until analysis.

Effect of Stimulation by fMLP on L-selectin and CD11b surface expression

To each 50µl of whole blood diluted 1:10 in sterile PBS (0.5%BSA), 33.5µl of PBS or PBS containing fMLP was added to give final fMLP concentrations (10^{-9}, 3x 10^{-9}, 10^{-8}, 3x10^{-8}, 10^{7}M), and then incubated at 37°C for 20 min. Tubes were then put on ice, and 10µl of a 50/50 mixture of anti-hu L-selectin-FITC and anti-hu CD11b-PE added. Staining was then carried out as described above.

Intracellular MPO

Cells were permeablized with 'Fix and Perm' according to the manufacturers protocol prior to addition of antibody to MPO as detailed below:

To each 50µl of whole blood diluted 1:10 in sterile PBS (0.5%BSA), 100µl of 'Reagent A' was added and incubated at room temperature for 15 min. PBS (4ml) was added, and tubes then centrifuged at 300g for 5 min. The supernatant was removed, and 100µl of 'Reagent B' (for permeabilized cells) or PBS (control) added to the cell pellet, followed by 20µl of Anti hu MPO-FITC. This was then incubated for 15 min. at room temperature, before washing twice in PBS (0.5%BSA). Red cells were lysed by adding 2.0ml of lysing solution, and incubated at room temp for 10 min. After washing with PBS, cells were resuspended in 0.5ml 1%PFA in PBS.

Calibration curve

Dako fluorospheres were run with each experiment, and used to determine molecules of equivalent fluorescence (MEF) from the mean fluorescence. A calibration curve was carried out with every experiment to allow for day-to-day variation of the FASCan.

FACS analysis

Each tube was put on the FACSscan to determine 10000 cells (events), and the data stored for later analysis using the specialised program 'winmdi'. Neutrophils were selected based on their forward and side scatter pattern, which left about 6000 cells (the others being monocytes and lymphocytes) for mean fluorescence calculation.

2.2.12 Adherence

Adherence was determined using a method by Thom *et al* [1997]. Nylon Columns were prepared by packing 62.5mg of scrubbed nylon fibre wool into the shoulder of a 3ml sterile plastic pasteur pipette to a length of exactly 15mm. 1ml of whole blood was then added per column and left to filter through the column by gravity. The leukocyte number and differential were measured in whole blood before and after passage down the nylon column using a Coulter counter. Lithium Heparin was found to be the best anticoagulant for these experiments. Inter-assay variation was only 5% (Table 2.17), however there was much greater intra-subject variation.

Table 2.17

Consistency of adherence obtained down nylon column

Subject 1			Subject 2		
Neutrophil number before	1.75 x 10^6/ml	Adherence (%)	Neutrophil number before	2.65 x 10^6/ml	Adherence (%)
Neutrophil number after	1.18	32	Neutrophil number after	2.35	11
	1.26	28		2.45	8
	1.23	30		2.45	8
	1.16	34		2.45	8
	1.18	32		2.54	6
	1.32	25		2.44	9
Mean	1.22	30	Mean	2.45	8.3
CV (%)	4.9	10.8	CV (%)	2.5	19.6

2.2.13 Analysis of Poly Unsaturated Fatty Acids (PUFA) by GC

Analysis was carried out on serum or EDTA plasma. All tubes were cleaned thoroughly, and rinsed with distilled water prior to use. High pressure liquid chromatography (HPLC) grade chemicals and water were used throughout. An internal standard of margaric acid (C17) was used as this was not present in biological samples and 50 µl was added to all assay tubes at the start of extraction.

Extraction

In addition to margaric acid either 250 µl of sample or 250µl of standard was added to a 10 ml glass tube. Methanol containing 0.01% Butylated hydroxytoluene (BHT) was added (2ml), followed by Chloroform containing 0.01% BHT (4ml) whilst mixing. The mixture was left for 20 min., with occasional mixing. 2ml solution of 0.2% $CaCl_2$ was added and the mixture whirlimixed for 1 min. before centrifugation for 5 min. at 2500rpm. The lower chloroform layer was removed and put in a clean test tube and dried down with N_2 gas at 30°C. The pellet containing fatty acids were reconstituted with 3ml methanol, and 100µl of conc. sulphuric acid was added, the tubes capped, whirlimixed and incubated at 65°C for 1 hour. After being allowed to cool 1ml of water, followed by 1ml of petroleum ether (40-60) was added. Tubes were whirlimixed and the phases allowed to separate. The upper layer was transferred to a dark chromatography vial and evaporated down with N_2 gas at room temperature. The samples could be stored at –20°C until analysis.

Gas Chromatography

The GC (Hewlett Packard 5890 Series II) was fitted with an Polyethyleneglycol polar column 25m x 0.25mm x 0.25µm film (BP21, SGE Europe Ltd. 1 Potters Lane, Kiln Farm, Milton Keynes MK11 3LA). Detection was by Flame ionisation detector (FID). GC set up was an initial flow rate of 3 ml/min., with Helium set to 40 and Air set to 400. Split was measured as 30, therefore 1:10. The injector was set to 220°C, and the detector to 280°C. The run had an initial temperature of 200°C, and this increased at a rate of 2°/min until 222°C, the rate was then increased to 4°/min. until a temperature of 260°C, which was held for 5 min. This gave a total run time of 25 min. per sample. A column compensation was run, and this was subtracted from sample runs.

Analysis

Each Fatty Acid was determined by its retention time relative to the internal standard (Margaric Acid, C17), and quantity determined by the size of peak relative to the internal standard for each run.

2.2.14 Blood glucose

Blood glucose was measured by a YSI2300 Stat Plus Glucose and Lactate Analyser which provides a quick measurement of glucose in whole blood. The instrument uses a glucose oxidise enzyme hydrogen peroxide sensor to determine glucose levels. This was carried out by the Diabetes Department.

2.2.15 Statistical analysis

The majority of data were not normally distributed and statistical analysis was carried out after log10 transformation. A paired student's 't' test was used to compare the pre and post antibiotic results. An unpaired 't' test (Welch's) was used to analyse all other differences. Spearman rank correlation was used to compare non-log transformed data. Statistical analysis was carried out using 'Instat' for Study A, and 'SPSS' for all other studies.

2.3.1 Clinical determinations

Spirometry (Forced Expiratory Volumne in 1 second (FEV_1), Forced Vital Capacity (FVC) and FEV_1/FVC ratio) was determined for both patients and healthy subjects in all studies. A portable spirometer (Vitalograph) was used and the best of 3 values accepted. The spirometer receives an annual service and calibration. Lung function values were expressed as % predicted for the age and height of the individual according to standard tables (ERS 1993).

Absolute neutrophil numbers were obtained using an automatic differential count by a COULTER MAXM (Beckman Coulter UK Ltd, Oakley Court, Kingsmead Business Park, High Wycombe, Buckinghamshire, UK) in the routine haematology laboratory at Llandough Hospital.

Sputum was sent to microbiology for culture of organisms and their sensitivity to antibiotics for patients at the start of exacerbation, and whilst clinically stable. The results were recorded. These are routine tests in the clinical management of these patients.

2.3.2 Protocol and Patient Involvement

All studies were approved by the local research ethics committee and all subjects gave consent as specified.

Study Group A - Results shown in chapters 3, 4, 5 and 6

Patients with CF were recruited into the study when presenting with an exacerbation of respiratory symptoms and were studied within 24 hours of the beginning of antibiotic treatment. These patients were studied again at the end of a course of *i.v.* antibiotics (10 to 14 days after start). Each patient was age and sex matched with a healthy subject, who was studied at the same time as the patient. A further group of patients were recruited who were considered clinically stable, and these were matched with a further group of healthy subjects. Each group consisted of 12 patients, and 12 matched healthy subjects.

A clinical exacerbation was defined as a combination of a drop in lung function (FEV_1) of greater than 10% of the best recorded value in the previous 12 months, an increase in purulent sputum, a worsening of other symptoms, and a clinical decision that a course of *i.v.* antibiotics was necessary. Clinically stable patients were defined as having no worsening of symptoms, free from *i.v.* antibiotic treatment for one month and a lung function within 10% of their best in the previous 12 months.

On each day of study blood and sputum was collected from a CF patient, and blood was collected from their matched healthy subject. Neutrophil functional studies for both the patient and healthy subjects were run in parallel to give inter-assay control and allow paired comparisons. Clinical data for both the patient and healthy subject were recorded (Appendix 1). This included age, sex, height weight and lung function and any medication taken. For the patients this included the antibiotic

treatment received, intercurrent illness and other medication. Bacteriological information was also recorded.

Blood was collected by venepunture, and from each subject the following was collected: 40ml into citrate buffer (for neutrophil separation); 10ml no anticoagulant (for serum); 8 ml into EDTA (for plasma), and 4ml into EDTA for full blood count (FBC) and differential. Neutrophils separation was started within 15 min of collection of blood. Some serum was kept for the phagocytosis and killing assays and the rest aliquoted and stored at -70°C. Plasma was also aliquoted and stored at -70°C. A fresh sputum sample from the patient was collected at the same time as the blood, and this sputum was used for both the patient and the healthy subject on that day. The overall plan is shown in Table 2.18.

Table 2.18 **Summary of protocol for Study A**

Paired	Start of a clinical exacerbation	After 2 weeks of treatment	Clinically stable
CF	Sputum from CF neutrophils for functional studies	Sputum from CF neutrophils for functional studies	Sputum from CF neutrophils for functional studies
non CF	Plasma / serum	Plasma / serum	Plasma / serum

Study Group A(2) Results shown in chapter 3

Patients with previously diagnosed COPD (history of cigarette consumption, airflow obstruction and reversibility of <10%) were recruited at the time of the diagnosis of an acute exacerbation of respiratory symptoms. An exacerbation was defined as an increase in symptoms including cough, sputum volume, purulence and breathlessness associated with a reduction of > 10 % at time of entry

into the study. Blood and sputum was collected at the time of diagnosis and after 2 weeks oral antibiotic (Clarithromycin 500mg bd) treatment.

Study Group B - Results shown in chapters 7 and 10

Twelve patients were recruited on presentation with a respiratory exacerbation as in A. These patients were studied again at the end of a course of *i.v.* antibiotics (10 to 14 days after start). Each patient matched with a healthy subject, who was also studied on the same days as the patient. Blood was collected and processed as previously. No clinically stable group was included as this group did not appear to provide additional information, and no sputum was collected in this or further studies.

Study Group C - Results shown in chapters 8

Eight patients were recruited into the study on presentation with a respiratory exacerbation and again at the end of a course of *i.v.* antibiotics (10 to 14 days after start of treatment). Each patient was age and sex matched with a healthy subject, who was also studied on the same days as the patient. Blood was obtained by venepuncture and collected into EDTA for both Flow Cytometry (chapter 8) and smear for morphological assessment of neutrophil maturity (chapter 10). Processing was started within 30 min. of collection. Additional blood was collected for plasma (EDTA) and serum (no coagulant), and aliquots were stored at –70°C, prior to the measurement of inflammatory markers. Lung function, FBC, height and weight were recorded for the patients, and FBC for the healthy subjects.

Study Group D - Results shown in chapter 9

Patients (n=29) on attendance at clinic or the ward were asked for a sample of blood collected by venepuncture into Li Heparin (for adherence) and no anticoagulant (for serum) and EDTA (for plasma). Patients were grouped according to clinical symptoms at the time, resulting in 18 patients classed as in a respiratory exacerbation, and 11 patients as clinically stable. A further 15 samples

were taken from non-CF healthy subjects. Serum was aliquoted and stored at -70°C for subsequent analysis.

Study group E - Results shown in chapter 10 and 11

Patients (n=15) were recruited when a glucose tolerance test was being carried out for clinical reasons (annual review). Patients with known diabetes were not recruited for the initial study, however, it was suspected that the study may have included patients with impaired glucose tolerance, or those where diabetes had not been diagnosed. All subjects fasted for 12 hours before the OGTT, which commenced at 08.00 hours. All medication was withheld on the morning of the test. The glucose load consisted of 1.72 g/kg up to a total of 75 g, usually in the form of 113 ml of Polycal drink diluted with up to 200 ml of water, and followed over the next 5 min. with 100ml of water.

Venous blood was taken into citrate for neutrophil separation at fasting (time 0) and 120 min. after glucose challenge. Additional blood was collected for plasma and serum from the time 0 sample. Aliquots were stored at –70°C for subsequent analysis of plasma lipids and fatty acids.

Chapter 3

Inflammatory mediators in the circulation and sputum and their relationship to clinical status in patients with cystic fibrosis

Aim

To compare circulating and sputum concentrations of inflammatory mediators in patients with CF and non-CF healthy subjects.

To determine the effect of an exacerbation of respiratory symptoms and its treatment on concentrations of both circulating and sputum inflammatory mediators.

To determine the relationship between the concentrations of inflammatory mediators in the circulation and sputum to clinical status in patients with CF.

Introduction

A variety of factors are likely to modulate neutrophil function in CF. These include those intrinsic to the neutrophil such as maturity, and external effects such as host mediators of inflammation and bacterial products resulting from chronic infection. In relation to chronic lung infection, the neutrophil experiences potential factors in three separate compartments: the circulation, airways parenchyma, and the airspace.

The combination of factors modulating neutrophil function are likely to be different within the three compartments. Host produced factors such as C-reactive protein (CRP), IL-6 and TNFα have the potential to affect neutrophil function, as do factors released by the neutrophil, such as elastase (hNE), which could act by negative feedback [McElvaney *et al* 1991]. Movement from the bloodstream to inflamed tissue is a sequence of events which depends on the activation of neutrophils. This process involves neutrophil margination, rolling, and adhesion mediated by L-selecting [Drost & MacNee2002].

In addition there may be effects on neutrophils by both the pathogen and its products. *P.aeruginosa* produces a number of cytotoxic substances including leukocidin, exotoxin A and hemolysin. In addition it produces enzymes capable of proteolysis of opsonic IgG molecules, reducing opsonin dependent phagocytosis [Buret *et al* 1993]. Another mechanism used by *P.aeruginosa* is the production of pyochelin which is lipophilic, and which by associating with cellular membranes is able to enhance neutrophil mediated injury to pulmonary vasculature. *In vitro* both pyochelin and pyocyanin have been shown to increase PMA stimulated neutrophil superoxide generation [Buret *et al* 1993, Muller *et al* 1997]. LPS is also capable of priming neutrophils for enhanced superoxide generation although LPS from some isolates of *P.aeruginosa* may not have this effect [Kharazmi *et al* 1991, Hughes *et al* 1997]. *In vivo* a study in sheep showed that the *P.aeruginosa* products pyocyanin and 1-hydroxphenazine were able to increase neutrophil recruitment to the airways and the airways inflammation including increased production of LTB_4 and IL-8 by alveolar macrophages, and increased concentration of albumin in BALF [Lauredo *et al* 1998].

Inflammatory leukocytes have for a long time been implicated in connective tissue damage during chronic inflammatory lung disease, and that this damage is mediated principally by serine proteases such as hNE [Brown *et al* 1988]. Circulating concentrations of neutrophil elastase α_1-antiproteinase complex (hNEAPC), and CRP have been shown to be increased in clinically stable patients Rayner *et al* [1991]. It has been argued that the concentration of hNEAPC is an indirect indicator of intravascular activation of neutrophils and relates to the margination and migration process in the vasculature of the airways [Banda *et al* 1998]. CRP can be considered as a downstream indicator of the functional levels of IL-6 and secondarily of pro-inflammatory primary cytokines such as TNFα, and therefore indicates elements of the regulators of the inflammatory response [Bauer & Hermann 1991]. The concentration of circulating CRP and hNEAPC in patients with CF is further increased at the time of worsening respiratory symptoms. Antibiotic treatment of such episodes reduces both mediators, but the reduction is usually incomplete with neither returning to within normal limits in the majority of patients [Hollsing *et al* 1987, Suter *et al* 1989, Norman *et al* 1991, McGraph *et al* 1999, Bell *et al* 2000, Ionescu *et al* 2000].

Various cytokines have also been proposed as indicators of the host inflammatory response in CF. IL-6, IL-8, TNFα and IL1β are the most commonly measured, and most studies have focused on airway inflammation. It is hypothesized that cytokine mediators generated in the lung have a role in co-ordinating physiologically appropriate aspects of the host response such as acute phase response, the catabolic response and the recruitment of neutrophils to the site of infection.

An increased circulating concentration of TNFα was reported in clinically stable patients with cystic fibrosis [Elborn *et al* 1995]. Similar to CRP and hNEAPC, TNFα concentration was reduced by antibiotic treatment of an exacerbation of respiratory symptoms [Suter *et al* 1989 , Norman *et al* 1991]. TNFα has been implicated in the acute phase response in both acute and chronic diseases. These are wide ranging and include HIV [Salazar-Gonzalez *et al* 1997], rheumatoid arthritis [de Bendetti 1997], TB [Poveda *et al* 1999], sepsis and ARDS [Skoutelis *et al* 2000, Bauer *et al* 2000]. It has been implicated in causing cachexia in HIV [Drexler 1995, Odeh 1990], chronic heart failure [Zhao & Zeng 1997] and Cancer [Tisdale 2001]. TNFα is also implicated in the rejection in transplants, particularly in those genetically disposed to production of greater amounts of TNFα [Azzawi *et al* 2001].

The concentration of IL-6 in serum has been shown to be raised compared with healthy subjects in various disorders including rheumatoid arthritis [Cohick *et al* 1994], surgery [Sweed *et al* 1992], sepsis [Friedland *et al* 1992] and a range of cancers [Yanagawa *et al* 1995, Gause *et al* 1991]. IL6 was the earliest cytokine to rise during surgery [Sweed *et al* 1992] and the best predictor of mortality in sepsis [Friedland *et al* 1992], suggesting it plays a key role in the host response to both stress and infection.

L-selectin is a cell surface glycoprotein expressed constitutively on various leukocytes. Soluble L-selectin is shed from the neutrophil surface on activation [Albelda 1994] and is linked to neutrophil recruitment [Hafezi-Moghdam *et al* 2001]. The role of this marker in neutrophil functioning and disease is discussed further in Chapters 8 and 9.

Measurements carried out in sputum and BALF are used to represent, at least in part, the constituents of the epithelial lining fluid. The epithelial lining fluid covers the surface epithelia

within the lung and aids gaseous exchange. The composition of this fluid represents the effective level of inflammation or degradation at the lung surface. Henig *et al* [2001] compared cell numbers and type and concentrations of TNFα and IL-8 and found no difference between spontaneously produced sputum, induced sputum and BALF in patients with CF. The concentrations of TNFα and LTB_4 in sputum were positively correlated and inversely related to FEV_1 and FVC suggesting a link to the severity of lung disease [Greally *et al* 1993]. Kronborg *et al* [1993] reported that the concentrations of IL-6, IL-1, TNFα and Interleukin 1 receptor were all much greater in sputum sol than plasma. Chest radiography often shows a variation of severity in different parts of the lung, and it has been shown that there are more neutrophils, and more active hNE, in the more severe areas [Meyer *et al* 1997]. The use of sputum and BALF to determine levels are mixture of these areas, which may be represented differently and proportionally. A summary of these findings are shown in Table 3.1

Table 3.1

Inflammatory markers in sputum and BALF

Author	Date	Matrix	increased	decreased	comments
Osika	1999	sputum	TNFα, IL-1β, IL-8		CF vs HS
Schuster	1995	sputum	IL-1b, IL-8, TNFα		CF vs HS
Dean	1993	BALF sputum	IL-8		CF related to severity
Wolter	1999	sputum	hNEAPC, protein, α_1PI	with treatment	CF in exacerbation
Bonfield	1995	BALF	IL-1, IL-8, IL-6, TNFα	IL-10	young CF vs HS
Armstrong	1997	BALF	hNE		young CF infected
Noah	1997	BALF	IL-8, IL-6, cell number		young CF infected
Pesci	1998	BALF	IL-8, MPO, ECP		COPD vs HS
Aaron	2001	induced sputum	TNFα, IL-8		in exacerbation compared to clinically stable
Keatings	1997	induced sputum	TNFα, IL-8, neutrophil number		COPD vs HS
Braun	1994	BALF	hNE, MPO		acute pneumonia

Abbreviations: CF: Patient with Cystic Fibrosis, COPD: Patient with Chronic Obstructive Pulmonary Disease, HS: Healthy Subject, IL-8: Interleukin-8, MPO: Myeloperoxidase, TNFα: Tumour Necrosis Factor alpha.

There are a number of possible sources of these cytokines, which may in some instances be produced in greater amounts by cells from patients with CF compared to those from healthy subjects. *In vitro* LPS from various bacteria have been shown to induce IL-6, IL-8 and TNFα production by whole human blood [Hutchinson *et al* 2000]. In addition human cystic fibrosis bronchial epithelial cells have been shown to produce greater amounts of IL-8 and IL-6 than control of CFTR corrected cells on stimulation with TNFα and IL1β [Black *et al* 1998, Stecenko *et al* 2001]. This release will also occur in un-stimulated cells, along with the production of IL-6, in lung epithelial cells in from patients with cystic fibrosis but not in controls [Bonfield *et al* 1999]. IL-10 has been highlighted as an anti-inflammatory cytokine. CF patients or their cells, were unable to produce unlimited amounts of Il-10 in response to antigen stimuli unlike controls [Bonfield *et al* 1999, Shmarina *et al* 2001]. In alveolar macrophages from patients with COPD increased production of IL-6 is seen in those with poor lung function [Song *et al* 2001].

The continuous presence of *P.aeruginosa* influences the nature of the environment of the airways and is a further potential factor in the modulation of neutrophil function. Chronic infection leads to the phenotypic switch to mucoid colony type and is associated with alginate production and the formation of microcolonies which act to protect the organism from host defences and antibiotics [Hoiby *et al* 2001]. Neutrophils will engulf alginate but cannot break it down, thus their phagocytic activity is reduced leading to an accumulation of inactive but frustrated neutrophils that soon die [Travis *et al* 1994]. *P.aeruginosa* also produces proteases, lipases and LPS which may act to stimulate neutrophils. Pyocyanin has been show to inactivate α_1 protease inhibitor [Britigan *et al* 1999], adding to the effect of the hNE on the host's protection from damage by proteases.

To gain an insight into the inflammatory environment our patient's neutrophils were likely to experience we studied the pattern of selected inflammatory mediators in the airways and circulatory compartments. The circulating inflammatory markers CRP, hNEAPC, IL-6 and TNFα were measured, and compared to the sputum levels of protein, NE, IL-6 and TNFα. Patients with CF were studied at a time of clinical exacerbation, after treatment of exacerbation and when clinically stable. Circulating markers were also determined in healthy subjects. For further comparison, levels of inflammatory markers were measured in patients with COPD to act as a disease control.

Method

Patient and control healthy subjects were collected as described for Study A (chapter 2.1.2). Plasma was assayed for hNEAPC (2.3.2) and TNFα (2.3.4); serum for CRP (2.3.1) and IL-6 (2.3.4); and sputum sol was assayed for NE, TNFα and IL-6 all by ELISA. Soluble L-selectin was assayed by ELISA (kit; R&D Systems) using the recommended protocol. Sputum sol was assayed for protein by a modified Lowry assay (2.5.1). Patient data not collected at the time of sampling was recorded from their notes.

Samples from patients with COPD were studied for the same markers, as well as a further group of age and sex matched healthy subjects. The only methodological difference was that the sputum had been frozen once prior to processing.

A 1g sputum sample was approximately 1ml which allowed for sputum sol and plasma to be compared.

Clinical Data

Sputum culture

In the exacerbation group, in addition to *P.aeruginosa* in the sputum culture, four patients showed evidence of the mucoid strain *P.aeruginosa*, one grew *Haemophilus influenzae*, and one *Aspergillus fumigatus*. In the clinically stable group, four patients showed evidence of the mucoid strain of *P.aeruginosa*, five grew *S.aureus*, one *H. influenzae*, and one *Candida albicans*.

Medication received by patients and controls

Each patient received two *i.v.* antibiotics for the two week treatment of their respiratory exacerbation, the antibiotics used were as follows: gentamicin (8), ceftazidime (7), tobramycin (4), azlocillin (4), aztreonam (1). Three patients in this group were on home O_2, and 5 received nocturnal feeds. Almost all patients were taking vitamin tablets (included Vitamins A, D, E, K, and

Multi vitamins). Other medication included: Pulmicort (4), Bricanyl (3), Ventolin (3), Nutrizym (3), omeprazole (2), sucralfate (1), temazepam (1), beclometasone (1), prednisolone (1), and DF-118 (1). Two patients were started on nebulised Colomycin at the start of their *i.v.* antibiotics.

In the clinically stable group almost all patients were taking vitamin tablets (included Vitamins A, D, E, K, and Multi vitamins). Other medication included: flucloxacillin (8), Creon (6), Ventolin (5), prednisolone (2), Netrizym (1), omeprazole (1), sucralfate (1), Colomycin (1), Pulmicort (1), Losec (1), ranitidine (1), Serevent (1) and DNase (1). Two patients received home O_2.

In the exacerbation study, one healthy subject received salbutamol for mild asthma, and one healthy subject developed a cold/chest infection after blood was taken for the 'start' and received a course of oral ampicillin between blood samples.

Results

CF: Lung function and clinical tests

All patient groups had significantly lower FEV_1 and FVC values than healthy subjects ($p<0.001$). There was an improvement in the FEV_1 and FVC after treatment with *i.v.* antibiotics ($p=0.004$ and $p=0.007$). The white blood cell count fell from 8.4 to 3.4 x 10^9/l ($p<0.001$) with antibiotic treatment, and was significantly raised at the start of antibiotic treatment compared with healthy subjects ($p=0.007$) and for the clinically stable group ($p=0.016$) (Table 3.1).

COPD: Lung function and clinical tests

The group of 25 COPD patients studied had a mean (SD) age of 66 (9.6). FEV_1 increased from 46.9 (19.7) to 52.2 (22.9) ($p=0.02$) and FVC from 45.7(18.6) to 55.3 (19.5) ($p=0.02$) following 2 weeks oral antibiotic treatment. There was no significant difference between FEV_1 and FVC in the CF compared to the COPD patient groups, although age was significantly greater in the COPD group ($p<0.001$).

Table 3.2

Clinical Data for Patients with CF and healthy subjects

	Exacerbation			Stable	
	CF before treatment	CF after treatment	healthy subjects	CF Clinically Stable	healthy subjects
Age (yr)	23 (20-26)		25 (23-27)	24 (21-28)	27 (23-30)
FEV_1 % predicted Sig. (pt to hs)	35.8 * (24-52) p<0.001	47.2 * $^{\$1}$ (31-72) p<0.001	104 (96-113)	41.1 * (30-57) p<0.001	103.8 (96-112)
FVC % predicted Sig. (pt to hs)	52.4 * (40-68) p<0.001	64.5 * $^{\$2}$ (48-87) p<0.001	103.2 (95-112)	57.7 * (43-77) p<0.001	105.3 (101-110)
Neutrophil count (10^9/l) Sig. (pt to hs)	8.4 * (6.5 –10.8) p=0.007	3.4 $^{\$3}$ (2.7 –4.4) ns	5.5 (4.2-6.9)	5.0 * (3.9-6.6) p=0.016	2.9 (2.2 – 3.8)

Values are geometric means with 95% confidence intervals shown in parentheses

* Patient compared with healthy subject using unpaired t test ($p<0.05$)

$ patient compared before and after antibiotic treatment using paired t test ($^{\$1}$ p=0.010, $^{\$2}$ p=0.005, $^{\$3}$ p<0.001)

Circulatory compartment

The concentration of both CRP and IL-6 was greater in all CF patient groups compared with healthy subjects ($p<0.001$) (Table 3.2 and Figures 3.1 and 3.2). For the patients commencing antibiotic treatment, both CRP and IL-6 concentrations were at their greatest. Concentrations of both mediators were reduced after antibiotic treatment ($p=0.001$ for both), but remained significantly greater than in healthy subjects. Clinically stable patients had significantly greater IL-6 concentrations than those after antibiotic treatment ($p=0.007$), and were not different from those at the start of antibiotic treatment. There were no differences between IL-6 concentrations for the healthy subject groups.

Both CRP and IL-6 were significantly reduced by antibiotic treatment in the patients with COPD ($p<0.001$, $p=0.017$ respectively) (Table 3.4). For the group of patients with COPD, CRP or IL-6 were not significantly different when compared to patients with CF at the commencement of, and after, antibiotic treatment. CRP was significantly greater in the patients with COPD both in exacerbation and after antibiotic treatment when compared to a group of age matched healthy subjects ($p<0.001$ and $p= 0.007$ respectively). IL-6 was significantly higher in patients with COPD at the start of exacerbation compared to a group of age matched healthy subjects ($p<0.001$), but not after antibiotic treatment. The older healthy subjects had significantly higher levels of CRP and IL-6 than the younger group of healthy subjects ($p<0.001$).

CRP and IL-6 levels were significantly related for both patients ($r=0.690$, $p<0.001$, $n=35$) and healthy subjects ($r=0.838$, $p<0.001$, $n=35$) (Figure 3.3), but were not significantly related in each patient group, which was probably due to group size (Table 3.5). The magnitude of reduction in the concentrations of IL-6 and CRP was also correlated ($r=0.6455$, $p=0.03$). Similarly in the COPD patients CRP and IL-6 were significantly related both in exacerbation ($r=0.508$ $p=0.008$) and after antibiotic treatment ($r=0.892$, $p<0.001$), but the falls in IL-6 and CRP were not quite significantly related ($r=0.370$, $p=0.083$).

The concentration of TNFα was not significantly greater in the patient groups compared with the healthy subjects. TNFα was reduced by antibiotic treatment of an exacerbation but this was not

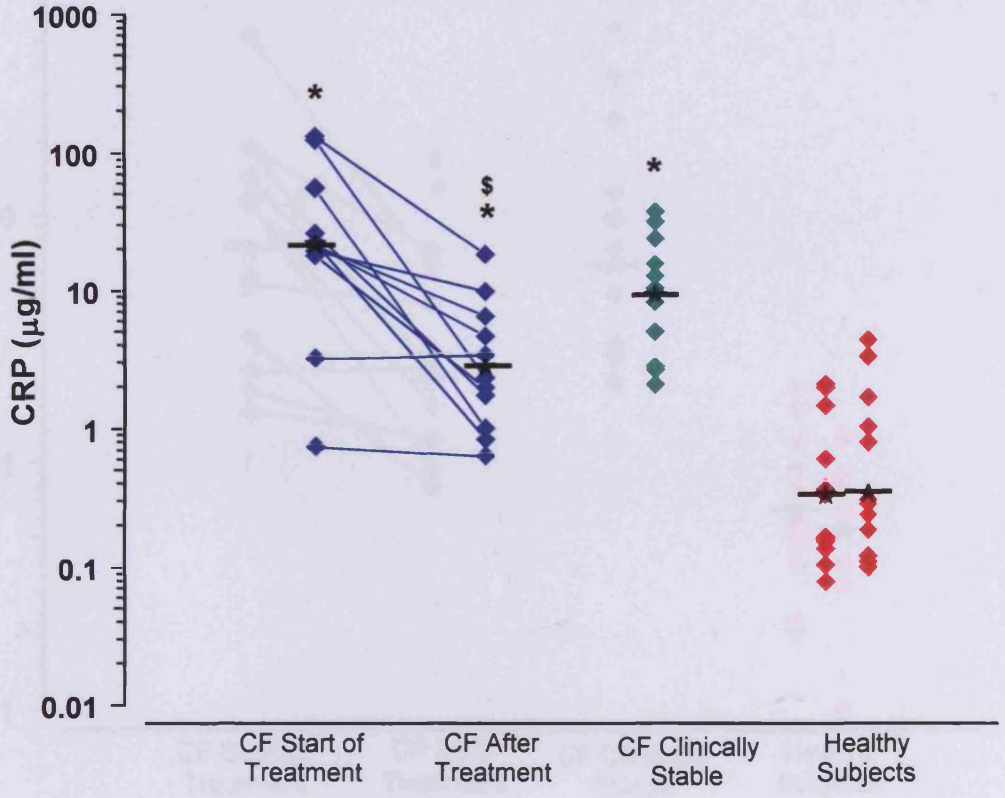
significant (p=0.09). The TNFα concentration of the clinically stable group was significantly greater than the patients after treatment (p=0.045). (Table 3.3). However, the healthy subjects matched to the stable group did show a significant difference when compared with the other two groups (p<0.05). The concentration of TNFα in the patients with COPD was not significantly altered by antibiotic treatment, and was not significantly different to the patients with CF at any clinical state (Table 3.3). The group of healthy subjects age matched to the COPD patients had a significantly lower concentration of circulating TNFα than COPD patients at both time points (p= 0.001 for both) and when compared to the other healthy subjects (p=0.019).

The concentration of hNEAPC was significantly greater in patients with CF at the start of antibiotic treatment and in clinically stable patients compared with their matched healthy subjects (p=0.002, p=0.01 respectively). In the patients undergoing antibiotic treatment there was a fall in the concentration of hNEAPC between start and end of treatment (p=0.013)(Table 3.3). There was no significant differences between hNEAPC levels for any of the healthy subject groups. Concentrations of hNEAPC in patients with COPD were significantly greater than in patients with CF (p<0.001 when exacerbation was compared to exacerbation, and treated compared to treated). There was not a significant decrease in hNEAPC with antibiotic treatment in patients with COPD.

Circulating sL-selectin concentrations, though they increased significantly following antibiotic treatment (p=0.0006) were not significantly different from healthy subject values at either time point (Figure 3.4). The concentration of sL-selectin corrected for the absolute neutrophil count was lower (p=0.03) at the beginning of treatment and increased (p=0.02) after treatment (Table 3.3). The concentration of sL-selectin was inversely related to the absolute neutrophil count (r=-0.57; p=0.07).

Figure 3.1

The circulating concentration of CRP related to clinical status in patients with CF and healthy subjects



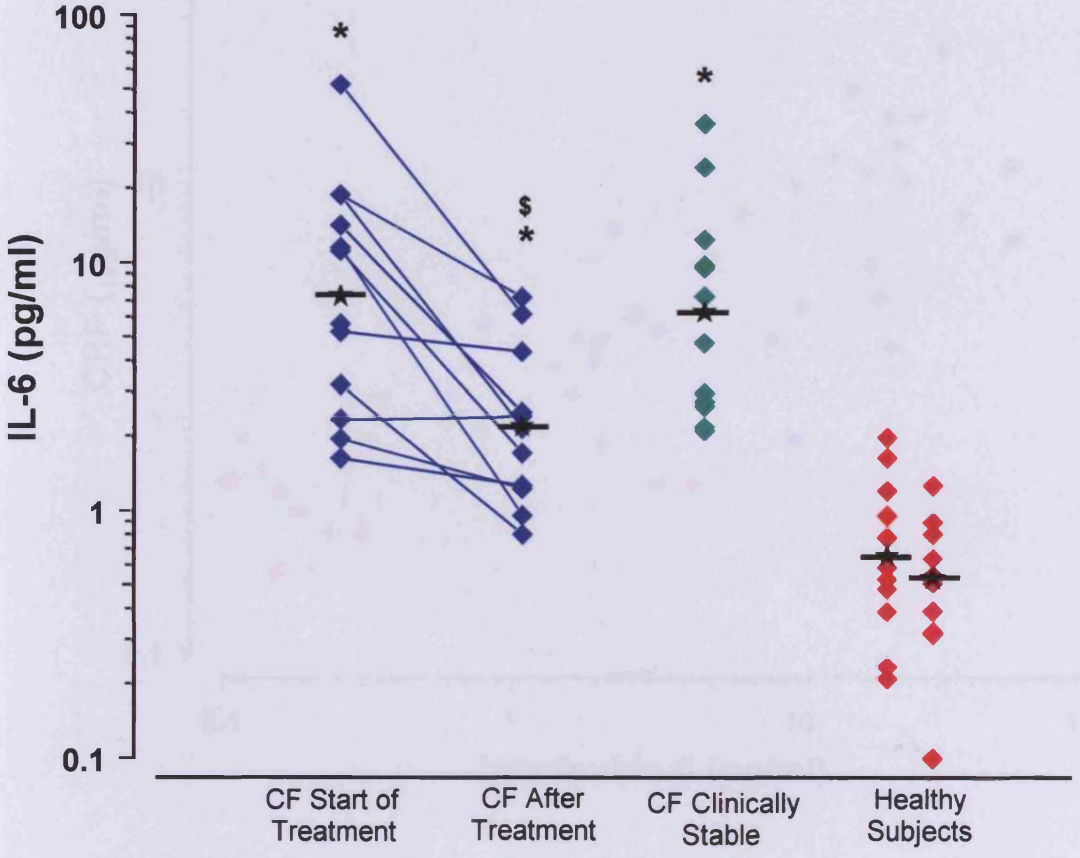
* p<0.001 Patients with CF compared to healthy subjects

$ p=0.001 Effect of antibiotic treatment

—✱— Geometric mean

Figure 3.2

The circulating concentration of IL-6 related to clinical status in patients with CF and healthy subjects



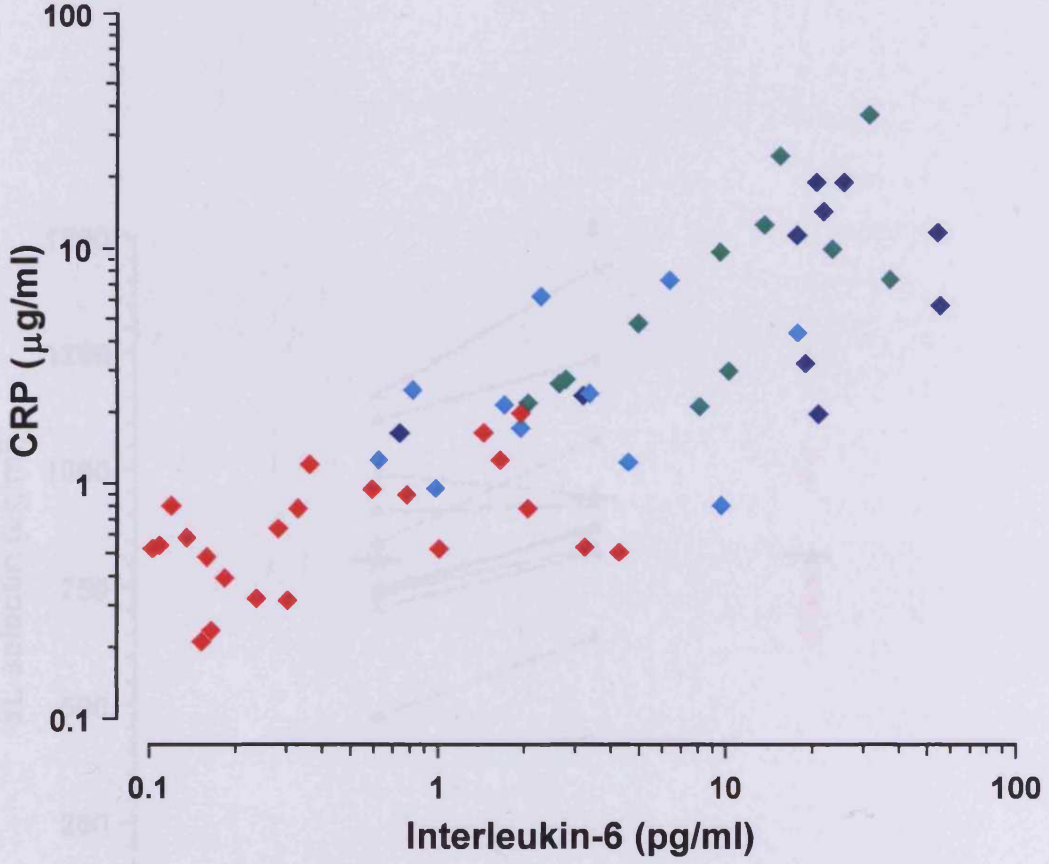
* p<0.000 Patients with CF compared to healthy subjects

$ p=0.001 Effect of antibiotic treatment

—✱— Geometric mean

Figure 3.3

Correlation of circulating levels of CRP and IL-6 in patients with CF and healthy subjects.



◆ CF start of treatment ◆ CF end of treatment

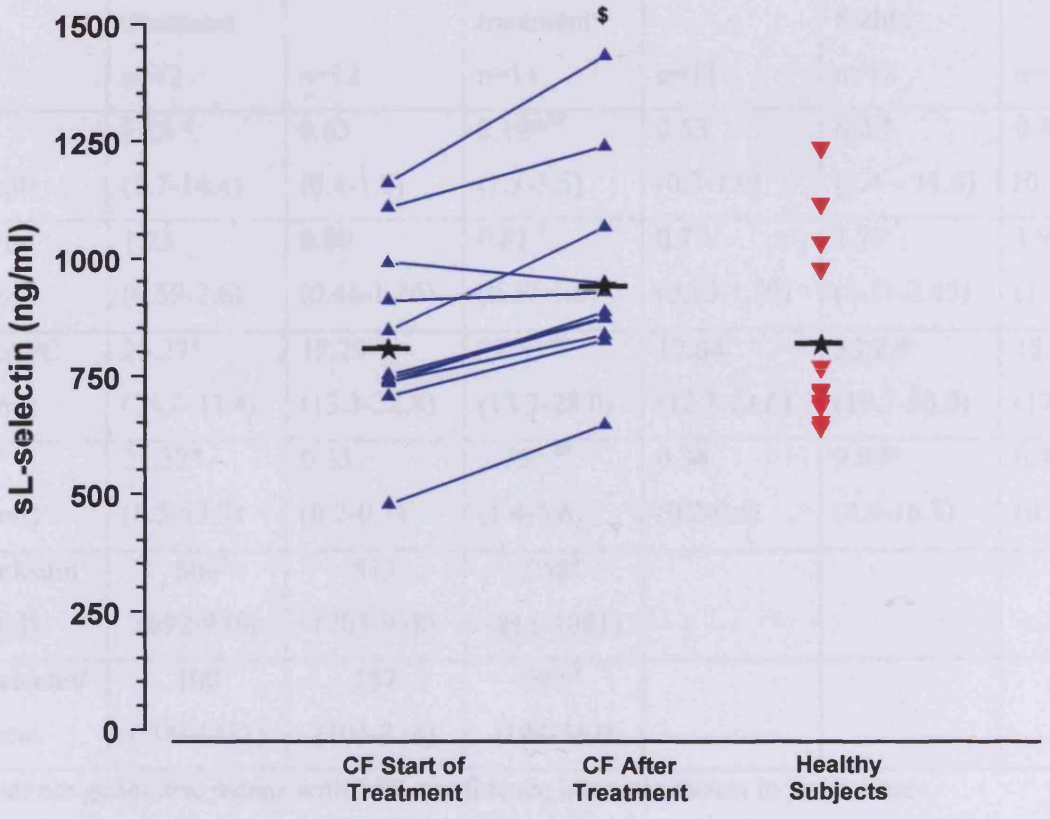
◆ CF clinically stable ◆ Healthy subject

Spearman correlation: Patients with CF (n=35); r=0.690, p<0.001

Healthy subjects (n=35): r=0.838, p<0.001

Figure 3.4

The circulating concentrations of sL-selectin related to clinical status in patients with CF and healthy subjects



$ p<0.001 Effect of treatment

—✱— Geometric mean

Table 3.3

Circulating markers of inflammation in patients with cystic fibrosis at the start of exacerbation, after antibiotic treatment and when clinically stable, and in healthy subjects

	Exacerbation		Exacerbation		Stable	
	Patients before treatment n=12	healthy subjects n=12	Patients after treatment n=11	healthy subjects n=11	Patients Clinically Stable n=12	healthy subjects n=12
IL-6 (pg/ml)	7.28 * (3.7-14.4)	0.65 (0.4-1.0)	2.16*$# (1.3-3.5)	0.53 (0.3-1.0)	6.2 * (3.4 – 11.4)	0.49 (0.3-0.7)
TNFα (pg/ml)	1.23 (0.59-2.6)	0.80 (0.46-1.36)	0.81 # (0.37-1.8)	0.77 (0.33-1.79)	1.79 (1.31-2.45)	1.98 (1.19-3.27)
hNEAPC (ng/ml)	29.27* (24.2-35.4)	17.29 (13.3-22.4)	22.63 $ (18.3-28.0)	17.04 (12.3-23.6)	33.2 * (19.7-56.0)	15.73 (12.2-20.3)
CRP (μg/ml)	21.32* (8.5-53.3)	0.33 (0.2-0.7)	2.75* $# (1.4-5.6)	0.34 (0.2-0.6)	9.09* (4.9-16.8)	0.46 (0.2-1.1)
sL-selectin (ng/ml)	804 (692-935)	812 (703-938)	958$ (811-1081)			
sL-selectin/ 10^6neut.	100 (709-1431)	157 (101-216)	265$ (194-362)			

Values are geometric means with 95% confidence intervals shown in parentheses

* $p<0.01$ Patients compared with healthy subjects (unpaired t test on log transformed data)

$ $p<0.01$ Effect of antibiotic treatment (paired t test on log transformed data)

# $p<0.05$ Patients after antibiotic treatment compared with clinically stable (unpaired t test on log transformed data)

Table 3.4

Circulating markers of inflammation in patients with COPD at the start of exacerbation, after antibiotic treatment and in age matched healthy subjects

	Exacerbation		
	Patients before treatment n=25	Patients after treatment n=23	healthy subjects n=26
IL-6 (pg/ml)	4.57 * (2.8-7.5)	2.75 $ (1.7-4.5)	1.86 (1.5-2.4)
TNFα (pg/ml)	1.58 * (0.97 -2.6)	1.348 * (0.91-2.0)	0.8 0 (0.46-1.36)
hNEAPC (ng/ml)	44.67 * (31.6-63.1)	46.77 * (34.8-62.9)	10.47 (7.1 –15.6)
CRP (μg/ml)	12.88 * (7.1-23.3)	4.17 * $ (1.9-9.2)	1.48 (0.95-2.3)

Values are geometric means with 95% confidence intervals shown in parentheses

* $p<0.05$ patient compared with healthy subjects (unpaired t test on log transformed data)

$ $p<0.01$ patient compared before and after antibiotic treatment (paired t test on log transformed data)

Airways compartment

Mean total protein concentration in sputum sol did not change with clinical state in patients with CF. The concentrations of inflammatory mediators were expressed per mg of total protein (Table 3.5). There were insufficient amounts of sputum-sol phase from some patients to carry out all the analyses. The concentration of protein was reduced by antibiotic treatment in the patients with COPD ($p=0.003$) (Table 3.5).

IL-6 values increased with antibiotic treatment in both the patients with CF and with COPD, although this was only significant in the COPD group ($p=0.046$) (Figure 3.5).

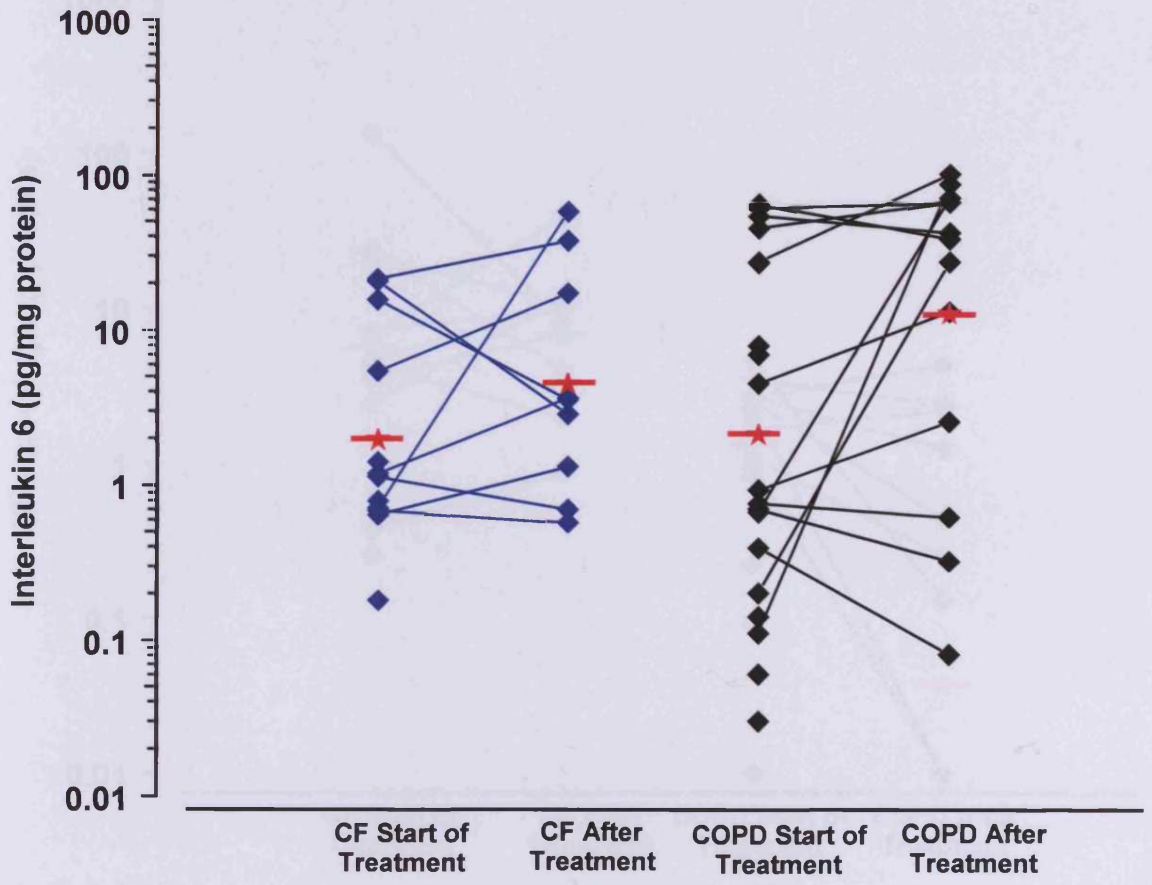
Sputum sol hNE and TNFα were not reduced with antibiotic treatment in patients with CF. Contrastingly a reduction was seen in the patients with COPD for both markers ($p=0.003$ and 0.024 respectively) (Figure 3.6, Table 3.5).

The concentrations of the markers measured were consistently greater per g sputum (equivalent to per ml) than in plasma or serum; mean IL-6 (x19), TNFα (x182), NE (x5800) for patients with CF and mean IL-6 (x73), TNFα (x52), NE (x340) for patients with COPD. This ratio was significantly different for hNE between patients with CF and COPD at the start ($p=0.006$) and end ($p<0.001$) of antibiotic treatment and for TNFα at the end of treatment ($p=0.018$) (Figure 3.7).

Antibiotic treatment of a respiratory exacerbation in 10 patients revealed that change in sputum NE and sputum IL-6 were inversely related ($r=-0.683$, $p=0.05$). Mean (per g sputum) NE fell from 18.8 to 10.1 μg, and IL-6 increased from 5.8 to 13.7 pg. Circulatory levels of hNEAPC and IL-6 both fell in the same group of patients.

Figure 3.5

IL-6 concentration in Sputum sol of patients with CF and COPD

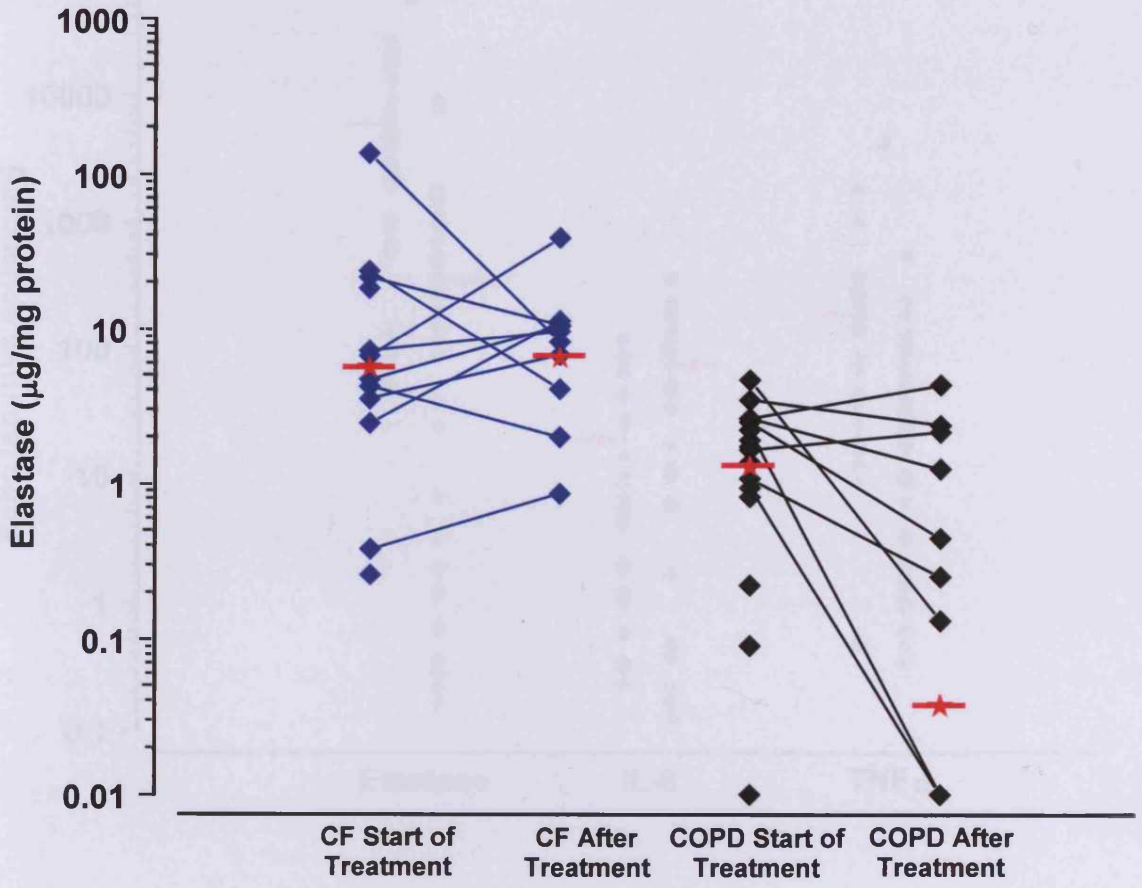


Geometric Mean

Significant increase in IL-6 with treatment in patients with COPD (p=0.046) (paired t test on log transformed data)

Figure 3.6

Elastase concentration in Sputum sol of patients with CF and COPD

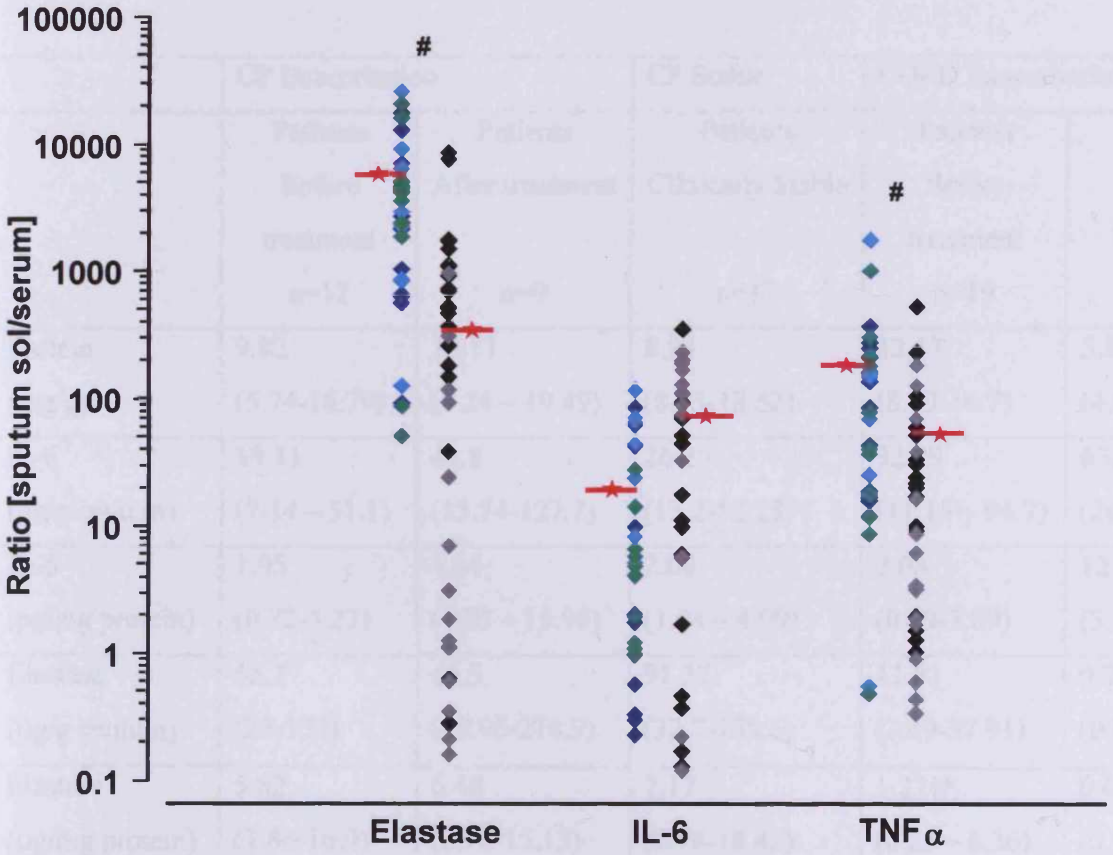


Geometric Mean

Significant decrease in elastase with treatment in patients with COPD (p=0.003) (paired t test on log transformed data)

Figure 3.7

Ratio of concentration in Sputum sol to the circulation in patients with CF and COPD



CF: ◆ Start of Treatment, ◆ After treatment, ◆ Clinically Stable

COPD: ◆Start of Treatment, ◆ After treatment

—✳— Geometric Mean

p ≤ 0.001 CF compared to COPD (Mann Whitney U Test)

Table 3.5

Sputum sol markers of inflammation in patients with cystic fibrosis and COPD at the start of exacerbation, after antibiotic treatment and when clinically stable

	CF Exacerbation		CF Stable	COPD Exacerbation	
	Patients Before treatment n=12	Patients After treatment n=9	Patients Clinically Stable n=11	Patients Before treatment n=19	Patients After treatment n=14
Protein (mg/g)	9.82 (5.74-16.79)	10.11 (5.24 – 19.49)	8.93 (8.73-18.62)	12.17 (8.83-16.7)	5.81[$] (4.06-8.30)
IL-6 (pg/g sputum)	19.11 (7.14 – 51.1)	44.8 (15.74-127.7)	26.25 (13.2-52.23)	32.49 (11.15 – 94.7)	63.2 (20.8-192.1)
IL-6 (pg/mg protein)	1.95 (0.72-5.27)	4.44 (1.23 – 15.98)	2.06 (1.04 – 4.09)	2.08 (0.59-7.09)	12.32 (3.16 - 47.96)
Elastase (μg/g sputum)	55.2 (23-132)	65.5 (19.96-214.9)	91.37 (32.7-255.6)	11.81 (3.69-37.91)	0.302[$#] (0.061 – 1.50)
Elastase (μg/mg protein)	5.62 (1.86-16.9)	6.48 (2.78-15.13)	7.17 (2.78-18.43)	1.271* (0.25 – 6.36)	0.0374[$#] (0.007-0.19)
TNFα (pg/g sputum)	89.6 (43.0-186.6)	60.9 (20.9-177.4)	77.1 (22.49-263.9)	47.4 (21.4-104.6)	6.92[$#] (2.2-21.4)
TNFα (pg/mg protein)	9.12 (4.16- 19.9)	6.02 (1.16 – 31.1)	6.04 (1.89-19.32)	3.50 (1.79 – 6.80)	0.952[#] (0.38 – 2.37)

Values are geometric means with 95% confidence intervals shown in parentheses

[$] $p<0.05$ patient compared before and after antibiotic treatment (paired t test on log transformed data)

[#] $p<0.05$ CF compared to COPD at same clinical state (unpaired t test on log transformed data)

Relationship of circulating inflammatory markers to clinical parameters

The reduction in both CRP and IL-6 was positively related to the increase in FEV_1 (r=0.682, p=0.026 and r=0.682, p=0.021 respectively) (Table 3.6). The neutrophil count was related to circulating IL-6 in patients with CF after antibiotic treatment (r=0.733, p=0.025).

Relationship of circulating inflammatory markers to sputum inflammatory markers

Sputum TNFα (pg/g sputum) was inversely related to circulating levels (r= -0.667 p=0.050) in patients with CF after treatment of an exacerbation, but at no other time. There were no other significant correlations between circulating and sputum markers.

Relationship of sputum inflammatory markers to clinical parameters

The values for spearman correlations are shown in Table 3.6. The numbers within each group are only just sufficient for significance in a spearman correlation, and greater numbers may strengthen the statistics. Therefore all samples were pooled to determine whether stronger relationships became apparent (Table 3.7). This did confirm the trend seen with the individual groups and was as follows:

Neutrophil number was;

-ve related to lung function +ve related to circulating CRP, IL-6 and hNEAPC

FEV1, FVC were

-ve related to CRP, IL-6, hNEAPC, TNFα

+ve related to sputum NE and sputum TNFα

+ve related to sputum IL-6

Circulating CRP and IL6 were

+ve related to sputum protein, NE and TNFα

-ve related to sputum IL6

Sputum protein was

+ve related to sputum NE and TNFα

ve related to sputum IL6

Sputum IL-6 was -ve related to sputum NE and TNFα

Table 3.6

The relationship between lung function, circulating and inflammatory markers in CF and COPD.

Spearman correlation		CF			COPD	
		Before Treatment	After treatment	Clinically stable	Before treatment	After treatment
FEV_1	Sputum elastase (μg/g sputum)	r= -0.175 p=0.587	r= -0.733 p=0.016	r= -0.694 p=0.012	r= -0.013 p=0.955	r=0.004 p=0.990
	Sputum TNFα	r= -0.266 p=0.404	r=0.550 p=0.125	r= -0.569 p=0.067	r= -0.544 p=0.020	r= -0.254 p=0.361
	Sputum IL-6	r=0.573 p=0.051	r= -0.576 p=0.082	r=0.310 p=0.354	r= -0.045 p=0.370	r=0.246 p=0.377
FVC	Sputum elastase	r= -0.144 p=0.656	r= -0.733 p=0.016	r= -0.720 p=0.008	r= -0.184 p= 0.400	r= -0.018 p=0.950
	Sputum TNFα	r= -0.291 p=0.359	r=0.550 p=0.125	r= -0.418 p=0.210	r= -0.155 p=0.639	r= -0.164 p=0.558
	Sputum IL-6	r=0.585 p=0.046	r= -0.576 p=0.082	r=0.300 p=0.370	r= 0.332 p=0.164	r=0.169 p=0.548
Sputum protein	Sputum elastase	r= -0.154 p=0.633	r=0.636 p=0.048	r= 0.627 p=0.039	r=0.797 p=0.000	r=0.600 p=0.023
	Sputum TNFα	r=0.573 p=0.051	r= -0.600 p=0.088	r=0.500 p=0.117	r=0.296 p=0.233	r=0.429 p=0.126
	Sputum IL-6	r=0.413 p=0.183	r= -0.083 p=0.831	r=0.309 p=0.355	r= -0.684 p=0.002	r= -0.298 p=0.301
Sputum IL-6	Sputum elastase	r= -0.476 p=0.118	r= -0.617 p=0.077	r= -0355 p=0.285	r= -0.420 p=0.074	r= -0.577 p=0.019
Circ. CRP	Circ IL-6	r=0.497 p=0.101	r=0.182 p=0.593	r=0.769 p=0.003	r=0.508 p=0.008	r=0.668 p=0.000

Table 3.7

Relationship of clinical, circulating and sputum measures in the study group as a whole including patients with CF, patients with COPD and healthy subjects

		NEUT	FEV1	FVC	CRP	hNEAPC	IL6	TNF	Spu PRO	Spu NE	Spu IL6	Spu TNF
NEUT	Corr. Coeff. Sig. (2-tailed) N	1.000 . 67	-.366 .006 55	-.438 .001 55	.517 .000 67	.437 .000 67	.559 .000 67	.106 .393 67	.196 .299 30	-.003 .986 31	-.124 .521 29	.266 .163 29
FEV1	Corr. Coeff. Sig. (2-tailed) N		1.000 . 101	.895 .000 101	-.673 .000 101	-.459 .000 100	-.630 .000 101	-.239 .016 101	-.240 .053 66	-.318 .007 70	.304 .014 65	-.345 .005 65
FVC	Corr. Coeff. Sig. (2-tailed) N			1.000 . 102	-.606 .000 102	-.504 .000 101	-.596 .000 102	-.184 .065 102	-.220 .074 67	-.100 .407 71	.299 .015 66	-.144 .249 66
CRP	Corr. Coeff. Sig. (2-tailed) N				1.000 . 146	.508 .000 144	.824 .000 119	.269 .001 145	.300 .012 70	.227 .055 72	-.294 .016 66	.311 .011 66
hNEAPC	Corr. Coeff. Sig. (2-tailed) N					1.000 . 144	.519 .000 118	.199 .017 144	.038 .754 69	-.396 .001 71	-.063 .616 65	-.121 .335 65
IL6	Corr. Coeff. Sig. (2-tailed) N						1.000 . 119	.296 .001 119	.407 .000 70	.430 .000 72	-.368 .002 66	.268 .030 66
TNF	Corr. Coeff. Sig. (2-tailed) N							1.000 . 145	.202 .094 70	.067 .575 72	-.103 .412 66	.060 .631 66
Spu Pro	Corr. Coeff. Sig. (2-tailed) N								1.000 . 70	.518 .000 67	-.286 .022 64	.285 .022 64
Spu NE	Corr. Coeff. Sig. (2-tailed) N									1.000 . 73	-.514 .000 67	.539 .000 66
Spu IL6	Corr. Coeff. Sig. (2-tailed) N										1.000 . 67	-.325 .008 65
Spu TNF	Corr. Coeff. Sig. (2-tailed) N											1.000 . 66

CRP in µg/ml, NE as ng/ml or µg/g spu, IL-6 and TNFα as pg/ml or pg/g sputum, and protein as mg/g sputum.

significant +ve correlation, significant –ve correlation.

Discussion

The results obtained with hNEAPC and CRP at a time of commencing antibiotics for an exacerbation of respiratory symptoms in our patients confirms earlier reports [Rayner *et al* 1991, Norman *et al* 1991, McGraph *et al* 1999]. The results from our patients with COPD have been confirmed in recent reports [Wouters *et al* 2002]. Circulating immuno-reactive IL-6 showed parallel changes with those of CRP and hNEAPC, being highest at a time of exacerbation of respiratory symptoms, as well as being raised compared to healthy subjects at a time of clinical stability. The raised circulating IL-6, CRP and hNEAPC concentrations following antibiotic treatment and at times of clinical stability supports evidence for a continued inflammatory process .

Circulatory compartment

IL-6 is an important cytokine in the host inflammatory response, in particular the acute phase response, as evidenced by increased CRP secretion through an endocrine action on hepatocytes . This relationship was supported by the correlation we found between circulating concentrations of IL-6 and CRP, and their parallel reduction following antibiotic treatment. A similar relationship between IL-6 and CRP occurred in rheumatoid arthritis and both decreased with treatment [Cohick *et al* 1994]. The raised circulating concentration of IL-6, a β-cell regulator, may also contribute to the hypergammaglobulinaemia that occurs in CF. Circulating cytokine concentrations have been linked to abnormalities in metabolism, including increased resting energy expenditure, weight loss, osteoporosis and altered metabolism is CF [Elborn *et al* 1993CS, Bell *et al* 2000, Ionescu *et al* 2000, Aris *et al* 2000]. We have previously reported increased circulating concentrations of TNFα in CF [Norman *et al* 1991, Bell *et al* 1995, Ionescu *et al* 2000] and COPD [Eid *et al* 2001]. The lack of significant changes found in this study in patients with CF and COPD may reflect the small group sizes. From this study IL-6 appears easier to measure reliably, and is more often detected in plasma than TNFα. The clear separation between patients and healthy subjects, the reduction in concentrations after antibiotic treatment, and its close correlation with CRP, supports the use of IL-6 as a circulating marker of inflammation in CF while confirming findings in other infections.

The origin of circulating immuno-reactive TNFα and IL-6 is unclear, because they are produced by a variety of cell types in various compartments, such as the lung interstitium, the airspace or the vascular compartment, and in response to many different stimuli, such as circulating antibody-bacterial endotoxin complexes and hypoxemia [Ertel *et al* 1995, Lang *et al* 2003]. It is possible that these cytokines represent activation signals from T and B lymphocytes, endothelial cells, fibroblasts, monocytes, macrophages [Song *et al* 2001] or epithelial cells. Whatever their origin they probably indicate lung injury is occurring. External to the lung they may act as regulators of the inflammatory, energy and metabolic responses to injury or infection. In trauma or systemic sepsis such cytokines are likely to regulate the acute phase inflammatory and catabolic responses [Majetschak *et al* 2002, Streetz *et al* 2001], and blockade using TNF Receptor improves outcome of sepsis [Ramick *et al* 2001]. In the chronic setting it is less clear how important such factors may be in systemic adaptations, such as cachexia and lesser degrees of weight loss, that characterize CF, despite appropriate pancreatic enzyme replacement and dietary supplementation, and COPD [Eid *et al* 2001, Schols *et al* 1996]. It is difficult to comment precisely on the role of cytokines in the context of this study, as we did not measure bio-activity, soluble receptors of cytokines, natural inhibitors or immunoglobulins, which may be important in modulating the biological impact of such cytokines in the circulation. However, this data, in the context of that linking the inflammatory and metabolic responses to Gram-negative sepsis, suggests that there are likely to be important inter-relationships.

L-selectin is shed from the cell surface on adherence prior to migration [Halfezi-Moghadam *et al* 2001], and therefore it may be assumed that sL-selectin levels would rise during infection. Contrastingly we have observed a lower level of sL-selectin in the circulation. Maekawa *et al* [1998] showed that sL-selectin peaked initially (by about 3 hours in trauma), then fell after 24 hours. It is likely that our data is reflective of the response after 24 hours, since the blood was taken the day after admission of the patient. This may be a reflection of chronic infection, and long term continuous low level exposure to stimulatory agents such as LPS. However, issues relating to stability in plasma, and other sources, such as endothelial cells [Collett *et al* 1999] must be also considered, as well as possible differences in the nature of the neutrophils present in chronic infection.

The airways compartment

The amount of protein in sputum remained relatively constant in patients with CF, but was reduced by antibiotic treatment in the patients with COPD. Wolter *et al* [1999] showed a small but significant increase in sputum protein with exacerbation in a group of patients with CF. In patients with COPD Stockley *et al* [2000T] showed inflammatory mediators, including hNE and IL-8, in sputum correlated positively with protein leak. We also saw a positive relationship between sputum protein and hNE. The pattern of change for hNE concentrations was similar to the change in protein concentration i.e. a reduction in hNE in the patients with COPD, but not in those with CF. There have been several papers linking increased hNE with increased bacterial load [Llobres 1992, Stockley *et al* 2000C, Armstrong *et al* 1997] and these data may reflect the more chronic colonisation of the patients with CF, and the continuous presence of bacteria such as mucoid *P.aeruginosa* colonies which are largely unaffected by antibiotic treatment [Drenkard & Ausubel 2002]. Increased cytokines have been shown in young patients with CF and no measurable infection [Bonfield *et al* 1995, Balough *et al* 1995], suggesting that inflammation may not be resolved by removal of bacteria, and that the continual presence of hNE is due to the genetic nature of CF. Host anti proteinases, such as α_1-antitrypsin are inactivated by binding to elastase [Brown *et al* 1994] and by pyocyanin produced by *P.aeruginosa* [Britigan *et al* 1999] allowing active hNE to break down the lung tissue [Cochrane *et al* 1988]. In addition there are genetic differences in the form of α_1-antitrypsin, the patients with the 1237A allele were found to have better indices of pulmonary disease progression, and a lesser propensity to infection than those without. This may account for some of the variation in lung disease between patients with the same CFTR genotype [Henry *et al* 2001], and also may explain some of the heterogeneity of patients with COPD particularly in involvement of emphysema [Cuvelier *et al* 2000]. From these results we speculate that for a given lung function, there may be more damage to the lungs of patients with CF, giving a more constant flow of protein into the lungs. Alternatively, this may be related to the nature of CF and the increased sputum production due the genetic nature of the disease. It is most likely that the protein content of sputum is due to a combination of inflammation, lung damage, and genotype.

Levels of IL-6 and TNFα showed a similar pattern in both patients with CF and those with COPD unlike the protein and hNE concentrations. The levels of IL-6 in the sputum increased with

antibiotic treatment, significant for the group as a whole. IL-6 is degraded by proteases at sites of inflammation [Bank 1999] where the predicted bioactivity was much lower than the immunoreactive measures of IL-6. This degradation was found to occur predominantly in the membrane and granule fractions of neutrophils. Elastase has also been show to inactivate IL-8 [Leavell *et al* 1997]. IL-6 was higher in patients with better lung function, negatively related to serum IL-6 and CRP and sputum hNE and TNFα. BALF IL-6 was increased in young patients with CF [Bonfield *et al* 1995, Noah *et al* 1997]. The differences between the circulating and sputum concentrations of IL-6 suggest IL-6 may have different roles at different stages of infection and in different parts of the body. Ulich *et al* [1991] using a rat model of lung inflammation suggested an anti inflammatory role for IL-6. Sputum TNFα showed a similar pattern to serum concentrations. We did not see a fall in sputum TNFα concentrations in patients with CF, however there was a significant fall in patients with COPD. This is similar to the pattern seen with hNE. Wolter *et al* [1999] also found that levels of TNFα in sputum of patients with CF were raised but did not change much with treatment of an exacerbation.

Use of sputum sol

Sputum is a heterogeneous material and has not been considered a particularly good medium for measurements of inflammatory mediators. Adults with CF do not tolerate broncheoalveolar lavage (BAL) well, and BALF which can be obtained contains thick mucus plugs. Performing BAL is also linked to a small risk, and is an additional procedure which this group of patients are reluctant to undergo without clinical relevance. Sputum can still give insight into the inflammatory processes within the lung, without the other drawbacks. Healthy subjects do not produce sputum, so comparisons are not easily made, but healthy subjects do not experience the lung infection and the associated inflammatory status of the lungs. Sputum production can be induced by nebulised saline however sputum obtained in this way may not be physiologically relevant. When expectorated sputum was compared with BALF and induced sputum similar results were obtained for cell number and type, IL-8 and TNFα (when corrected by urea concentration) in the expectorated and induced sputum [Henig 2001]. This has been confirmed by other studies comparing induced and spontaneously produced sputum [Sagel 2001] and sputum and BALF [Dean 1993]. In young children, where there may not be copious purulent sputum production, BALF has given insights into

airway inflammation [Noah 1997, Bonfield 1995]. In older patients, and healthy subjects who do not produce sputum, induced sputum provides both a greater volume in producers, and a suitably sized sample in subjects who do not spontaneously expectorate sputum [Keatings 1997, Belda 2000]. In this study we were particularly interested on the influence of sputum from patients with CF on neutrophil functioning, rather than the comparison with healthy subjects, and our patient group were spontaneous sputum producers.

In this study no chemicals were used to treat the sputum. Some studies looking at sputum have included enzymes such as dithiothreitol (DTT) and dornase alfa which act to solubolize sputum. It has been reported that dornase alfa (DNAse) increases cationic enzymes such as hNE by solubilizing anionic extracellular DNA which removes the source of anionic-cationic binding complexes [Kim *et al* 2001]. This study also showed that addition of DTT resulted in inaccurately low concentrations of inflammatory markers when measured by ELISA. Patients using DNAse may have lower levels of DNA and higher levels of free elastase. Only one patient in the study was on DNAse (in the CF clinically stable group).

The findings of high concentrations of cytokines and neutrophil elastase in sputum-sol supports and extends reported data from sputum and bronchoalveolar lavage. It is unclear from these data as to which compartment strictly reflects inflammatory activity in the lung. Both compartments give indirect information relating to lung tissue. The high levels of neutrophil elastase shown in sputum has implications in the degradation of lung tissue, which would have a detrimental effect on lung function. High concentrations of cytokines in sputum may reflect production either in the tissue compartment or by airways cells including macrophages. Levels of both TNFα and IL-6 were found to be higher in sputum than in blood despite the high levels of proteases in sputum which could potentially break down these cytokines. There is a difference in matrix, but the concentrations are different enough to draw conclusions that there is a significant inflammatory load in the lungs. It has been argued because of this, that the airways compartment is a better index of the inflammatory response than the circulation. Various factors including sampling factors and heterogeneity of sputum need to be considered. The lack of relationship between circulating and sputum concentrations for elastase and IL-6 suggests that there is local 'production' rather than leak between the circulation and the lungs. The difference (negative correlation) between the circulating

and sputum concentration of IL-6 seems to quite clearly indicate that there is a different source, and possibly a different stimulus for production.

Comparison of CF and COPD

Many parallels can be drawn between these two diseases. By including patients with COPD into this section of the study we were able to draw parallels with CF and identify some differences. The two groups were comparable, although the patients with CF were slightly more severe having a mean FEV_1 around 10% predicted lower than the COPD group.

The majority of inflammatory markers measured behaved similarly in the two disease states, reflecting the impact of chronic lung infection. Circulating CRP, IL-6 and hNE were all raised at a time of pulmonary exacerbation compared to healthy subjects, and fell with antibiotic treatment. Mean values were higher for CRP and IL-6 in the patients with CF compared to COPD although not significantly so. and this is probably a reflection of the slightly more severe nature of the CF patient group.

The circulating hNEAPC was greater in COPD than CF and antibiotic treatment did not reduce the concentration in COPD as had occurred in CF. Sputum elastase also behaved differently between the two groups. Sputum sol elastase was much higher in patients with CF than COPD and unaffected by treatment. The sputum elastase in COPD was significantly reduced by treatment. This pattern was also seen for sputum TNFα. Sputum protein was also reduced with treatment of an exacerbation of COPD by not in CF. This suggests that there is less resolution of the ongoing neutrophil mediated inflammatory response in patients with CF than COPD. With only a small difference in severity of lung disease assessed by lung function of the groups it seems unlikely that this is the sole case. We therefore conclude that the neutrophil mediated inflammation in CF is not effectively resolved in the way seen in patients with COPD. This highlights a potential difference in the two diseases with regard to neutrophil functioning or behaviour. The elastase appears to be continuously released by neutrophils into the lungs of the patients with CF, not reduced with antibiotic treatment, as if the recruitment of neutrophils to the lungs is not reduced even when systemically inflammatory markers are. This could be related to the nature of the infections, as

P.aeruginosa is able to induce elastase release [Jensen *et al* 1990], however a reduction of *P.aeruginosa* should have occurred with antibiotic treatment.

The neutrophil will experience the effects of inflammatory markers in all compartments as it travels from the circulation into the lungs. CRP has been shown to reduce superoxide generation which is likely to be via p47-phox, a component of the NADPH oxidase complex [Mortensen 2000]. Neutrophil products such as lysozyme and elastase are thought to act by a negative feedback [Gordon *et al* 1979, McElvaney *et al* 1992] and also cleaves some cell surface proteins [ref]. IL-6 bioactivity is considerably reduced compared to IL-6 concentration predicted by ELISA. Elastase, proteinase 3 and cathepsin G can all lead to this degradation, and IL6SR is protective against the effects of proteinase 3 but not elastase [Bank *et al* 2000]. TNFα and IL-8 have been reported to increase bactericidal activity of neutrophils [Simms *et al* 1997]. Hence the information obtained in this chapter should be considered in further chapters of this thesis.

The host inflammatory response is a co-ordinated host defence process. It acts to protect the local site of injury or insult as well as to protect the whole organism, therefore it is not strictly compartmentalised although it is conceptually easier to think that way. It is understood that there is constant movement of markers both from the circulation through the interstitium and into the airspace, and of markers from the airspace arriving back in the circulation. A lot of the control of the host response via the circulation is through the production of various hepatic proteins. The amount of movement that occurs, the rate, and the time spent and effect on the interstitium is not understood. It is not possible to study this directly in humans, therefore we have relied on the comparison of the two compartments. Further pieces of this complex jigsaw are completed with the help of animal studies and in vitro cell culture methods. Data from this study suggests that both compartments reflect the inflammatory response, but give different information likely to be dependent on different cellular components of each compartment and on temporal and spatial factors involved in regulation of the inflammatory response.

We have shown evidence for increased concentrations of the circulating inflammatory markers CRP, hNEAPC, IL-6 and TNFα in patients with CF which have the potential to affect the functioning of circulating neutrophils. Concentrations of CRP, IL-6 and TNFα were reduced by

antibiotic treatment and therefore their effects may be less on neutrophils after treatment. Concentrations of hNE, IL-6 and TNFα were higher in sputum sol that in the circulation, indicating local production or movement into the lungs. A similar pattern of inflammatory markers was seen in the patients with COPD, with some small differences, which suggests that much of the host inflammatory response is the result of chronic pulmonary infection.

Chapter 4

Phagocytosis and Intracellular Killing of *Pseudomonas aeruginosa* by human neutrophils and the influence of cystic fibrosis

Aim

To determine whether neutrophils from patients with CF can phagocytose and kill *P.aeruginosa* in vitro and compare this to the effectiveness of neutrophils from healthy subjects.

To determine whether the presence of sputum sol affects the phagocytosis and killing of *P.aeruginosa by* neutrophils from patients with CF and healthy subjects.

Introduction

Phagocytosis is a process where foreign particles are bound by specific phagocytic receptors and are very rapidly engulfed by circulating or tissue based phagocytic cells. Phagocytosis is triggered by the binding of serum-opsonized microorganisms through opsonin receptors or by the binding of non-opsonized micro organisms mostly through lectin-sugar recognition. After phagocytosis, the bacteria are exposed to a number of cytotoxic products, the process of intracellular killing. This is primarily through two microbial processes; NADPH oxidase-dependent production of O_2^-, a precursor of other reactive oxygen species (ROS), and degranulation, which involves the release of azurophil granule contents and other granule types into phagosomes [Cougoule *et al* 2002]. Neutrophils account for the majority of phagocytosis and killing of bacteria in the body, however monocytes and macrophages are also capable of this function. The fact that killing is primarily by ROS has been supported by the fact that neutrophils from patients with chronic granulomatous disease are unable to produce ROS, or to kill microbes efficiently [Reeves *et al* 2002]. Elastase has been shown to be necessary in the killing of *E.coli*, acting by degradation of the bacterial surface protein [Belaaouja *et al* 2000].

Multiple pathways are involved in activating the phagocytic and killing mechanisms. If neutrophil function were completely controlled by a single signalling system, then a defect in that system

would have devastating consequences. In addition, the cytotoxic products are highly lethal towards host tissues. The pathogen will try and overcome host defences, which adds further weight to the need for multiple pathways. Complex intracellular signalling mechanisms to activate these cytotoxic pathways also guard against non-specific activation, which could lead to host tissue damage. Frustrated phagocytosis is the release of cytotoxic products extracellularly if the pathogen is too large to be fully enclosed within a phagocytic vesicle, and occurs with *P.aeruginosa* microcolonies and in biofilm [Govan 1996]. This extracellular release may also occur if the vesicles do not adequately seal during phagocytosis. Release of cytotoxic agents into the surrounding area may also occur when the neutrophil is exposed to certain stimulants. The cytotoxic factors involved are discussed in chapters 1, 5 and 6.

Two distinct receptor-linked phagocytic pathways, one with antibody receptors (FcRs, FcR, Fc receptor) and the other with complement receptors (CRs), mediate binding and ingestion of pathogens by human neutrophils [Kobayashi *et al* 2002]. Complement receptor 3 (CR3) is also able to mediate both opsonic and nonopsonic phagocytosis of *P.aeruginosa* [Heale *et al* 2001].

Bacterial Factors involved in colonisation of patients with cystic fibrosis

Patients with CF experience bacterial infections of the airways from an early age. The main colonizing bacteria are *P.aeruginosa, Staphylococcus aureus* and *Haemophilus influenzae* [Lyczak *et al* 2002]. In patients with CF 80% are colonised by the age of 20 with *P.aeruginosa* [Hutchinson 1999], hence for this study *P.aeruginosa* was thought to be the most relevant organism to use. Pulmonary colonisation with *P.aeruginosa* is usually preceded by infection with bacteria such as *S.aureus* and *H.influenzae*, but there is increasing evidence that *P.aeruginosa* binds preferentially to the surface of epithelial cells from the lungs of patients with CF [Saiman *et al* 1993], supported by reduction of binding following in vitro gene transfer [Davies *et al* 1997]. This binding is thought to take place via pili on the epithelial cells supported by the fact that antibody to purified pili prevents the adherence of organisms to buccal epithelial cells [Woods *et al* 1980], although binding to mucin may also play a role [Scharfman *et al* 1996]. *P.aeruginosa* is also thought to take advantage of the mucus rich environment in the lungs [Deretic *et al* 1994], and its products may act as a secretagogue in airways [Kishioka *et al* 1999, Li *et al* 1998]. The susceptibility of patients with CF has also been

directly linked to to the CFTR protein which acts as a receptor for *P.aerugniosa*, and its enhanced binding to epithelial cells and allowing internalization of the microbe [Goldberg & Pier 2000].

Why the lungs of patients with CF become infected and stay infected not only with pathogens, but with pathogens unique to the disease is gradually becoming understood. A number of local host and bacterial factors combine to allow the establishment of the pathogen. In *P.aeruginosa* alginate biosynthesis is usually repressed, hence typical isolates are non mucoid when cultured on agar media. A number of genes are known to be involved in the control of mucoidy including AlgU and Alg D [Govan *et al* 1992]. Chronic colonization of the CF respiratory tract with *P.aeruginosa* is characterized by a gradual transit of the pathogen from a non-mucoid to a mucoid phenotype. Mucoidy is the result of increased transcription from the algD gene and is activated by the transcriptional regulator AlgR [Lizewski *et al* 2002]. Initially invading non-mucoid strains convert into the mucoid, exopolysaccharide alginate overproducing strain. This allows the *P.aeruginosa* to form biofilm microcolonies, where the exopolysaccharide barrier provides protection from host defences and antibiotics. This conversion is selected preferentially in the presence of toxic oxygen products produced by neutrophils [Mathee *et al* 1999]. The biofilm mode of growth is the survival strategy of environmental bacteria like *P.aeruginosa* [Hoiby *et al* 2001]. Mucoid strains of *Klebsiella ozaenae* and *Eschericia coli* have also been recovered from the sputum of patients with CF suggesting that the environment of the CF lung is conductive to the induction of a mucoid state by gram-negative bacilli. Although colonisation of the CF respiratory tract by bacteria can reach substantial numbers (10^8 bacteria /ml sputum) the infection does not spread to other sites, which indicates that systemic immunity against the organism is intact. Colonization of the lungs often leads to changes in strain to mucoid, and the switching 'on or off' of a number of virulence factors and enzymes. It also seems likely that antibiotic resistance and the formation of biofilms may be linked [Drenkard *et al* 2002], and these authors propose that environment driven phenotypic switching explains how antibiotic-resistant biofilm variants of *P.aeruginosa* arise.

Colonization of the lungs with *P.aeruginosa* is associated with a marked antibody response. IgG antibodies, IgA and secretary IgA have been seen to be fragmented in the bronchial secretions of patients with CF. *Pseudomonas* elastase (not hNE) and alkaline protease have been shown to cleave IgG and IgA respectively *in vitro* [Fick *et al* 1985]. These findings imply that specific antibodies to

P.aeruginosa (IgG and IgA) although appropriately increased in patients with CF, may be rendered useless by the microorganisim. The proteolysis of antibodies by bacteria may induce an increase in antibody production, giving a local increase in concentration of immune complexes, which in turn may enhance the promotion of tissue destruction in the CF lung [Buret *et al* 1993]. This has also been shown by Doring [Doring *et al* 1984, Kharazmi *et al* 1984]. Other mechanisms must be involved in the apparent ineffectiveness of the antibody response to *P.aeruginosa* in CF; shielding by bacterial biofilm / mucoid exopolysaccharide, the unusual amount and consistent production of host mucus in CF [Buret *et al* 1993].

P.aeruginosa has been shown to bind preferentially the pilli on CF bronchial epithelium when compared to controls [Saiman *et al* 1992]. AsialoGM1 is a receptor on the surface of respiratory epithelial cells for pilli, a major *P.aeruginosa* adhesion. The numbers of asialoGM1 were greater on CF epithelial cells than controls, and numbers were seen to be further increased by exposure of CF cells to *P.aeruginosa* exoproducts [Saiman *et al* 1993]. *B.cepacia* isolates have also been reported to bind to respiratory epithelial cells, probably through polar pili [Kuehn *et al* 1988]. Complement receptor 3 (CR3) mediates both opsonic and nonopsoinc phagocytosis of bacteria. In patients with leukocyte adhesion deficiency 50% of the *P.aeruginosa* strains could not be ingested by these CR3 deficient cells. The remainder of phagocytosis was blocked by antibodies to CD14 [Heale *et al* 2001]. *P.aeruginosa* aids its survival when it forms biofilms, as these only induce 25% of the respiratory burst than for free bacteria [Jensen *et al* 1990].

P.aeruginosa is able to produce a number of cell-associated and extracellular virulence factors which include lipopolysaccharide, alginate, pilli, exotoxin A, exoenzyme S, rhamnolipid and elastase [Fogle *et al* 2002]. Rhamnolipid, although a cellular toxic substance, has recently been proposed as playing an important role in the maintenance of biofilm architecture [Davey *et al* 2003]. Many of these are greatly reduced in alginate-secreting mucoid cells isolated from the lungs of CF patients [Kamath *et al* 1998]. Pyocyanin and 1-hydroxyphenazine are low molecular-weight phenazine redox pigments produced by *P.aeruginosa*, and are found in the sputum of infected patients. 1-hydroxyphenazine potentiates the release of primary granule enzymes with the potential of chronic futile inflammatory responses, resulting in inflammation-mediated tissue damage [Ramafi *et al* 1999].

Smith *et al* [1996] showed that epithelial cells in culture were able to kill up to 10^3 cfu *P.aeruginosa* in culture, however epithelial cells with the CFTR defect were unable to kill bacteria under the same experimental conditions. When CFTR was corrected on CF epithelia using a recombinant adenovirus the defect in killing of *P.aeruginosa* was corrected. When epithelia were washed the fluid from CF and normal epithelia killed the organisms, suggesting that CF epithelia do not in fact lack a bactericidal factor. They found that as the concentration of NaCl increased, the ability to kill both *P.aeruginosa* and *S.aureus* was significantly depressed or even abolished.

The *P.aeruginosa* induced reduction in immune efficacy may not dramatically affect the eradication of the pathogen in a subject with normal pulmonary function. In the CF lung however, this impairment of immunity may be sufficient to compromise protection because of the other 'abnormal' elements of the environment such as the usual viscosity of the host mucus, the enhanced bactericidal adherence to CF epithelia, the impairment of mucocilliary clearance, and perhaps other factors.

Method

Samples were collected from patients and healthy subjects (Study A, Chapter 2.1.2). The inflammatory status of the patients was described in Chapter 3. Phagocytosis and intracellular killing were determined as described in 2.2.9 and 2.2.10 respectively.

Protocol

Phagocytosis rates were obtained in triplicate in the presence of buffer and three concentrations of sputum-sol. The control for assay conditions in the absence of neutrophils consisted of duplicate determinations for each component (absence, and presence of 3 concentrations of sputum). Each incubation mixture was cultured separately to obtain a colony count representing the number of colony forming units.

Initially it was hoped to use sputum sol concentrations of 1:2, 1:5, and 1:10. The amount of sputum available meant that higher dilutions (1:20 and 1:40) were carried out in some instances (Table 4.1). Three dilutions were tested for each patient. The methodology meant that the final dilution in the assay for sputum was double that added (i.e. 1:4, 1:10, 1:20, 1:40, 1:80).

Table 4.1

The numbers studied for each sputum dilution.

Sputum-sol dilution added	1:2	1:5	1:10	1:20	1:40
Final sputum-sol dilution	1:4	1:10	1:20	1:40	1:80
Start of Antibiotic Treatment	4	9	12	8	3
End of Antibiotic Treatment	1	9	9	9	1
Clinically Stable	4	10	12	9	2

The final sputum-sol dilution will be quoted throughout these results.

Calculation of Results for Phagocytosis

$$\% \text{ phagocytosis} = \frac{\text{mean (n=3) colony counts in presence of neutrophils}}{\text{mean (n=2) colony counts in absence of neutrophils}}$$

From this the effect of sputum was determined by calculating the change in percentage phagocytosis

% change in phagocytosis = % phagocytosis in absence of sputum sol - % phagocytosis in presence of spuutm sol

Examples are given in appendix 4.2

Calculation of Results for Intracellular killing

Intracellular killing was determined in triplicate at 0 and after 30 min. incubation in the absence and presence at each concentration of sputum-sol. The mean colony count for each triplicate was used to determine the amount of killing where the cfu for time 0 was taken as no killing. Differences were seen in the number of colonies at time '0' in different experiments, and therefore this value was used to calculate killing as a percentage. Mean colony counts are shown in Table 4.3. The effect of sputum was determined by taking the difference in % killing in the absence and presence of sputum.

Results - Phagocytosis

In the absence of sputum-sol there was no difference in phagocytosis by neutrophils from healthy subjects and those from patients with CF at any of the clinical states studied. There was no effect of sputum-sol at any clinical state or dilution studied (Table 4.2).

Table 4.2

Phagocytosis by neutrophils isolated from patients with CF and healthy subjects in the absence and presence of sputum-sol

	Start of Treatment		End of Treatment		Clinically Stable	
	Patient	Healthy subject	Patient	Healthy subject	Patient	Healthy subject
% Phagocytosis (95% CI)	65.2 (53 – 77)	56.8 (43 – 71)	52.4 (47 – 72)	51.3 (38 – 71)	63.1 (53 – 73)	57.3 (49 – 66)
1:40 sol	2.5	4.8	-0.11	-6.67	-2.0	-2.5
1:20 sol	-2.0	3.1	4.1	-3.56	0.91	2.5
1:10 sol	-4.7	5.89	-1.6	-3.2	4.8	5.7

Numbers in each group are shown in Table 4.1, and calculation of the effect of sputum sol is as described earlier.

Results - Intracellular killing

In the absence of sputum there was no difference in intracellular killing by neutrophils from healthy subjects and those from patients with CF at any of the clinical states studied. The variation between the six groups is shown in Table 4.3. The variation between the group means was much less when killing was calculated as a % of time '0' (also shown). The inter-assay Coefficient of Variation as a percentage (CV) of actual killing i.e. colony counts from the same experiment (no sputum) was 10.0 %, which gave a % killing CV of 2.7 %.

The effect of sputum was determined by Wilcoxon signed rank test of the percentage killing values for each concentration of sputum sol. Percentage killing was normalised to 100% for no sputum to allow comparison between patients with CF and healthy subjects. A Mann-Whitney U test was used to compare groups of 'normalised' data to compare the effect of sputum in patients and healthy subjects. Insufficient values were available for analysis of the 1:4 and 1:80 dilutions of sputum.

Start of antibiotic treatment

The presence of sputum sol at 1:40, 1:20 and 1:10 dilutions significantly reduced the killing of *P.aeruginosa* by neutrophils from patients with CF ($p=0.012$, 0.010, 0.008 respectively) (Figure 4.1). The percentage killing was then normalised to 100% to compare effects of sputum on killing by neutrophils from patients with CF and healthy subjects. All dilutions of sputum sol (1:40, 1:20, 1:10) affected the killing by neutrophils from patients with CF more than for healthy subjects ($p<0.001$, $p=0.045$, $p=0.011$ respectively).

After antibiotic treatment

The presence of sputum sol at 1:40, 1:20 and 1:10 dilutions significantly reduced the killing of P.aeruginosa by neutrophils from patients with CF (p=0.021, 0.008, 0.015 respectively). There was no significant reduction by sputum on the killing by neutrophils from healthy subjects (Figure 4.2). The percentage killing was then normalised to 100% to compare effects of sputum on killing by neutrophils from patients with CF and healthy subjects. Sputum sol at 1:20 and 1:10 affected the killing by neutrophils from the patients with CF more than for healthy subjects (p=0.011, p=0.024 respectively).

Clinically stable

The presence of sputum sol at 1:40, 1:20 and 1:10 dilutions significantly reduced the killing of P.aeruginosa by neutrophils from patients with CF (p=0.008, 0.028, 0.005 respectively). There was no significant reduction by sputum on the killing by control neutrophils at 1:5 dilution (p=0.038)(Figure 4.3). The percentage killing was then normalised to 100% to compare effects of sputum on killing by neutrophils from patients with CF and healthy subjects and there was no significant difference between patients with CF and healthy subjects.

Patients with CF compared to healthy subjects

There was no difference between each of the three patient groups, or each of the three healthy subject groups with regard to the effect of sputum. When data was split into 2 groups, patients with CF and healthy subjects, there was a significant difference between the two groups at all but the 1:2 dilution of sputum (Figure 4.4). This indicates that there is a difference in the way neutrophils from patients with CF are affected by sputum sol compared to neutrophils from healthy subjects exposed to the same sputum sol.

Table 4.3

Variation in the number of colonies and % killing in the patient and healthy subject groups in the absence of sputum-sol

Mean number of colonies (time 0) in each group were:			SD	CV %
Start of treatment	patient	188	63	63
After treatment		213	221	104
Clinically stable		325	206	63
Start of treatment	healthy subject	233	148	64
After treatment		200	197	98
Clinically stable		214	138	65
Mean % killing was:				
Start of treatment	patient	86	5.5	6.4
After treatment		81	10.6	13.1
Clinically stable		82	8.6	10.5
Start of treatment	healthy subject	82	9.2	11.2
After treatment		77	13.4	17.5
Clinically stable		82	6.6	8.1

Table 4.4

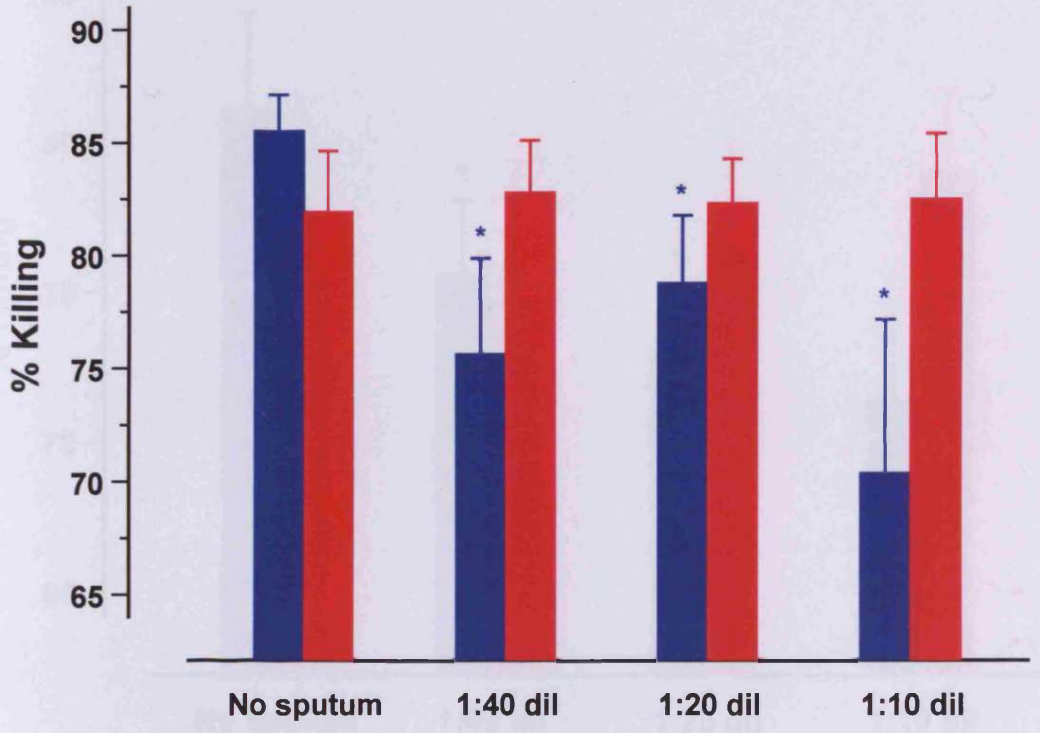
Intracellular killing of *P.aeruginosa* by neutrophils isolated from patients with CF and healthy subjects in the absence and presence of sputum-sol

	Start of Treatment		End of Treatment		Clinically Stable	
	Patient	Healthy subject	Patient	Healthy subject	Patient	Healthy subject
% killing	85.5	81.9	81.1	75.6	81.9	81.8
GM (95% CI)	(82, 89)	(76, 88)	(74, 88)	(76, 87)	(76, 87)	(78, 86)
Data 'Normalised' so killing in buffer is 100%						
1:40 sol	-10.7	4.7	-9.3	-4.5	-6.9	-2.8
	(-20.9, 1.0)	(-0.7, 10.5)	(-14.7, -3.4)	(-11.6, 3.3)	(-10.6, -3.1)	(-9.8, 4.8)
1:20 sol	-9.1	1.1	-13.1	-1.9	-6.1	-4.7
	(-14.6, -3.2)	(-5.2, 7.7)	(-19.4, -6.3)	(-7.3, 3.8)	(-10.9, -1.1)	(-11.5, 2.7)
1:10 sol	-26.4	0.6	-15.4	0.7	-9.8	-8.3
	(-45.6, -0.4)	(-10.4, 12.9)	(-24.3, -5.4)	(-5.0, 6.7)	(-13.2, -6.4)	(-16.4, 0.7)

Numbers in each group are shown in Table 4.1, and calculation of the effect of sputum sol as described earlier.

Figure 4.1

The effect of sputum sol on patients and healthy subjects neutrophils when patients are at the start of treatment for a pulmonary exacerbation

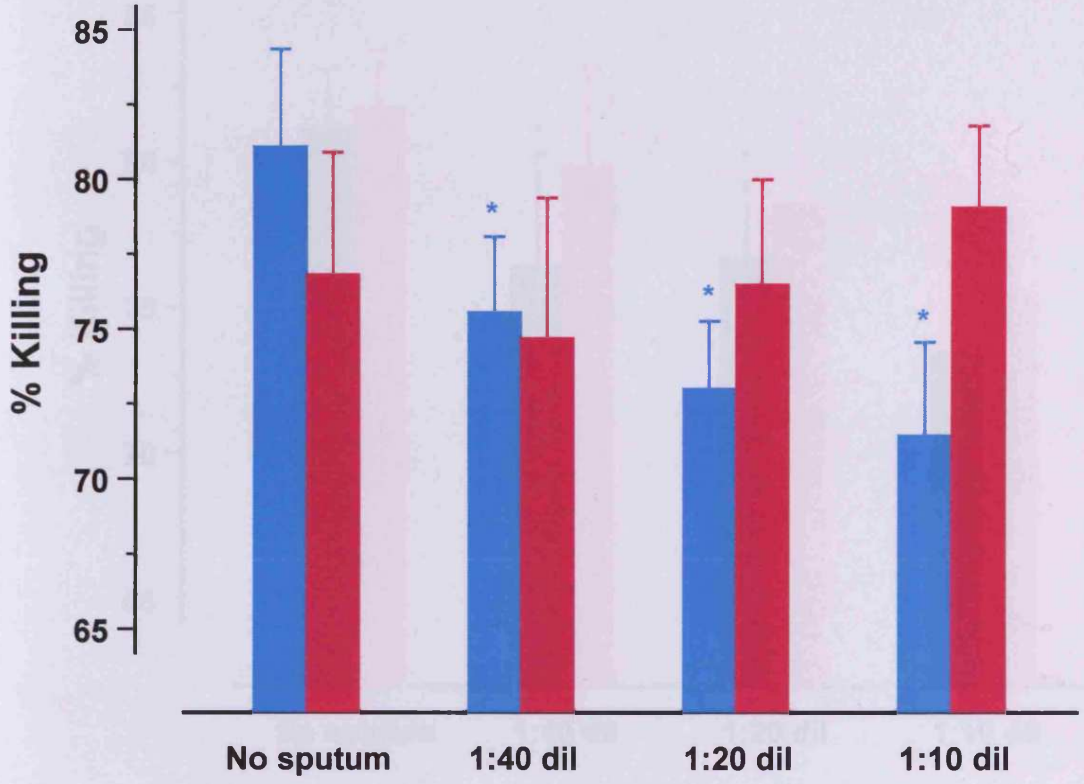


■ Patients with CF * $p<0.05$ effect of sputum sol

■ Healthy subjects

Figure 4.2

The effect of sputum sol on neutrophils from patients with CF after treatment of an exacerbation and healthy subjects

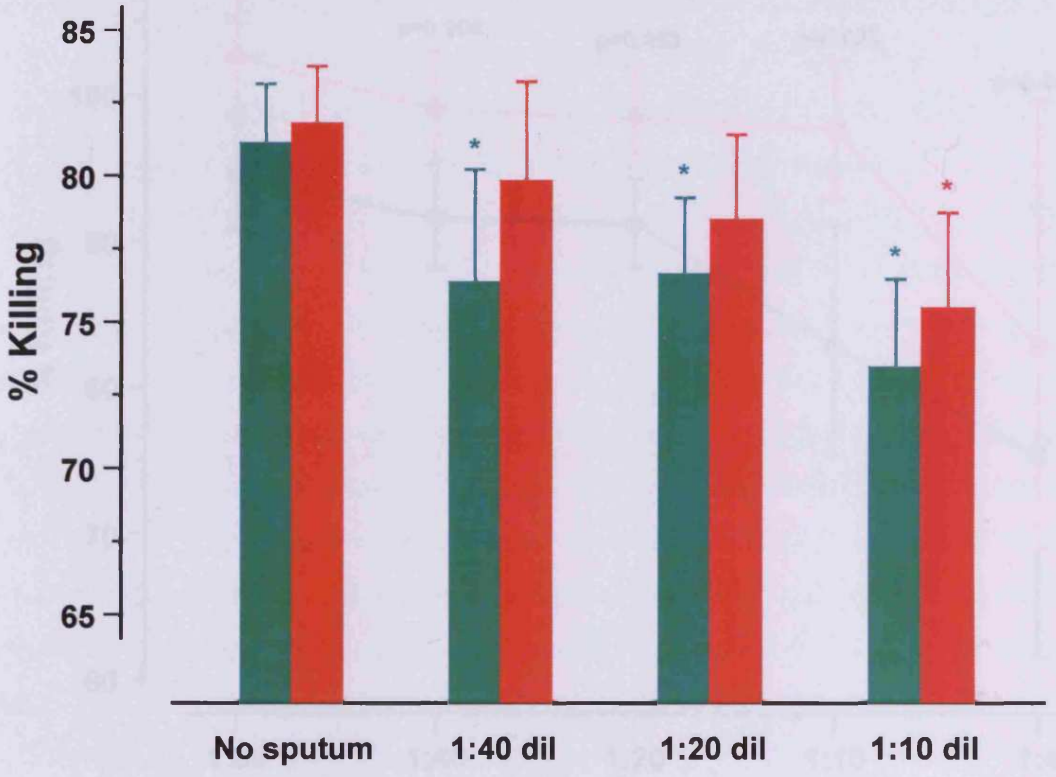


Patients with CF, * $p<0.05$ effect of sputum sol

Healthy subjects

Figure 4.3

The effect of sputum sol on neutrophils from patients with CF when clinically stable and healthy subjects

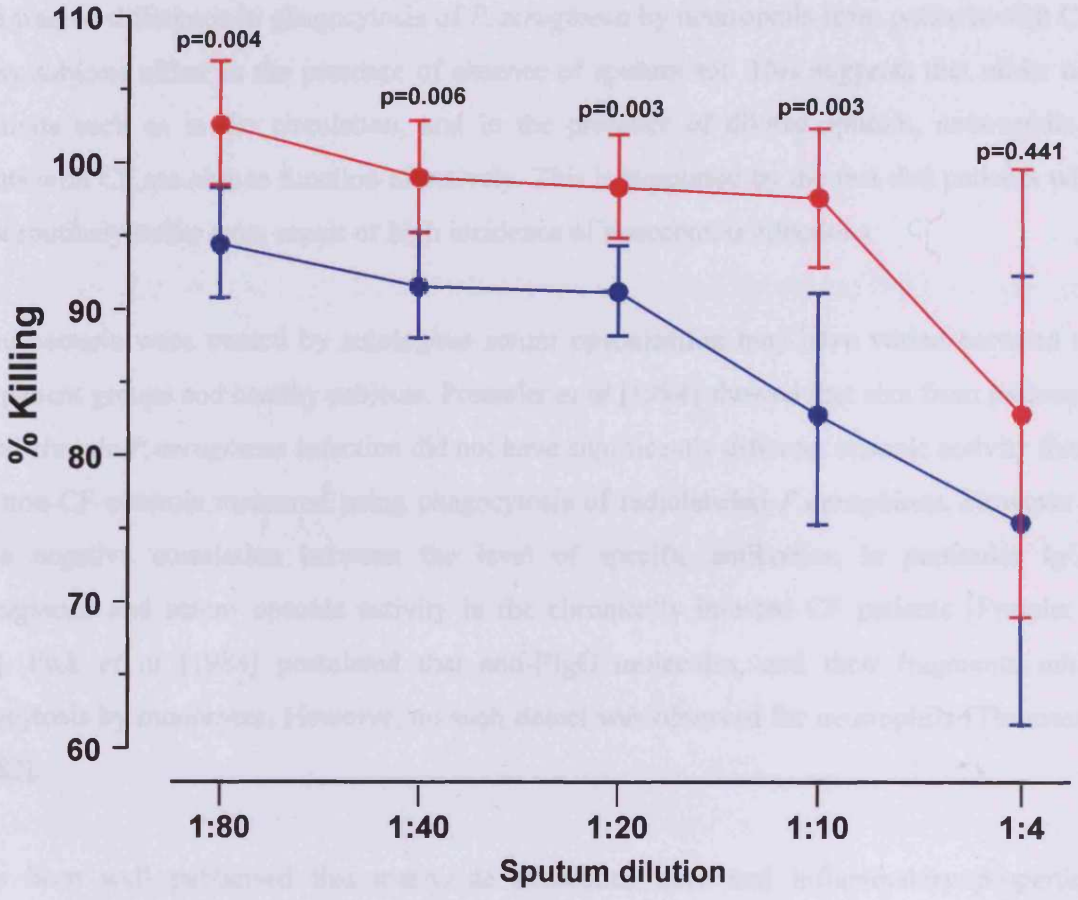


Patients with CF, * $p<0.05$ effect of sputum sol

Healthy subjects, * $p<0.05$ effect of sputum sol

Figure 4.4

Intracellular killing of *P.aeruginosa* by neutrophils from patients with CF and healthy subjects, showing the effect of sputum sol.



Geometric mean of normalised data (95% Confidence Intervals)

● Patients with CF, ● Healthy subjects

1:80 n=2, 1:40 n=9, 1:20 n=12, 1:10 n=10, 1:4 n=4

Discussion

Phagocytosis of P.aeruginosa

There was no difference in phagocytosis of *P.aeruginosa* by neutrophils from patients with CF and healthy subjects either in the presence of absence of sputum-sol. This suggests that under normal conditions such as in the circulation, and in the presence of diluted sputum, neutrophils from patients with CF are able to function effectively. This is supported by the fact that patients with CF do not routinely suffer from sepsis or high incidence of nosocomial infections.

As the bacteria were treated by autologous serum opsonization may have varied between serum from patient groups and healthy subjects. Presseler *et al* [1994] showed that sera from patients with CF and chronic *P.aeruginosa* infection did not have significantly different opsonic activity than sera from non-CF controls measured using phagocytosis of radiolabeled *P.aeruginosa*. However there was a negative correlation between the level of specific antibodies, in particular IgG1 to *P.aeruginosa* and serum opsonic activity in the chronically infected CF patients [Pressler *et al* 1992]. Fick *et al* [1984] postulated that anti-PIgG molecules, and their fragments inhibited phagocytosis by monocytes. However, no such defect was observed for neutrophils [Thomassen *et al* 1982].

It has been well publicised that macrolide antibiotics have anti inflammatory properties, in particular clarithromycin [Labro 1998], but none of the patients were taking this group of antibiotics. In contrast, Cefodizime has been shown to enhance phagocytosis and intracellular killing of *S.aureus*, but not influence neutrophil response to fMLP [Bialasiewicz *et al* 2000]. None of the patients were taking Cefodizime, however, 7 of the 12 exacerbation patients were treated with Ceftazidime, which is also a 3rd generation cephalosporin and therefore may have similar effects. This may have resulted in the reduced responsiveness of the neutrophils counteracting any beneficial effect of antibodies to *P.aeruginosa* in the serum, resulting in no net difference, and therefore no difference in phagocytosis between groups being observed.

Inter-assay variation, donor to donor variation, and assay sensitivity may have hidden small differences, but if such differences were that small, the physiological impact is unlikely to be of importance. More recently more sensitive methods have been developed to determine phagocytosis using Flow Cytometry (FACS), however, many of these studies have used external stimulation for phagocytosis of beads. The advantage of these more controlled conditions is outweighed by the lack of a relationship to the *in vivo* situation [Bassoe 2000].

Intracellular Killing of P.aeruginosa

There was no difference in intracellular killing of *P.aeruginosa* by neutrophils from patients with CF compared with healthy subjects under basal conditions. Sputum-sol reduced the bacterial killing capacity of neutrophils from the patients with CF in all three clinical states.

Concentration related effects of sputum-sol on intracellular killing by our patients' neutrophils may reflect the effects of gradients of inhibitory and stimulatory factors that occur within the lungs. Reducing the ability of the neutrophil to respond to infection will enhance bacterial retention in the airways and could be interpreted as a bacterial mechanism to evade host defences. However, this does not explain differences between CF and non-CF individuals and in this context suggests an intrinsic reduction in killing ability of the neutrophils from patients with CF. This may be an intrinsic effect of the CFTR gene defect on the neutrophil making it more sensitive to inhibitory components in sputum-sol than neutrophils from non-CF subjects. Alternatively it may be related to the pre-exposure of the neutrophil to inflammatory mediators when in the circulation.

Serum factors can kill bacteria through complement-mediated lysis, although clinical isolates of *P.aeruginosa* are normally resistant to this process [Sorensen *et al* 1991]. The *P.aeruginosa* strain used in these experiments was a clinical isolate. In addition comparison of *P.aeruginosa* killing was compared to a control value in the presence of the same concentration of serum, and therefore serum did not kill this strain of *P.aeruginosa.* Similarly there was no observed difference in phagocytosis in the CF and healthy subject groups. Such an effect is unlikely to explain the difference in killing in the presence of sputum-sol. Sub-inhibitory concentrations of the antibiotics cefepime, ceftazidime and imipenem have been shown to increase killing of some strains of

P.aeruginosa [Darveau *et al* 1990], and there is a possibility that this could have aided the neutrophils from patients with CF. However, patients in the clinically stable group were not on antibiotics, and behaved similarly to those on antibiotic treatment.

Use of autologous serum was not thought to have affected the results as similar levels of opsonic activity are likely to have occurred both in healthy and CF affected individuals becuase a difference would have been evident in the phagocytosis between the groups. However, CF derived cells came from blood and were mixed with autologous serum containing varying quantities of a variety of potentially active factors (Chapter 3). The prior exposure of CF cells to circulating regulatory factors or mediators may have changed their reactivity in favour of inhibition of killing when new host or bacterial factors were added. This suggests the effect comes from circulating factors with sputum having specific effects to which CF cells may be more sensitive than non-CF cells. The lack of effect of sputum on phagocytosis may be linked to phagocytosis occurring through a different mechanism to killing. Phagocytosis still occurs in Chronic Granulatomous Disease, even though killing of bacteria is substantially reduced [Burg *et al* 2001].

There is some evidence to suggest an intrinsic effect of the CF gene defect on neutrophil homeostasis. The intracellular pH of resting neutrophils is 7.0 and this is reduced upon stimulation such as with fMLP and PMA [Coakley *et al* 2000] or bacteria [Coakely *et al* 2002]. The fMLP and PMA induced acidification is smaller in neutrophils from patients with CF. Intracellular pH and the potassium ion (K^+) concentrations are involved in the killing process [Reeves *et al* 2002], so lack of CFTR function and therefore of Na^+/K^+ transport may be a factor. However, this is speculative as CFTR has not yet been shown to be present in neutrophils. The killing of neutrophils is dependent not on the intracellular pH, but on the phagosomal pH, which has been reported to undergo a biphasic change. Alkalinization during the first few minutes, is slowly followed by a modest acidification to pH 6.5 [Jankowisk *et al* 2002]. The alkalosis was attributed to consumption of H^+ during the process of dismutation of superoxide to H_2O_2, and the secondary acidification the ongoing activity of the V-ATPase after the respiratory burst subsides [Segal *et al* 1981]. A H^+ conductive pathway of unknown molecular identity affects both the cytosolic and phagosomal pH. NADPH oxidase controls phagosomal pH by multiple mechanisms which include this H^+ pump

[Jankowski *et al* 2002]. A Na^+/H^+ exchanger, which is susceptible to inhibition by amiloride and its analogues is driven by the concentration gradients of these ions [Hackman *et al* 1997].

The finding of a concentration related inhibition of bacterial killing indicates the effect was due to one or more constituents of the sputum sol. We produced a relatively crude sputum sol to mimic the likely mixture of products derived from both host and bacteria present in the airways in CF. Neutrophils produce defensins which have antimicrobial action, and this effect is enhanced in the presence of lung tissue by inducing H_2O_2 production [Porro *et al* 2001]. Defensins also interfere with the activation of neutrophil NADPH oxidase [Tal *et al* 1998]. Higher levels of defensins may be one mechanism by which a difference may be seen between CF and non-CF subjects. Kaplan and Simmons [2001] showed a marked reduction in phagocytic killing of *S.aureus* on a second exposure of the neutrophils, which also occurred with bystander cells (those not stimulated by exposure to the supernatant). This group suggest a primarly role for a cytotoxic effect of defensins, the activity of which could be reduced by plasma/matrix proteins. The key link to our work is the reduced killing on second exposure to the inoculum which affected bystander cells. Whether or not this effect is mediated by defensins, the potential presence of it in the serum as a result of travel down the concentration gradient in the lungs of patients with CF would explain the differences seen between patients with CF (who have had prior exposure) and healthy subjects (who have not had prior exposure).

Human airway secretions can kill bacteria by antibacterial proteins and peptides such as lysozyme, lactoferrin and secretory leukoproteinase inhibitor [Basbaum *et al* 1990]. Elevated NaCl concentration, which is thought to be present in the airways surface liquid, inhibits such antibacterial factors [Travis *et al* 1999]. This cannot account for the differences we saw between killing by neutrophils from patients with CF and healthy subjects in the presence of sputum because the same sputum was used on each pair. Whether NaCl concentrations are hyper- or hypo-tonic in CF remains a topic of debate, although the evidence for raised NaCl concentration seems to be slightly stronger at the current time [Krouse 2001]. Direct measurements in mice gave an ASL concentration of 97mM for Na^+ and 118mM for Cl^- (combined 110mM), similar to 103 for Na^+ and 92 for Cl^- (combined 96mM) in CFTR-null mice [Jayaram 2001] suggesting no difference. This agreed with another study in humans which found Na^+ 109mM and Cl^- 125mM (combined 119mM)

in both normal and CF subjects [Knowles 1997]. It has been proposed that NaCl concentration is linked to an abnormality in the volume of CF airway fluids or that abnormalities in the composition of these fluids are key pathogenic features in CF pathology [Boucher 2002]. In this study saline, with a molarity of 155mM, was used to dilute samples. This may have diluted any effects of NaCl concentrations in sputum, but was the same both for neutrophils from patients and healthy subjects. It is also likely that other ions are present in the ASF, and the values of these are being diluted. Antibacterial action against *P.aeruginosa* of ASF in the absence of cells has been shown *in vitro*, and this is increased when the ASF is desalted [Bals *et al* 2001]. As such, the effect of defensins is likely to be low as a result of the NaCl concentration used.

Grutuski *et al* [2002] have shown that phagocytosis of *E.coli* was significantly reduced when neutrophils were exposed to a culture medium where other neutrophils had been pre-exposed to TNFα, and suggests a negative feedback loop. It is very possible that something similar could be happening to this when the sputum-sol was added to the neutrophils, and a reduction of killing observed. In contrast, TNFα, IL-8 and IL1β have been shown to increase the bactericidal activity of neutrophils [Simms *et al* 1997]. There is also evidence that elastase is able to decrease the ability of neutrophils to kill opsonized *P.aeruginosa in vitro* [Berger *et al* 1989].

Mucoid strains of *P.aeruginosa* produce alginate which can reduce phagocytosis [Pederson *et al* 1990]. Neutrophil elastase may inhibit the killing of bacteria, particularly *P.aeruginosa* which may be corrected when adequate antiproteinase capacity is present [McElvaney *et al* 1991]. The pyocyanin produced by *P.aeruginosa* is able to increase IL-8 production by neutrophils [Hachicha 1998] and epithelial cells, as well as increasing reactive oxygen species production by neutrophils [Muller *et al* 1997, Denning 1998]. Pyocyanin has also been shown to induce apoptosis in neutrophils [Usher 2002]. Alginate is also able to increase the stimulated production of reactive oxygen species [Pederson 1990] whilst haemolytic phospholipase C reduces the neutrophil respiratory burst [Terada *et al* 1999]. *P.aeruginosa* is also able to secrete cytotoxic proteins (ExoS, ExoT, ExoU and ExoY). Some strains cause death by lysis of neutrophils [Dacheux 2000] and exotoxin A induces death of epithelial cells [Plotkowski *et al* 2002]. These examples of host and bacterial factors that inhibit intracellular killing by neutrophils do not explain the difference we

found between non-CF and CF individuals, although they do suggest that there is a complex mechanism of *P.aeruginosa* control occurring.

It has been shown that the rate of bacterial killing is dependent on the concentration of neutrophils and not on the ratio of neutrophils to bacteria [Li *et al* 2002]. In these experiments both the number of neutrophils and the number of bacteria were kept constant therefore this is unlikely to be an area for differences to be observed. It is possible that there was no observed difference between killing in the absence of sputum because the assay was not sensitive enough to detect slight variations. However small differences between neutrophils from patients with CF and healthy subjects were magnified by putting the neutrophils under stress by the addition of sputum sol, to produce the observed reduction in killing. Combining the patients and healthy subjects into two groups could be considered scientifically incorrect, since the patients are in different clinical states, and those in the after treatment the same as those in the before treatment. Despite this it shows that there is a clear significant split between the patients and healthy subjects due to the larger numbers. In hindsight, this part of the study was slightly underpowered in this respect.

Our *in vitro* model may not accurately reflect the relationships between sputum components and neutrophils found in the lung, though we used a range of dilutions to mirror possible concentration gradients for inhibitory and stimulatory factors between the airway lumen and the airways tissues. If impairment of killing capacity occurs *in vivo* it may be a factor in establishing and maintaining bacterial colonisation of the lung and the long term consequences of chronic pulmonary infection in CF. This study adds further evidence that subtle alterations in the inflammatory response in CF may be detrimental to the host and suggests more knowledge of host-bacterial interactions in CF is required to enable potential anti-inflammatory therapeutic interventions to be made. The nature of the mechanism underlying reduced intracellular killing of bacteria remains unknown, as does its pathophysiological and clinical importance.

Chapter 5

Superoxide Generation by isolated neutrophils in patients with cystic fibrosis and healthy subjects

Aims

To determine whether superoxide generation by neutrophils from patients with CF is affected by their clinical status and whether it is different to neutrophils from healthy non-CF subjects.

To determine the effect of sputum-sol on the superoxide generation by isolated neutrophils.

Introduction

Baboir *et al* [1973] showed that phagocytosing neutrophils generate O_2^-. In aqueous solutions O_2^- can act as a reducing agent by donating an electron. This forms the basis for an assay to detect O_2^- production because it is able to donate an electron to, and hence reduce, ferri- (oxidised) cytochrome C. Reduced cytochrome C has an absorption maximum at λ550nm, and this increase in absorption can be quantified spectrophotometrically.

Reactive oxygen species, and therefore O_2^-, are produced in response to specific stimuli in the environment. NADPH becomes activated in response to signals which include fMLP, PAF, LTB_4, PMA, IgG-immune complexes, opsonized zymosan and opsonized bacteria. Here we use fMLP to induce neutrophil activation which acts by binding to a G-protein fMLP cell surface receptor. When neutrophils are stimulated in this way a relatively small amount of superoxide is generated. This can be increased signficantly by the use of cytochalasin B (CB), which is commonly used in neutrophil functional studies. CB increases oxygen metabolite production (shortens actin filaments and hence does not restrain movement of granules) probably by an up-regulation of some receptors and CB molecules on the cell surface. We therefore included CB in our incubation buffer.

The potency of these signals can be modified by the environment that the neutrophil has been exposed to. Thus the past history of the neutrophil in terms of its *in vivo* or *in vitro* exposure to agents that can cause such an up-regulation, will affect its ability to generate reactive oxidants in response to specific agonists. Up-regulation, known as priming, has been shown to occur with GM-CSF and TNFα in human neutrophils [Weisbart 1987, McColl *et al* 1990, English *et al* 1988]. Thus primed neutrophils generate enhanced levels of reactive oxidants and have higher levels of degranulation and greater phagocytic activity compared to untreated cells. Hence they are more potent in killing many types of microbial pathogens. In addition to the short term priming effects, which do not involve *de novo* protein biosynthesis, priming agents such as cytokines induce some functional changes in neutrophils that are detectable many hours, or even days, after exposure. Activated gene expression is required to enhance and extend the functional lifespan of neutrophils and also to generate molecules that will actively regulate the progress of an inflammatory reaction.

Due to the importance of superoxide generation in the anit-microbial action of the neutrophil an alteration in this mechanism may affect the ability of neutrophils to clear infection *in vivo*. We therefore determined the superoxide generation of neutrophils in patients with CF at different clinical states, and compared the response to that of neutrophils from healthy subjects. We also investigated this in the presence of sputum sol, as this is present in the environment in which impaired clearance of micro-organisms occurs.

Method

Neutrophils were isolated as described (Chapter 2, 2.3.7). Superoxide generation was determined by reduction of cytochrome C as described (Chapter 2, 2.3.8). The change in optical density at λ550nm was converted to superoxide produced using the calculation in Appendix 4.

Dose response curves were carried out for fMLP (10^{-9}M to $3x10^{-7}$M). The effect of sputum sol (1:5, 1:10 and 1:20) on resting neutrophils was determined. The sputum collected on each study day from the patient was used that day for the patient and matched healthy subject for neutrophil functional studies. The sputum was effectively diluted by a further factor of 10 in the final incubation mixture. For each sputum sol concentration a blank containing no neutrophils was included, and compared to blank in the absence of sputum sol. The effect of each sputum-sol concentration was also determined in the presence of fMLP. A concentration of 10^{-8}M fMLP was chosen for this, as it is approximately 50 % of the maximum response. This allowed for both inhibition and enhancement of the response by the added sputum-sol. Each measurement was carried out in triplicate, and the mean obtained. The value of the blank was removed from all tubes and sputum control values were removed from those tubes containing sputum.

Statistics

The study design meant that the healthy subjects were studied in parallel with each patient. This meant that results were paired with respect to buffers, exact times of separation and incubation, and the sputum. Data was not normally distributed and therefore log transformed for analysis. For the fMLP does response curves, a multiple ANOVA was used to determine differences between patient and healthy subject groups to allow for problems of analysis associated with sequential t-tests. In addition, the EC_{50} (concentration of fMLP to produce 50 % of the response) was determined, and the maximal response of the control was taken as 100% and the percentage for the patient 'pair' for this calculated.

$$\textit{Patient response as \% of healthy subject response} = \frac{\textit{Patient SO release}}{\textit{Healthy subject SO release}} \times 100$$

Results

Effect of stimulation

Circulating neutrophils from patients with CF in exacerbation generated less superoxide in response to a range of doses of fMLP compared with healthy subjects (P=0.002) (Figure 5.1). There was return towards healthy subject responsiveness after antibiotic treatment although this did not reach significance (p= 0.295) and the difference between the patients after treatment and healthy subjects was no longer signifcant. A similar reduced responsiveness was found in neutrophils from clinically stable patients compared with non-CF healthy subjects (p=0.049) (Figure 5.2).

When calculated as a percentage of the maximum control response for the paired healthy subject, patients at the start of treatment of an exacerbation had a maximal response of 62% (p=0.0003), and 82% (ns) after treatment. An improvement in 8 out of 11 patients was observed (Figure 5.3). A paired t test showed the improvement was not quite significant (p=0.068). When calculated as a percentage of the maximum control response, the group of clinically stable patients with CF had a maximal response of 72% (p=0.04). The percentage of healthy subject control values for each patient fMLP concentration point was consistent across the range of fMLP concentrations (Table 5.1).

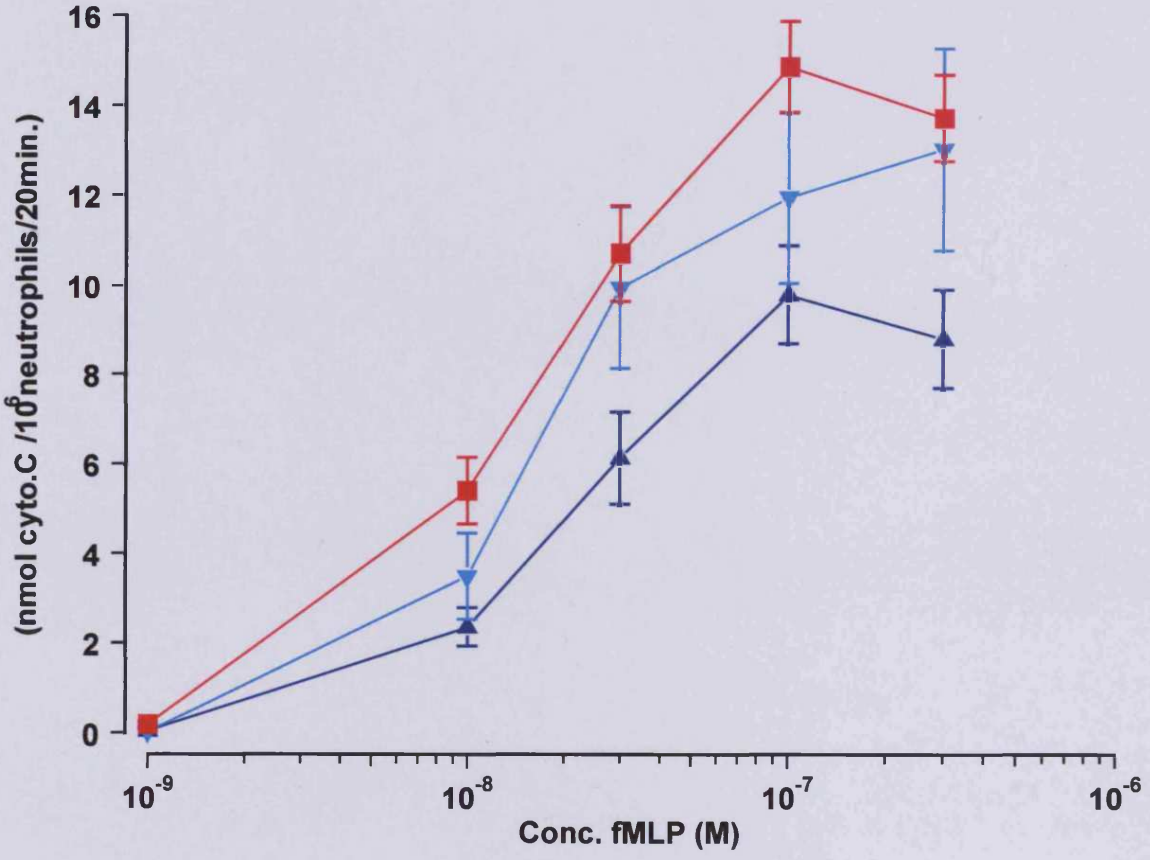
Effect of sputum-sol

On the unstimulated cells, the sputum-sol caused an increase in the generation of superoxide in all six groups, and this was significant for at least one of the sputum concentrations. Each group consisted of the mean of the effect of 12 different sputum samples (Table 5.2).

The presence of sputum sol at a final concentration of 1:100 increased superoxide generation by unstimulated cells from both patients with CF and healthy subjects (all $p<0.05$), but not their response to 10^{-8}M fMLP (Figure 5.4). Sputum sol appeared to have little effect on the stimulated response to fMLP. Where sputum-sol significantly altered the response to fMLP, the effect was a reduction in the stimulated superoxide generation (Table 5.2).

Figure 5.1

Superoxide generation by neutrophils in patients with CF at the start of an exacerbation, after treatment, and in healthy subjects.



▲ Start of treatment of patients with CF (n=12), ▼ End of treatment of patients with CF (n=11),

■ Healthy subjects matched CF start (n=12)

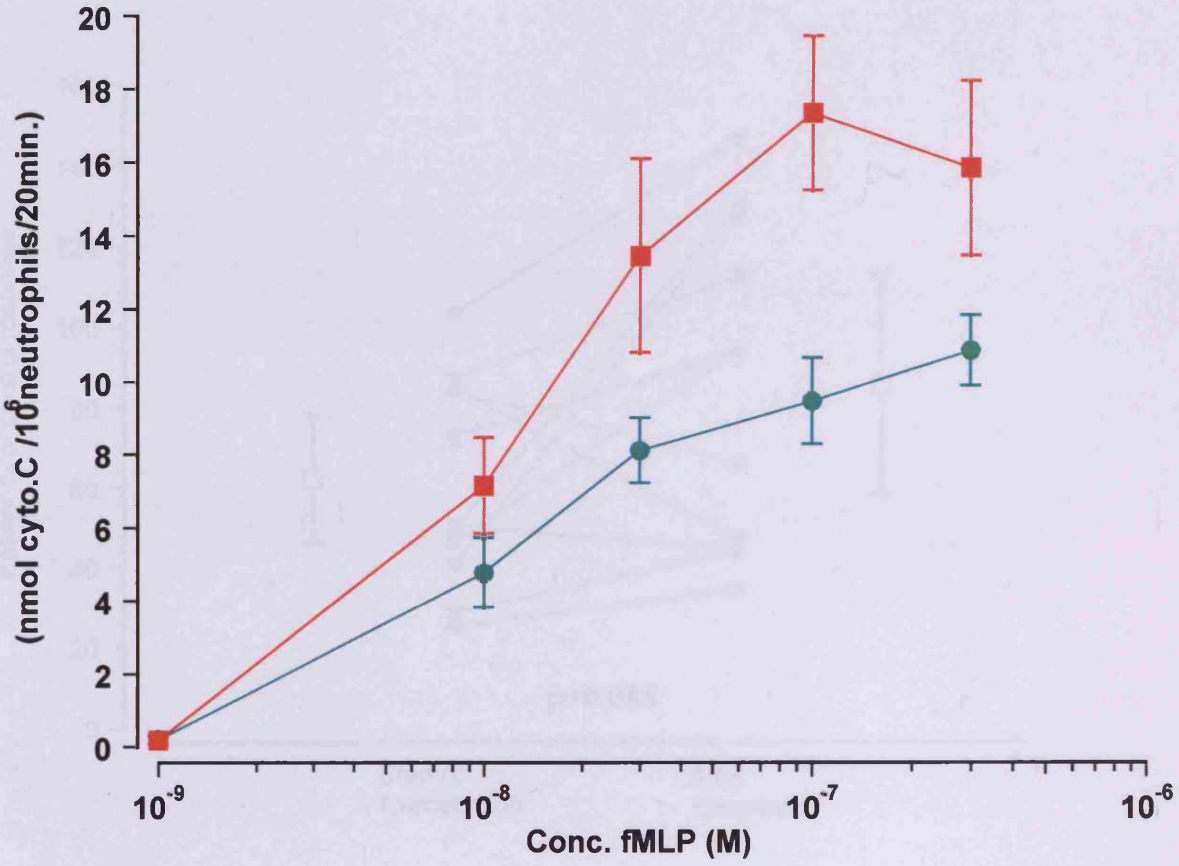
Arithmetic mean ± S.E.M.

CF compared to healthy subjects p=0.002 by MANOVA on $\log_{10}$ transformed data

(Healthy subjects matched CF end omitted for clarity)

Figure 5.2

Superoxide generation by neutrophils in patients with CF when clinically stable and in healthy subjects.



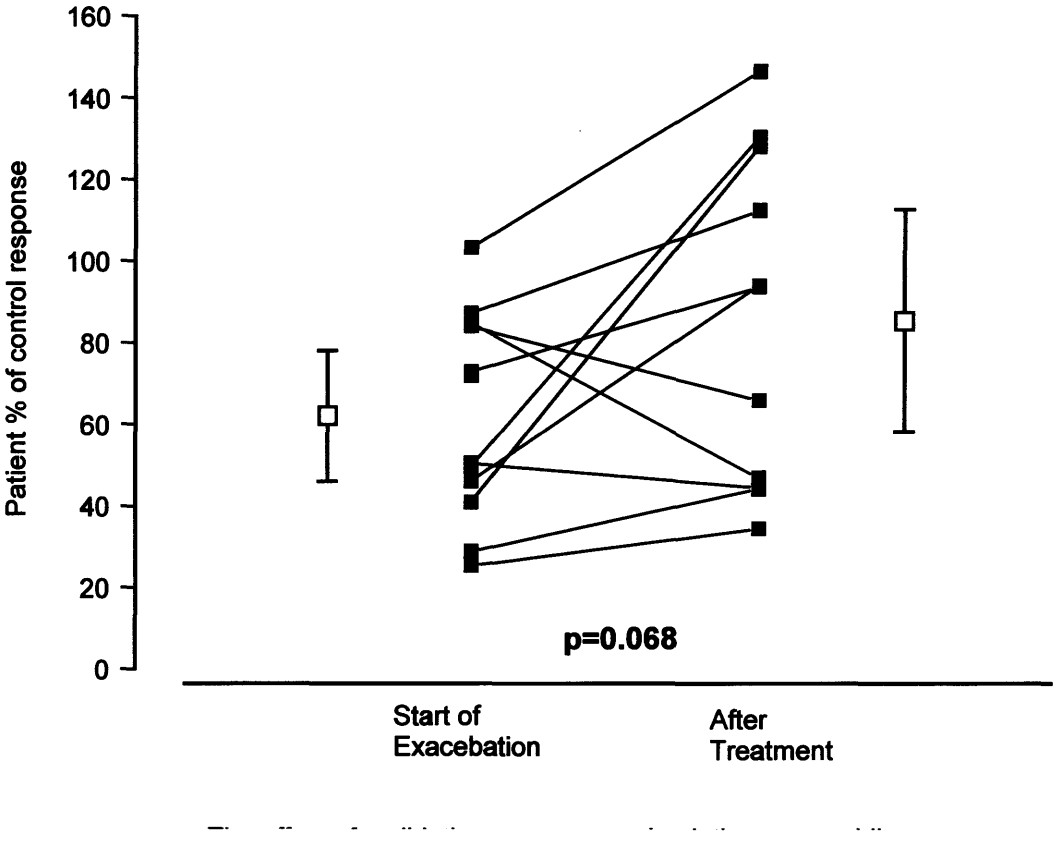
● Clinically stable patients with CF (n=12), ■ Healthy subjects (n=12)

Arithmetic mean ± S.E.M.

CF compared to healthy subjects p=0.049 by MANOVA on $\log_{10}$ transformed data

Figure 5.3

The effect of antibiotic treatment on circulating neutrophils on superoxide generation, expressed as a % of the response by neutrophils from matched healthy subjects



☐ Arithmetic Mean ± S.E.M., ■ Individual values

Table 5.1

The variation of patient percentage of healthy subject neutrophil superoxide generation for different fMLP concentrations

FMLP concentration	10^{-9}M	10^{-8}M	$3x10^{-8}$M	10^{-7}M	$3x10^{-7}$M
Start of Exacerbation	62.37 (35.2-110.7)	58.54 (26.9-127.4)	53.41 (32.8-86.9)	62.72 (51.4-76.6)	63.85 (45.7-89.1)
After treatment	135.9 (26.9-590.2)	42.05 (24.9-70.8)	76.87 (34.7-171)	80.5 (58.3-111)	97.35 (68.2-139)
Clinically stable	173.8 (81.5-371)	64.15 (3.2-116.9)	58.51 (41.7-82.2)	69.08 (44.4-108)	79.21 (51.9-121)

Geometric mean of % (95% CI)

Table 5.2

Effect of sputum sol on superoxide generation

	Start of Treatment		After Treatment		Clinically stable	
	Patient	Healthy subject	Patient	Healthy subject	Patient	Healthy subject
Background	1.81	2.38	1.88	2.65	1.96	2.60
with 1:20 sputum sol	2.09	2.44	2.09	3.13	2.32	3.08
(p=)	ns	ns	(0.008)	(0.02)	(0.03)	(0.08)
with 1:10 sputum sol	2.36	3.02	2.26	3.35	2.47	3.06
(p=)	(0.04)	(0.02)	(3.35)	(0.004)	(0.005)	(0.09)
with 1:5 sputum sol	2.45	3.90	2.15	3.50	2.18	2.76
(p=)	ns	(0.008)	ns	(0.06)	ns	ns
fMLP 10^{-8} M	4.64	7.16	5.36	8.68	6.71	9.82
with 1:20 sputum sol	4.88	6.13	4.69	7.67	6.95	8.60
(p=)	(0.04)	ns	ns	ns	ns	(0.07)
with 1:10 sputum sol	5.18	7.45	5.23	8.66	6.05	7.72
(p=)	ns	ns	ns	ns	ns	(0.02)
with 1:5 sputum sol	4.25	7.50	4.63	8.30	5.12	5.53
(p=)	ns	ns	ns	ns	ns	(0.006)

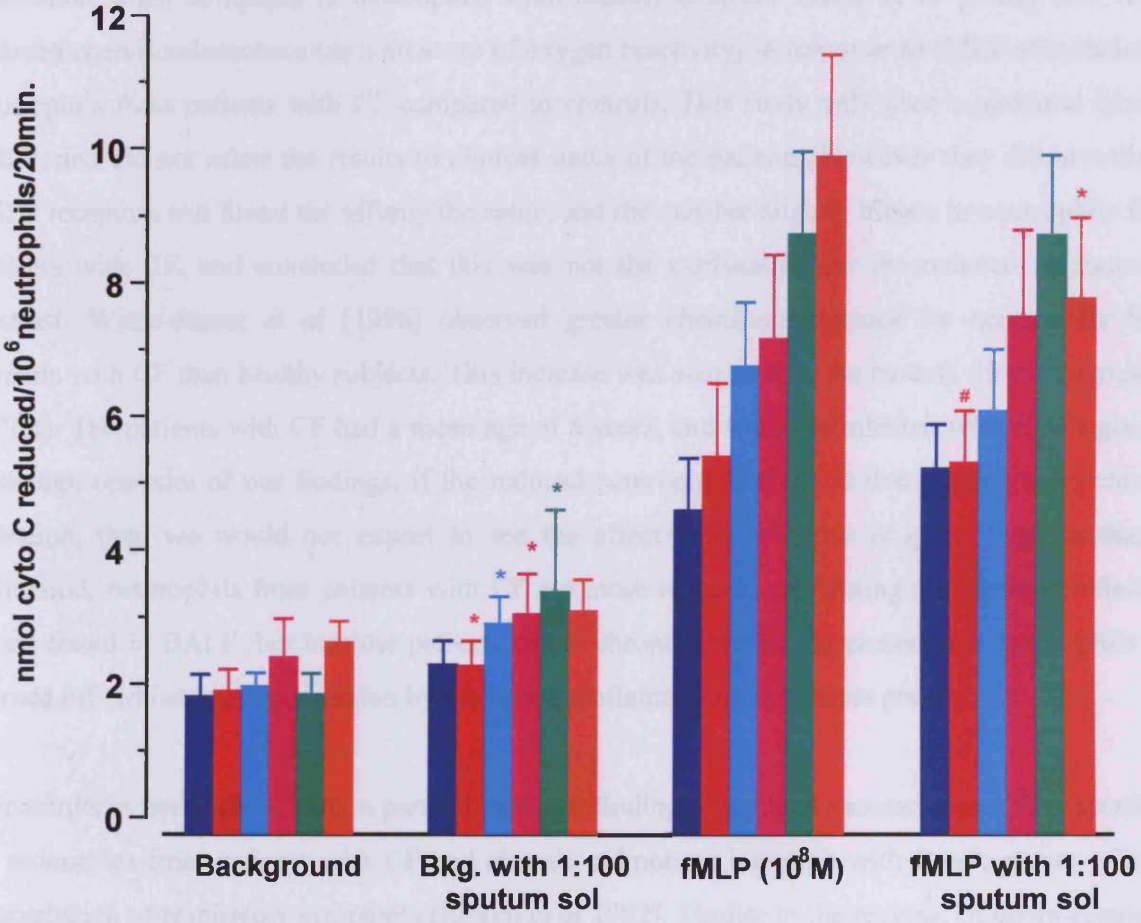
Arithmetic mean of the superoxide generation.

(p values represent significant effect of sputum; ns is where $p>0.1$)

Final sputum sol concentration is 10 times value quoted, and each sputum sol used once for the patient and matched healthy subject.

Figure 5.4

The effect of sputum sol on superoxide generation from neutrophils of CF patients and healthy subjects.



Arithmetic Mean ± S.E.M.

CF Start of treatment (n=12), CF after treatment (n=9), CF clinically stable (n=12)

healthy subject matched CF start (n=12) , healthy subject matched CF end (n=9),

healthy subject matched CF stable (n=12)

* $p<0.05$ effect of sputum by Wilcoxon signed rank test

# $p<0.05$ between patients and healthy subjects by Mann Whitney U Test

Discussion

Circulating neutrophils from patients with CF showed a reduced response to fMLP for superoxide generation when compared to neutrophils from healthy subjects. Kemp *et al* [1986] also found reduced chemiluminescence (as a measure of oxygen reactivity) in response to fMLP stimulation in neutrophils from patients with CF compared to controls. This study only used a maximal dose of fMLP, and did not relate the results to clinical status of the patients. However they did investigate fMLP receptors and found the affinity the same, and the number slightly higher in neutrophils from patients with CF, and concluded that this was not the explanation for the reduced response. In contrast, Witko-Sarsat *et al* [1996] observed greater chemiluminescence by neutrophils from patients with CF than healthy subjects. This increase was also seen in the parents (heterozygous for CFTR). The patients with CF had a mean age of 8 years, and were not infected with *P.aeruginosa.* Although opposite of our findings, if the reduced neutrophil activity is due to long term chronic infection, then we would not expect to see the effect in children. It is quite possible that in childhood, neutrophils from patients with CF are more reactive, explaining the increased elastase levels found in BALF, but in older patients where chronic infection is present, the neutrophils are 'turned off' whilst in the circulation by the various inflammatory mediators present.

Our results in neutrophil function parallel previous finding of reduced spontaneous TNFα secretion by monocytes from patients with CF and chronic pulmonary infection with *P.aeruginosa* with an exacerbation of respiratory symptoms [Elborn *et al* 1992]. Similar to the reversal of down-regulated neutrophil function reported here when circulating and airways inflammation is reduced (Chapter 3), there was a return to normal spontaneous rate of secretion of TNFα at the end of a two week period of antibiotic treatment, which was associated with a reduction in plasma immunoreactive TNFα concentration [Elborn 1992]. More recently a study by Coakley *et al* [2000] showed that fMLP stimulated intracellular pH is more acidic in CF cells that controls, and suggested this was due to a passive protein conductance abnormality, resulting in abnormal anion transport. This could subsequently affect functioning. We were able to relate the reduced responsiveness of neutrophils to clinical state, which would not be explained by the mechanism proposed by Coakely *et al* [2002] although such effects would not be ruled out by the results of our study.

Lipopolysaccharide (LPS) elicits a variety of neutrophil responses, including up-regulation, actin assembly, and adherence, and primes the cell for enhanced release of superoxide in response to other stimuli such as n-formylated bacterial peptides (e.g. fMLP) [DeLeo 1998]. It would therefore be likely to suppose that neutrophils from patents chronically infected would be exposed to LPS and therefore primed for enhanced response to fMLP. This was not the case, and there are clearly other control mechanisms in place. It is also possible that following treatment, the increased killed bacteria in the lung would liberate a higher LPS load which would get into the circulation system with an increased priming effect. At the same time the reduced bacterial load in the lung might reduce the rate of neutrophil production allowing them to mature more. These two conditions might explain the results seen.

Despite the reduction in superoxide generation in response to fMLP there was no loss of the capacity of the circulating neutrophils from the same patients to phagocytose or kill living *P.aeruginosa in vitro* when compared with subjects without CF (Chapter 4). The reduced response to fMLP was related to the clinical states of exacerbation and clinical stability, and was reversed by antibiotic treatment indicating a clear linkage to exacerbation of the pulmonary chronic infection. The acute phase serum protein amyloid A has been shown to reduce the oxidative response of neutrophils to fMLP [Linke 1991], and although amyloid A was not measured it can be assumed to increase in infection in the same way as CRP.

We used the addition of sputum sol in a range of doses to explore the possibility of secondary inhibitory effects on neutrophil function. Augmentation of basal superoxide generation was observed, however there was a lack of augmentation of fMLP stimulated superoxide generation, particularly in neutrophils from patients with CF, which contrasts with the reported priming effect of sputum sol on superoxide generation [Kharazmi 1987], though our study was not designed to determine priming effects. However, we cannot rule out the possibility of inactivation of some of the reactive oxygen species by the sputum-sol present. Superoxide is a major determinant of intracellular bacterial killing and the failure of sputum sol to augment the response to fMLP may indicate an advantage for bacteria, and may derive from bacterial products such as pyocyanin [Muller & Sorrell 1997]. The mechanism of this effect is unknown but combined with the reduced primary granule exocytic function in response to fMLP of circulating neutrophils it may be a factor

in the impaired bacterial killing by neutrophils from patients with CF in the presence of sputum sol. The fractionation of sputum sol phase might help in resolving whether there are separate priming effects such as soluble LPS and inhibitory effects present.

Hypertonic stress (1mol/l which is equivalent to 5.8%) has been shown to reduce the superoxide generation in response to fMLP via the ERK and p38 pathway [Orlic *et al* 2002]. The salt molarity of the sputum-sol was not measured, but was made up in saline (0.9% NaCl) but this may still have had some influence on the response.

We have shown a decrease in the superoxide generation in by neutrophils from patients with CF after stimulation with fMLP at a time of exacerbation of respiratory symptoms. This effect improved with anitbiotic treatment. In addition sputum sol augmented the non stimulated superoxide generation, but attenuated the fMLP stimulated superoxide generation.

Chapter 6

Elastase release by isolated neutrophils in patients with cystic fibrosis and healthy subjects

Aim

To determine whether the elastase release by neutrophils from patients with CF is affected by their clinical state and whether it is significantly different to neutrophils from healthy subjects.

To determine the effect of sputum sol on the elastase release by isolated neutrophils from patients with CF and healthy subjects.

Introduction

Elastase is important in the ability to kill micro-organisms, although this may not be as important as the production of reactive oxygen species. There is no doubt that elastase is a key mediator in the tissue damage which occurs in the lungs of patients with CF where its concentration is at such a high level that the antiproteinases cannot successfully inhibit elastase, and are actually inactivated by the elastase [Piccioni *et al* 1992, Suter & Chevallier 1991, Tetley 1993]. Where inhibitors are rendered ineffective, enzymes in addition to neutrophil elastase such as cathepsin G, plasminogen activator, collagenase and gelatinase can also degrade host tissues in an unmolested fashion. They have the ability to degrade almost all components of the extracellular matrix, and cleave a variety of plasma proteins [Weiss 1989].

Elastase is contained in the azurophilic granules of neutrophils, and is a good marker of degranulation. The degranulation of such granules occurs in response to stimulation, in particular by stimulants relevant to fighting infection, such as LPS. Since fMLP is a synthetic peptide which acts in a simialar way, we used this to mimic the *in vivo* situation of infection. We also used CB in the incubation buffer to experimentally increase the response as in the experiments described in Chapter 5.

The role elastase plays in host tissue damage in inflammatory conditions, as well as its bactericidal activities made it an important aspect of neutrophil function to study. We therefore compared neutrophils isolated from patients with CF at different clinical states with the responsiveness of neutrophils from healthy subjects. Similarly to chapter 5 we also investigated the effect of sputum sol to reflect the conditions which the neutrophil would be likely to be exposed to at the site of infection.

Method

Neutrophils were isolated as described (Chapter 2, 2.3.7). Elastase release was determined by ELISA as described (Chapter 2, 2.2.5).

Dose response curves were carried out for fMLP (10^{-9}M to $3x10^{-7}$M). The effect of sputum sol (1:5, 1:10 and 1:20) on resting neutrophils was determined, as carried out in chapter 5. The supernatant in which the superoxide generation was determined was frozen prior to determination of the concentration of elastase. A concentration of 10^{-8}M fMLP gave approximately 50 % of the maximum response, which was the same as for superoxide generation.

Statistics

The study design meant that a healthy subject was studied in parallel with each patient. Thus results were paired in respect to buffers, exact times of separation and incubation, and the sputum. Data was not normally distributed and therefore log transformed for analysis. For the fMLP dose response curves, a multiple ANOVA was used to determine differences between patient and healthy subject groups to allow for problems of analysis associated with sequential t-tests. In addition the maximal response of the control was taken as 100% and the percentage for the patient 'pair' for this calculated.

$$\textit{Patient response as \% of healthy subject response} = \frac{\textit{Patient NE release}}{\textit{Control NE release}} \times 100$$

Results

Effect of stimulation

Circulating neutrophils from patients with CF in an exacerbation released less elastase in response to a range of doses of fMLP compared with healthy subjects (p=0.032) (Figure 6.1). There was a return towards the level of responsiveness of the healthy subjects after antibiotic treatment although this did not reach significance and the difference between the patients after treatment and healthy subjects was no longer significant. A similar reduced responsiveness was found in neutrophils from clinically stable patients compared with healthy subjects (p=0.032) (Figure 6.2).

When calculated as a percentage of the maximum response for the paired healthy subject, patients at the start of treatment of an exacerbation had a maximal response of 74% (p=0.04), and 96% (ns) after treatment. An improvement in 8 out of 11 patients was observed, and a paired t test showed an improvement in the group (p=0.023) (Figure 6.3). When calculated as a percentage of the maximum response of the healthy subjects, the group of clinically stable patients with CF had a maximal response of 75% (p=0.09). The response of the neutrophils from the patients with CF at each concentration of fMLP was expressed as a percentage of the healthy subject value was consistent across the range of fMLP concentrations (Table 6.1).

Effect of sputum-sol

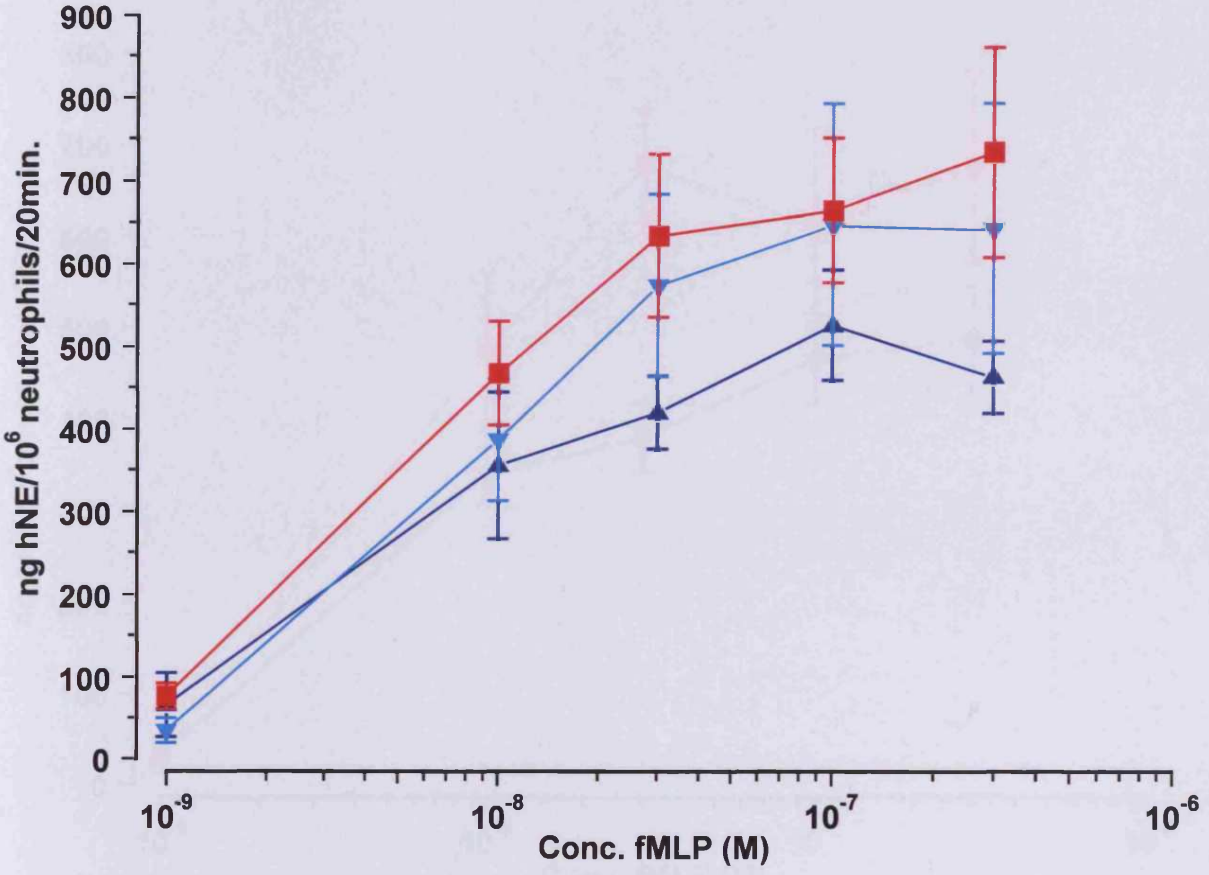
With the unstimulated cells (no fMLP) sputum-sol had no effect on elastase release, but produced significant augmentation of the response to 10^{-8} M fMLP stimulation in both CF and non-CF subjects (Table 6.2). Analysis on the effect of sputum showed no difference between groups at a different clinical state. The groups were then combined into patients and healthy subjects (Figure 6.5), which indicates that there is some increase in elastase generation by the sputum sol on unstimulated neutrophils, and a significant increase in the elastase release of the stimulated neutrophils at the majority of concentrations of sputum sol.

Comparison with superoxide generation

The fMLP stimulated increase in superoxide generation and elastase release were significantly correlated for all patient groups, but not for healthy subjects. (Figure 6.6).

Figure 6.1

Elastase release by neutrophils from patients with CF when in exacerbation and after antibiotic treatment, and in healthy subjects



▲ Start of treatment of patients with CF (n=12), ▼ End of treatment of patients with CF (n=11),

■ Healthy subjects matched CF start (n=12)

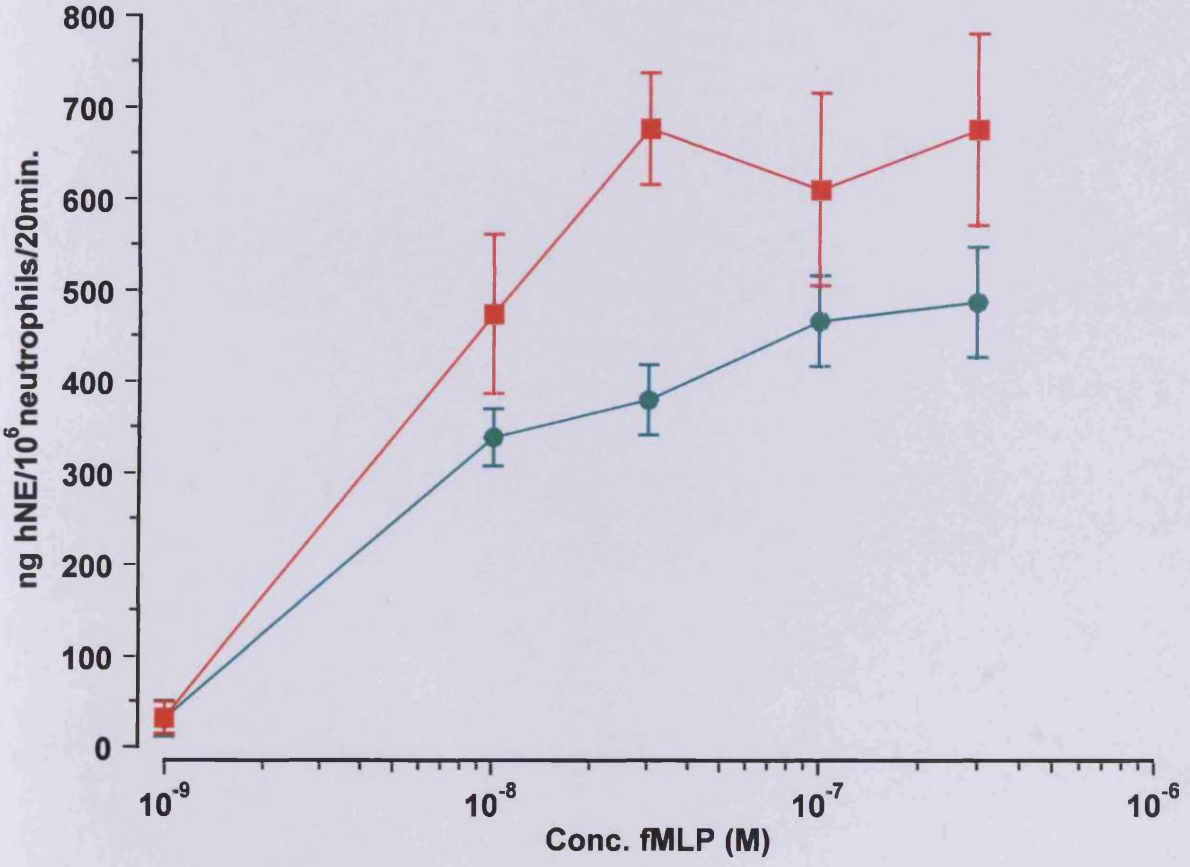
Arithmetic mean ± S.E.M.

CF compared to healthy subjects p=0.032 by MANOVA on $\log_{10}$ transformed data

(Healthy subjects matched CF end omitted for clarity)

Figure 6.2

Elastase release by neutrophils from patients with CF when clinically stable, and for healthy subjects



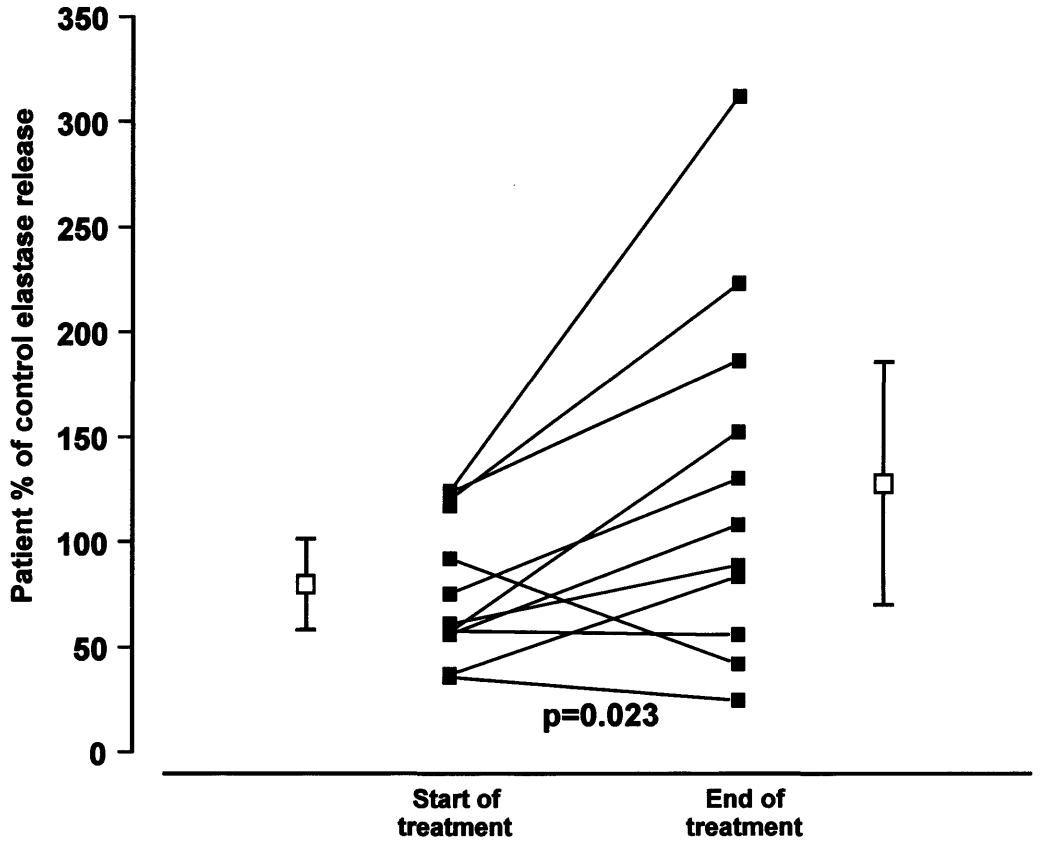
● Clinically stable patients with CF (n=12), ■ Healthy subjects (n=12)

Arithmetic mean ± S.E.M.

CF compared to healthy subjects p=0.032 by MANOVA on $\log_{10}$ transformed data

Figure 6.3

The effect of antibiotic treatment on elastase release by neutrophils from patients with CF expressed as a percentage of the response by neutrophils from matched healthy subjects



☐ Arithmetic Mean ± S.E.M., ■ Individual % values

Signifcance by paired t test

Table 6.1

The variation of patient percentage of control neutrophil elastase release for different fMLP concentrations

fMLP concentration	10^{-9}M	10^{-8}M	$3x10^{-8}$M	10^{-7}M	$3x10^{-7}$M
Start of Exacerbation	42.76 (21.3-85.7)	63.57 (41.6-97.3)	74.87 (53.7-104.5)	78.82 (58.1-106.9)	70.36 (51.1-97.1)
After Treatment	32.48 (12.2-86.7)	100.5 (55.9-180.7)	98.34 (98.3-173.8)	110.9 (71.9-171.0)	101.7 (55.6-186.2)
Clinically Stable	84.13 (17.7-398.1)	88.50 (45.8-171.0)	60.5 (41.0-89.1)	83.67 (55.9-125.0)	77.04 (52.1-114.0)

Geometric Mean of % (95%CI)

Table 6.2

Effect of Sputum sol on elastase release by neutrophils

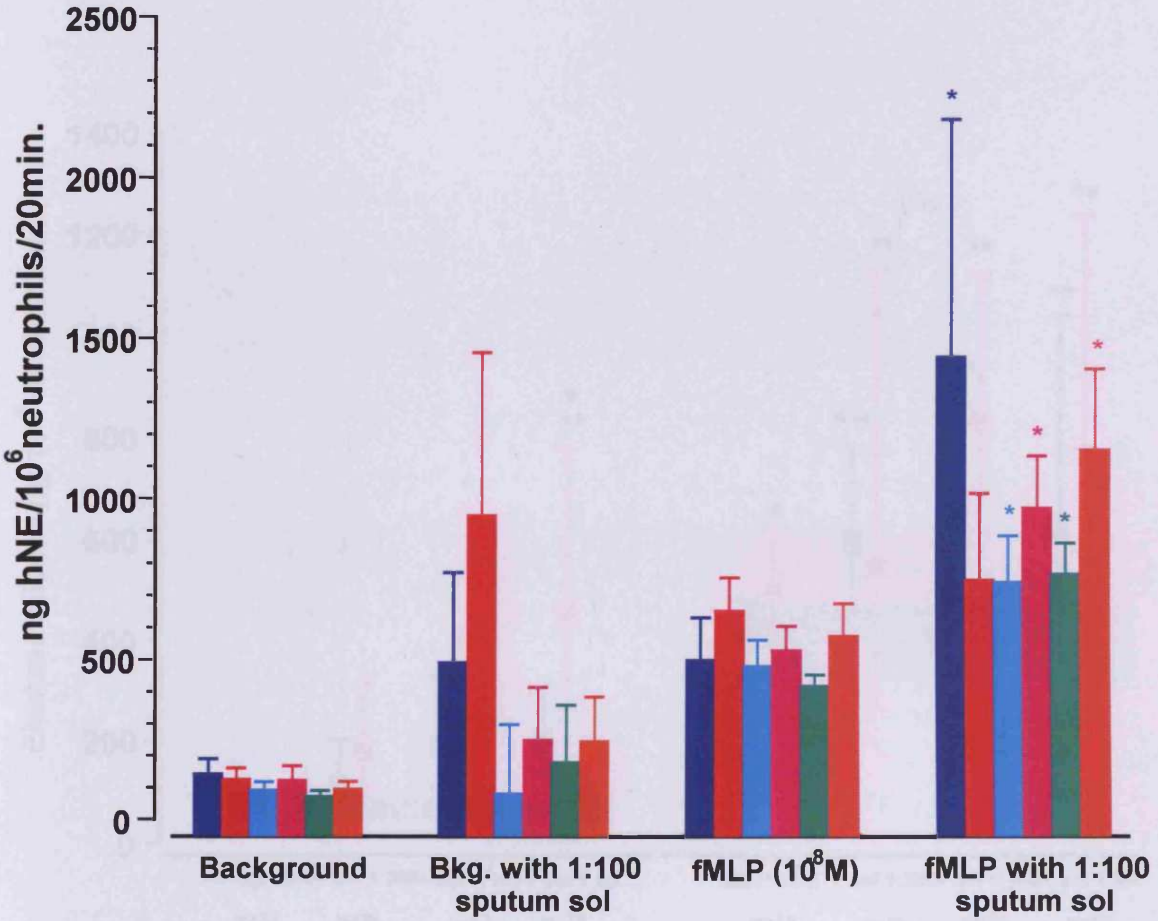
	Start of treatment		End of treatment		Clinically stable	
	patient with CF	healthy subject	patient with CF	healthy subject	patient with CF	healthy subject
Background	142	125	91	120	79	98
with 1:200 sputum sol	88	27	140	254	106	275
(p=)	ns	ns	ns	ns	ns	ns
with 1:100 sputum sol	489	957	-71	246	178	242
(p=)	ns	ns	ns	ns	ns	ns
with 1:50 sputum sol	322	811	-297	394	39	494
(p=)	ns	ns	ns	ns	ns	(0.047)
fMLP 10^{-8}M	496	647	477	525	416	570
with 1:200 sputum sol	690	743	762	858	712	1149
(p=)	(0.025)	ns	(0.011)	(0.051)	(0.021)	(0.008)
with 1:100 sputum sol	1473	788	737	996	764	1146
(p=)	(0.034)	ns	(0.024)	(0.021)	(0.006)	(0.012)
with 1:50 sputum sol	1440	1289	1119	1008	877	1229
(p=)	(0.011)	ns	ns	(0.021)	ns	ns

Arithmetic mean (significant effect of sputum sol, Wilcoxon signed rant test)

Sputum sol quoted is final dilution

Figure 6.4

The effect of sputum sol on elastase release by neutrophils from patients with CF and healthy subjects



Arithmetic Mean ± S.E.M.

CF Start of treatment (n=12), CF after treatment (n=9), CF clinically stable (n=12)

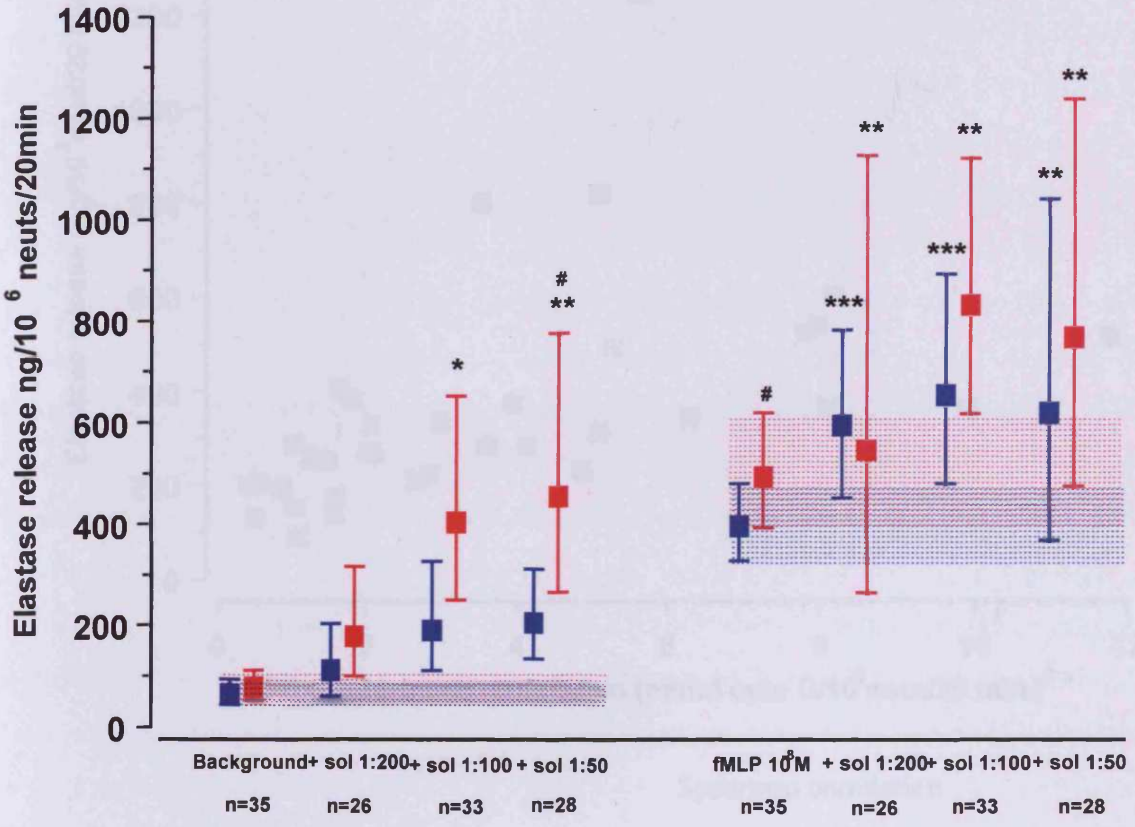
healthy subject matched CF start (n=12) , healthy subject matched CF end (n=9),

healthy subject matched CF stable (n=12)

* $p<0.05$ effect of sputum by Wilcoxon signed rank test

Figure 6.5

The effect of sputum sol on elastase release by neutrophils from patients with CF and healthy subjects where groups have been combined



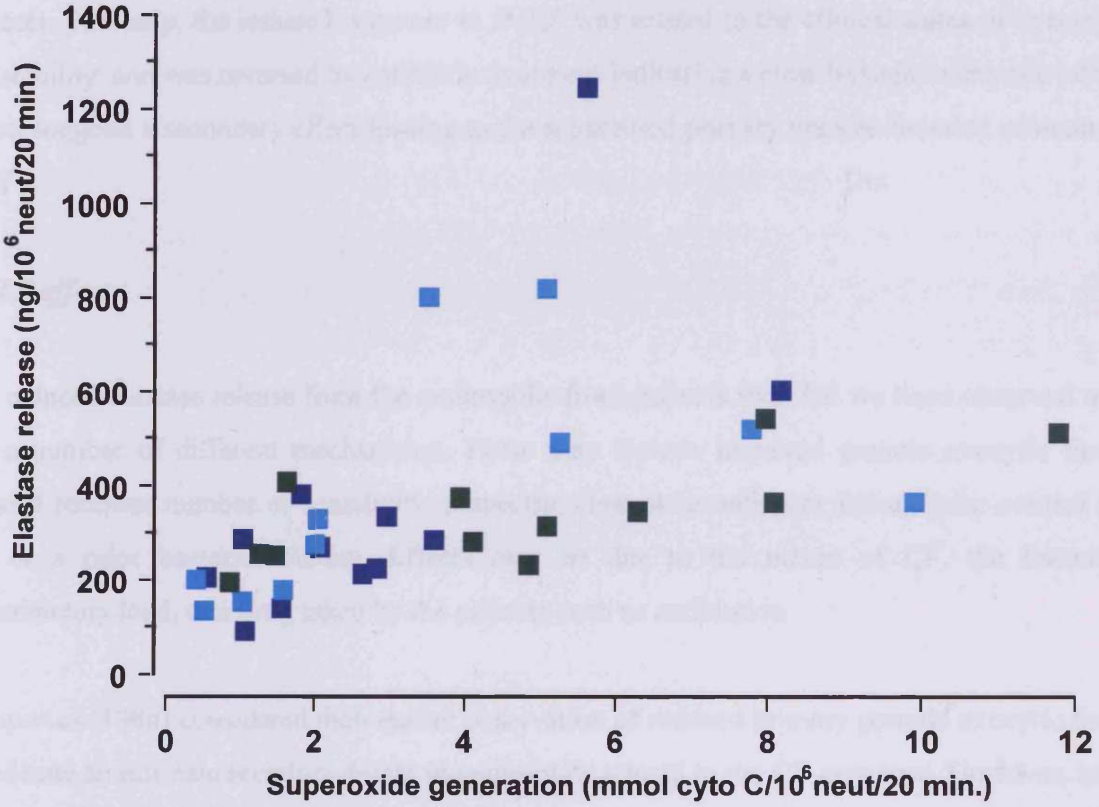
* p<0.05, ** p<0.01, *** p<0.001, Significant effect of sputum sol by Wilcoxon signed rank test

Significant difference between patients and controls by Mann Whitney U test.

■ Patients with CF: ■ Healthy subjects

Figure 6.6

Superoxide generation and elastase release in response to 10^{-8} M fMLP were related



		Spearman correlation
■	Patients at start of antibiotic treatment	r=0.643, p=0.024
■	Patients after antibiotic treatment	r=0.782, p=0.004
■	Patients clinically stable	r=0.636, p=0.026

Discussion

Circulating neutrophils from patients with CF showed a reduced response to fMLP for elastase release, as had been seen with superoxide generation compared with cells from paired healthy subjects. Similarly, the reduced response to fMLP was related to the clinical states of exacerbation and stability, and was reversed by antibiotic treatment indicating a clear linkage to chronic infection, which suggests a secondary effect leading to the suppressed primary granule function of neutrophils in CF.

FMLP effect

The reduced elastase release from the neutrophils from patients with CF we have observed may be due a number of different mechanisms. These may include impaired granule exocytic function, reduced receptor number or sensitivity, a specific chemokine effect or intracellular control of the cell or a prior bacterial burden. Effects may be due to the nature of CF, the bacterial or inflammatory load, or a drug taken by the patients such as antibiotics.

Kemp *et al* [1986] considered their earlier observation of reduced primary granule exocytic function to indicate an intrinsic secretory defect in neutrophils related to the CF genotype. However, here we have seen the reduced response alter with the clinical state of the patient suggesting that the effect is related to the current clinical state and its effect on the neutrophil rather than the underlying genetic effect of CF.

It has been reported that there are reduced numbers of IL-8 receptors on circulating neutrophils from patients with CF [Dai *et al* 1994] and this may also occur for fMLP receptors, although this was not measured. Receptor desensitisation was observed in the reduced chemotactic response to leukotriene B_4 of neutrophils from subjects with CF [Lawrence & Sorrell 1992]. In contrast, Russell *et al* [1998] discounted *in vivo* desensitisation in their demonstration of reduced L-selectin shedding in response to fMLP and IL-8 by neutrophils from subjects with CF compared with healthy and bronchiectatic subjects without CF. Although these potential mechanisms are largely speculative

they indicate the potential complexity of interactions that might underlie the down-regulation of neutrophil function in chronically infected patients with CF.

Neutrophil function may also be related to bacterial burden with the number of viable bacteria falling after antibiotic treatment [Regelmann *et al* 1990]. However, for this to show the effect we observed here such bacteria would have to be acting via a negative feedback mechanism for the responsiveness to be least at the time of greatest bacterial load. This does not appear to occur in the acute setting as neutrophils from patients with periodontitis have increased superoxide generation and elastase release *in vitro* [Figueredo *et al* 1999]. It has been suggested that neutrophils that are hyperreactive because of an *in vivo* priming may be less responsive to a second *in vitro* priming. Such impaired responsiveness to *in vitro* priming has been demonstrated in neutrophils in patients with obstructive jaundice [Jiang *et al* 1994] and AIDS [Elbim *et al* 1994]. A study in multiple trauma patients showed a reduced superoxide generation and MPO release accompanied by an increased basal intracellular calcium concentration which followed an increased in plasma IL-8 and elastase [Rose *et al* 2000] which may be one explanation for the observed results here. The study in trauma patients did not address the issue of priming.

It is possible that exposure to one or more regulators or mediators of the inflammatory response to chronic pulmonary infection, such as cytokines or cytokine networks, may have resulted in the down-regulation observed in this study. Such an effect is most likely to have occurred in the circulatory compartment. Antibiotic treatment changes the pattern of inflammatory mediators both in the circulation and airways, as shown in chapter 3, but it is unknown how bioactive such agents are in the circulation. CRP has been shown to act as an anti-inflammatory regulator by the attenuation of chemotaxis and superoxide generation by neutrophils [Morensen 2000]. These effects were observed at clinically relevant concentration. Correlations of our data were not found to be significant, except when all groups were pooled together when both the maximal response to fMLP for both superoxide generation and neutrophil elastase release were significantly correlated to circulating CRP levels. This implies that individual group size was too small for sufficient statistical power with respect to this parameter, not surprising considering the many other factors which could affect neutrophil reactivity.

Antibiotic treatment itself may have an effect, as neutrophils released more elastase after infusion with erythromycin *i.v.*, followed by stimulation *in vitro*. However, erythromycin had the effect of reducing the production of IL-8, a possible way in which macrolides may exert an anti inflammatory effect [Schultz *et al* 2000]. Neutrophils incubated with platelet *in vitro* showed a reduced elastase secretion both in the basal state and after stimulation with fMLP. These neutrophils had a much higher phagocytic activity, suggesting neutrophil-platelet interaction which promotes phagocytosis may lead to an inhibition of neutrophil functions [Losche 1996]. Since platelets have also been shown to stimulate neutrophils to generate superoxide [Nagata 1993] this indicates neutrophils are regulated by a complex balance of stimulation and inhibition. In these experiments, platelets had been removed by the cell separation procedure, but interactions of neutrophils and platelets prior to isolation cannot be ruled out.

Effect of sputum sol

In the basal state sputum sol had the tendency to increase the elastase release, although this was only significant in five of the measurements, and none of these at the 1:10 dilution. When stimulated with fMLP there was a significantly greater release in the majority of measurements. This increase following stimulation was greater than the sum of the fMLP response in the presence of buffer and the elastase release in response to sputum sol in the absence of fMLP which is suggestive of a priming effect of the sputum. TNFα, IL-8 and GM-CSF are known to prime neutrophils, and TNFα has been shown to be present in the sputum sol (Chapter 3). LPS is also know to produce a priming effect, and this would also have been present from the known bacterial load, although the quantities were not determined.

We used the addition of sputum sol in a range of doses to explore the possibility of secondary inhibitory effects on neutrophil function. Augmentation of elastase release by sputum sol by neutrophils from subjects with and without CF suggests a common responsiveness to factors in sputum-sol. The potency of LPS is increased by the presence of 1% serum, which may act via the LPS binding protein [Ottenolo *et al* 1999], a protein that is raised in serum at times of infection [Blairon 2003]. Neutrophils released more elastase in response to LPS when in the presence of endothelial cells than in the absence of EC [Houston *et al* 1997] via a calcium dependent

mechanism. TNFα release was reduced under the same circumstances, and elastase release can be inhibited by the calcium blocker Verapimil [Khalfi *et al* 1998].

After release elastase is expressed on the cell surface of neutrophils following stimulation with fMLP at the range of doses used here, and this expression was increased when the cells were incubated in the presence of serum [Owen 1995]. It is possible that the incubation of neutrophils with sputum-sol lead to an increase in the cell surface elastase, and serum proteins are also present in the sputum sol which may have led to the effect seen by Owen *et al* [1995]. The elastase which is bound to the cell surface of neutrophils is catalytically active, yet substantially resistant to inhibition by naturally occurring proteinase inhibitors. The presence of cell surface elastase persisted for at least 3hs at 37°C. It was unclear from the work of Owen *et al* whether the presence of elastase on the cell surface affected the magnitude of the response to fMLP. If this was the case this would have definite implications in the responses seen in this study. The up-regulation of cell surface elastase is also up-regulated by other pro-inflammatory mediators including TNFα and IL-8 [Owen *et al* 1997]. Not only is it unclear whether the presence of surface bound elastase affects the responsiveness of neutrophils, but also whether the presence of extracellular elastase affects the response magnitude or type of response. We added a high concentration of elastase due to its presence in many of the sputum-sols.

The inconclusive significance in many cases in the sputum-sol effects may be partly due to a large variability seen in the response, which is thought to be due to experimental factors. The amount of elastase present in the sputum was much greater than the elastase released by the neutrophils during the incubation. This resulted in a large value being removed to leave a small value. e.g. NVP08A; there was 45.341μg/ml elastase present in the incubation tube from the sputum sol, and only an additional 900ng/ml released by the neutrophils stimulated with 10^{-8}M fMLP. In addition the high concentration of sputum elastase meant large dilutions were carried out for the concentration to be within the range of the standard curve of the hNE ELISA, a further source of possible experimental variation. Despite this there were significant effects seen when the patient and controls were grouped in Figure 6.5, suggesting that larger numbers gave the study greater power, and overcame some of the limitations of experimental and subject variation.

The significant correlation between the superoxide generation and elastase release in the patient groups was expected more than the lack of relationship in the healthy subjects. There are large numbers of mechanisms which control the activity and responsiveness of neutrophils, and the results here do not provide sufficient information to even speculate on why such differences have been observed. A number of studies have looked at superoxide generation and granule release, but most have determined lactoferrin or MPO [Rose *et al* 2000] release rather than elastase release. Those who have determined both superoxide generation and granule release have not reported whether the two are related.

The observed pattern of down-regulation in both neutrophil superoxide generation and primary granule function, being at its greatest when in exacerbation and similar in clinically stable phases, and least after antibiotic treatment, mirrors the pattern of levels of circulating inflammatory markers, where exacerbation and clinically stable phases are more similar, with significant reduction observed after a course of antibiotic treatment. This adds further weight to the argument that the changes found may represent a pathophysiologically relevant mechanism linking neutrophil responsiveness to the level of infection and inflammatory activity in the lungs and further supports the possibility of regulation by CRP. Comparisons can be made to observations in rat airways exposed to repeated inhalation of *P.aeruginosa* LPS which resulted in a reduction of neutrophil recruitment. The continuous stimuli appeared to mitigate the accumulation of inflammatory cells in the airway by reducing chemokine production with a consequent change in the appearance of local inflammation to a chronic state [Shimada 2000].

Chapter 7

Possible mechanisms for the reduced responsiveness in neutrophils from patients with cystic fibrosis

Aim

To investigate possible mechanisms leading to the reduced superoxide generation and elastase release observed in patients with CF and healthy subjects by comparison of superoxide generation and elastase release in response to PMA and fMLP.

Introduction

From the results of reduced superoxide generation and elastase release in patients with CF at a time of exacerbation (chapters 5 & 6) we decided to look further into the mechanism responsible. The previous results used fMLP in the presence of Cytochalasin B (CB), and we decided to look at the response to a further stimulus, PMA and both of these in the absence of CB. These two stimuli act in different ways. In simple terms, PMA activates protein kinase C (PKC) producing NADPH oxidase [Olson *et al* 1990] activation while fMLP that binds to a specific cell surface receptor and induces, via GTP, activation of phosphodiesterases [Wolfson *et al* 1985]. The current understanding of how these two stimuli affect the intracellular activation of neutrophils is shown in Figure 7.1.

FMLP is a bacterial cell wall product which stimulates neutrophils via a seven transmembrane spanning receptor which is G-protein linked. G proteins are activated when GTP is bound and inactivated when GTP is hydrolysed [Fulop *et al* 1989]. This process initiates a sequence of protein phosphorylation events via a number of different pathways which are interrelated in a complex way and responsible for the regulation of the neutrophil.

The response to fMLP is both augmented and prolonged in the presence of CB, which inhibits and re-organizes actin polymers.

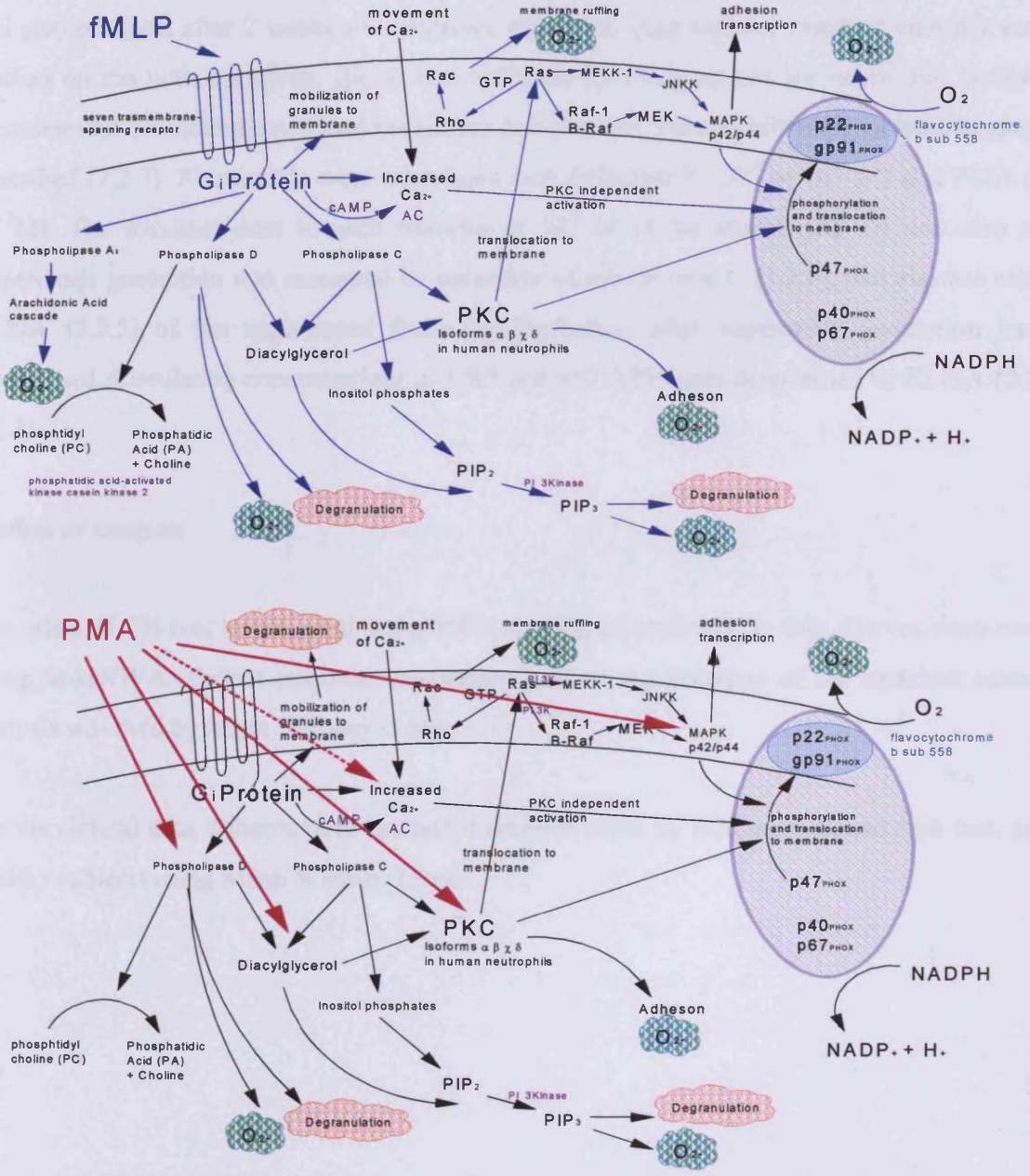
PMA is a phorbol ester which is able to induce its effect by direct movement into and across the cell plasma membrane. The direct stimulation of PKC has been suggested to activate the NADPH-oxidase through redistribution of PKC and phosphorylation of several proteins, including the cytosolic NADPH-oxidase component p47phox, and to induce extracellular release of oxygen metabolites. In addition assembly of the oxidase occurs in the specific granule membrane giving rise to intracellular oxygen metabolites [Karlsson *et al* 2000]. In particular, PKC-β has been shown to be recruited to the plasma membrane upon stimulation with phorbol ester and to the phagosomal membrane upon phagocytosis of IgG-coated particles (Fcγ-receptor stimulus), and this response to PMA is inhibited by the PKC-beta specific inhibitor 379196 [Dekker *et al* 2001].

In the previous experiments we included CB in the incubation buffer as this is regularly used in neutrophil *in vitro* experiments to provide measurable responses. Kawakami *et al* [2000] suggested that pre-treatment of neutrophils with CB dramatically alters the PMA response of the cells. CB has also been shown to enhance the fMLP-induced release of primary and secondary granules [Mocsai *et al* 2000]. One proposed mechanism is that the protein kinase C-dependent pathway is largely depressed, and a phospholipase D-dependent one for NADPH oxidase activation appears in CB-treated cells [Kawakami *et al* 2000]. CB has also been show to inhibit actin filament formation, which may be the mechanism responsible for potentiation of fMLP induced superoxide generation. Other possible mechanisms are by reducing the acid-resistant binding of fMLP, reducing the internalization of fMLP receptor complexs, and leading to the enhancement of second messengers such as diacylglyerol (DAG) [Bengtsson *et al* 1991]. It has also been reported that fMLP activation of phospholipase D leading to degranulation does not occur unless CB is present [DeLeo 1998].

In order to determine more information about the mechanism of responsiveness in neutrophils from patients with CF, we compared the superoxide generation and elastase release in response to fMLP to that obtained by PMA stimulation, and also looked at the effect of fMLP and PMA stimulation in the absence of CB, to determine whether there was a difference between neutrophils from patients with CF and healthy subjects. If the response to PMA was similarly reduced in patients with CF, then we could deduce that an inability of the neutrophils to respond exists.

Figure 7.1

Mechanisms involved in activation of neutrophils by fMLP and PMA



Additional information from Figure 1.3 collated from; Avdi [1996], Kent [1996], Gay [1997], Kular [1997], Burg [2001], Karlosson [2002], PithonCuri [2002], Chen [2003].

Method

Twelve patients with CF were recruited on admission for treatment of a pulmonary exacerbation, and studied again after 2 weeks *i.v.* antibiotic treatment. Age and sex matched controls were also studied on the both occasions. Blood was collected by venapuncture for neutrophil isolation and measurement of inflammatory markers (serum and plasma). Neutrophils were isolated as previously described (2.2.7). Neutrophils were stimulated with both fMLP (10^{-9} to 10^{-7} M) and PMA (10^{-9} to 10^{-7}M). The maximal dose to each stimulus at 10^{-7} M in the absence of CB was also studied. Superoxide generation was measured by reduction of cytochrome C (2.2.8), and elastase release by ELISA (2.2.5) of the supernatant from the incubation after superoxide generation had been determined. Circulating concentrations of CRP and hNEAPC were determined by ELISA (2.2.4 and 2.2.3).

Statistical analysis

The effect of CB was investigated using Wilcoxon signed rank test on data. Curves were compared using MANOVA. Patient response was determined as a percentage of the matched control and analysis was then by Mann Whitney U test.

For the clinical data, patients were compared between visits by Wilcoxon signed rank test, and with healthy subjects using Mann Whitney U test.

Results

Clinical

The patients with CF had a mean age of 23 (S.D. 4) years and a FEV_1 of 46.9 (27) % predicted before treatment which improved to 47.7 (28) after treatment (p=0.028). Healthy subjects had a mean age of 27 (SD 4) years. The mean serum CRP of the patients was 10.176 (6.32 - 22.09) {Geometric Mean (95%CI)} μg/ml at the start of antibiotic treatment and reduced to 3.938 (1.505 - 10.301) (p=0.005) after treatment. The healthy subjects CRP was 0.616 (0.264 - 1.437) which was less than the patients at the start and after antibiotic treatment (p=0.001, p=0.018 respectively). hNEAPC was not altered by antibiotic treatment with 34.70 (22.4 - 53.8) ng/ml before treatment and 41.8 (25.9 - 67.4) after treatment, but was different to the healthy subject value of 11.81 (6.3 - 20.1) ng/ml (p<0.001).

Superoxide generation

CB had no effect on the basal superoxide generation of cells in any of the groups. It caused a significant increase in the response to fMLP in all groups (p=0.002). CB had no effect on the superoxide generation in response to PMA (Figure 7.3a).

There was a significant difference between the response to fMLP (p= 0.004 by MANOVA) between patients at the start of a pulmonary exacerbation, and controls (Figure 7.2a). There was no significant difference in the response to fMLP between patients after treatment and healthy subjects. There was no significant difference between patients and healthy subjects in superoxide generation in response to PMA at either time point.

When the response of neutrophils from patients was determined as a percentage of the matched healthy subject, this again showed that patients had a reduced response to fMLP but not to PMA at a time of respiratory exacerbation (Figure 7.4a). When patients were compared before and after treatment, the response to fMLP in the absence of CB increased with treatment (p=0.034).

Elastase release

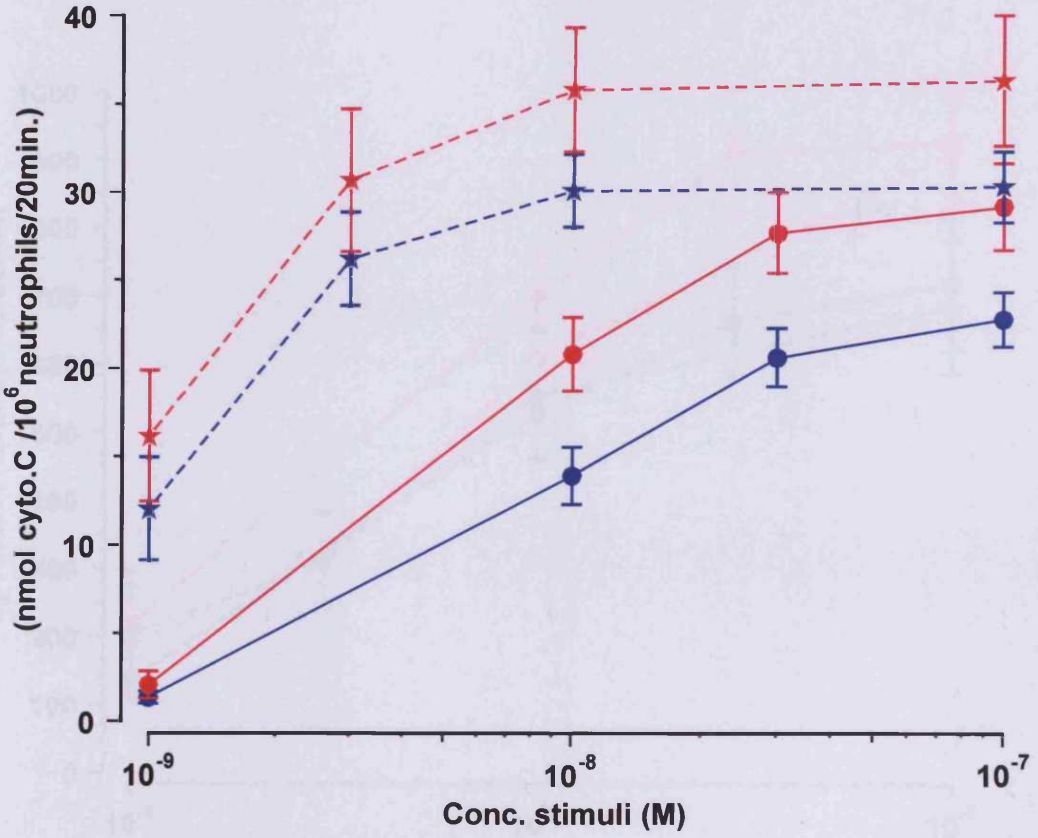
CB caused an increase in basal elastase release which was significant in the healthy subjects matched to the patients at the start of antibiotic treatment ($p=0.043$). It caused a significant increase in the response to fMLP ($p<0.02$) and PMA in all groups ($p<0.04$) (Figure 7.3b).

There was a difference between the response to fMLP ($p=0.004$ by MANOVA) between patients at the start of a pulmonary exacerbation, and healthy subjects (Figure 7.2b). There was also a difference in the response to PMA between patients before and after treatment ($p=0.015$ by MANOVA).

When the response of neutrophils from patients was determined as a percentage of the matched healthy subject, this again showed that patients had a reduced response to fMLP but not to PMA at a time of respiratory exacerbation (Figure 7.4b). When patients were compared before and after treatment, the response to PMA in the presence of CB increased with treatment ($p=0.012$).

Figure 7.2a

Superoxide generation in response to fMLP and PMA by neutrophils from patients with CF at the start of antibiotic treatment and healthy subjects



Values Arithmetic mean ± SEM (basal generation removed), n=12

fMLP stimulation p=0.004 by MANOVA Patient at start of treatment compared to healthy subject.

● patients with CF at start of antibiotic treatment

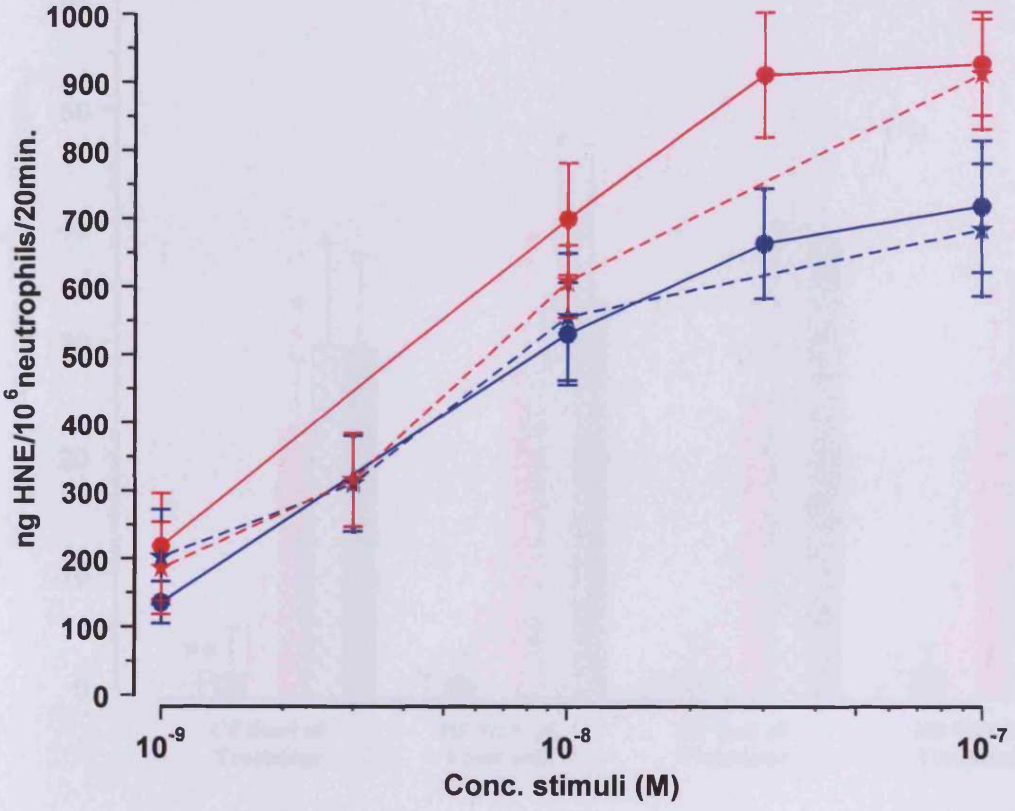
● healthy subjects

PMA stimulation, ★ patients with CF at start of antibiotic treatment

★ healthy subjects

Figure 7.2b

Elastase release in response to fMLP and PMA by neutrophils from patients with CF at the start of antibiotic treatment and healthy subjects



Values arithmetic mean ± SEM (basal release removed), n=12

Patient compared to healthy treatment; fMLP stimulation p=0.004 by MANOVA

● patients with CF at start of antibiotic treatment

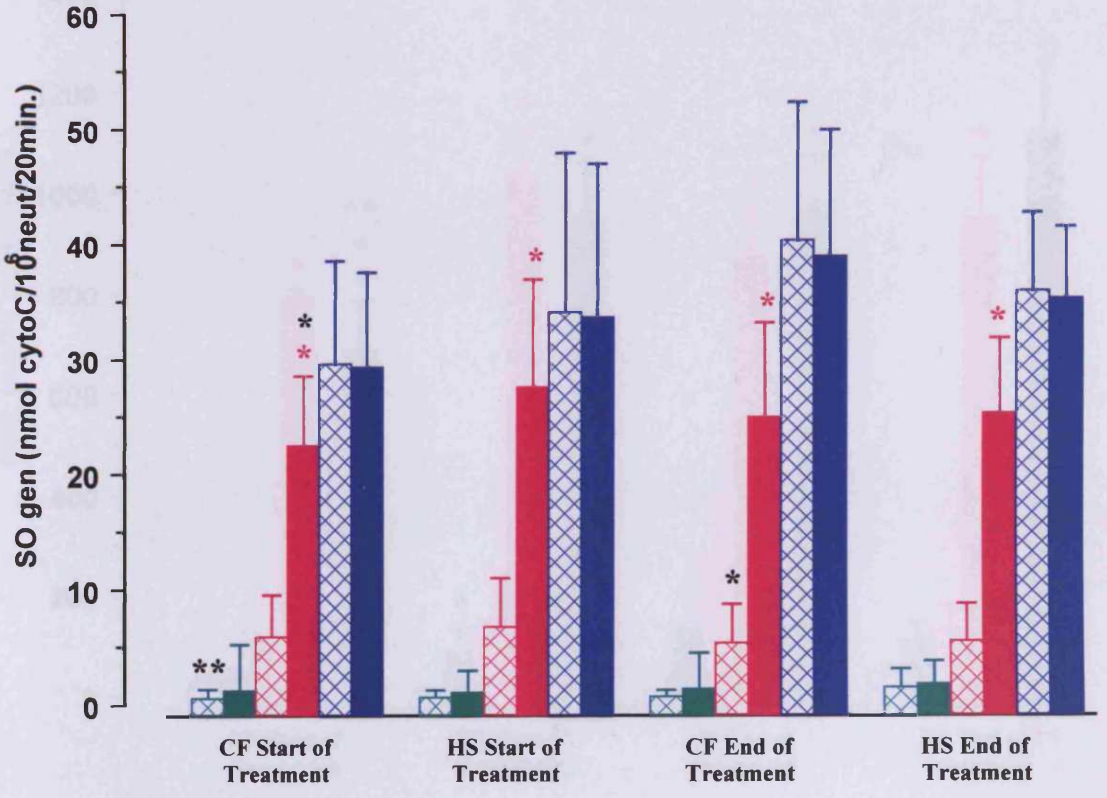
● healthy subjects

PMA stimulation, ★ patients with CF at start of antibiotic treatment

★ healthy subjects

Figure 7.3a

Superoxide generation of neutrophils when unstimulated, and stimulated with fMLP and PMA in the absence and presence of CB from patients with CF and healthy subjects



Basal generation ⊠ absence ■ presence of CB

fMLP (10^{-7}M) stimulation ⊠ absence ■ presence of CB

PMA (10^{-7}M) stimulation ⊠ absence ■ presence of CB

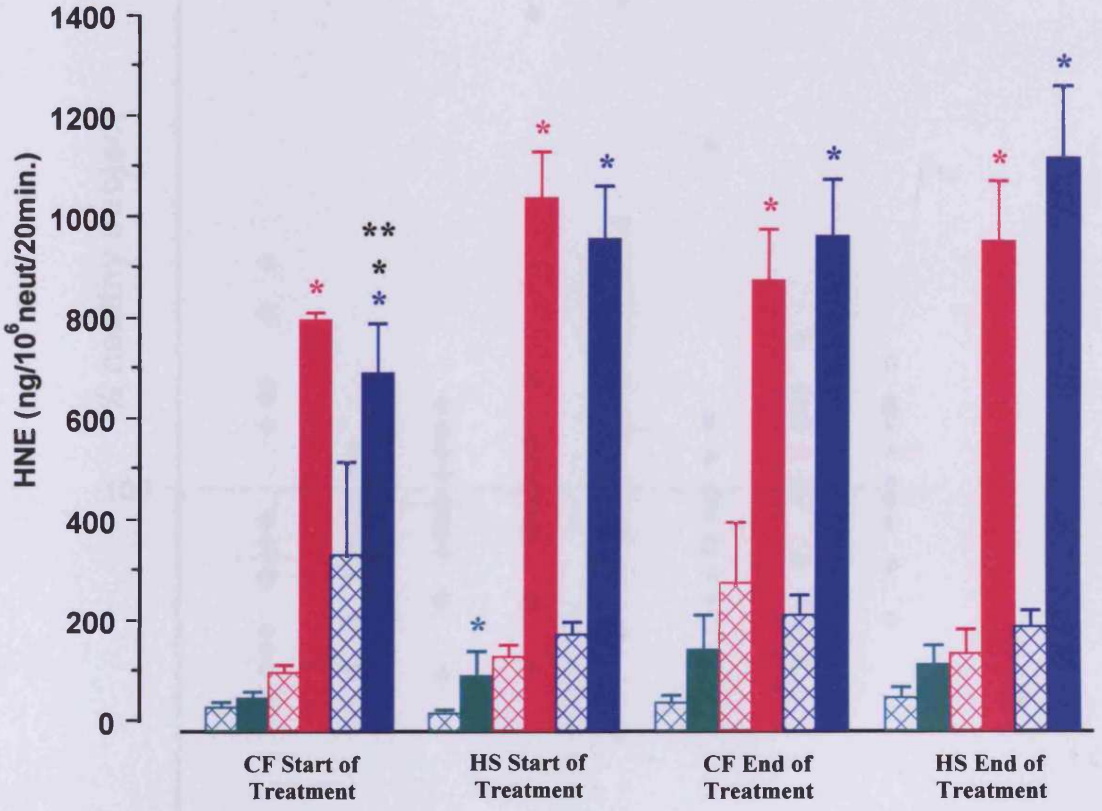
Arithmetic Mean ± S.E.M. n=12, analysis by Wilcoxon signed rank test.

✳ $p<0.05$ Effect of CB, ✳✳ $p<0.05$ Effect of treatment,

✳ $p<0.05$ Patient with CF compared to healthy subject.

Figure 7.3b

Elastase release of neutrophils when unstimulated, and stimulated with fMLP and PMA in the absence and presence of CB from patients with CF and healthy subjects



Basal release ☒ absence ■ presence of CB

fMLP (10^{-7}M) stimulation ☒ absence ■ presence of CB

PMA (10^{-7}M) stimulation ☒ absence ■ presence of CB

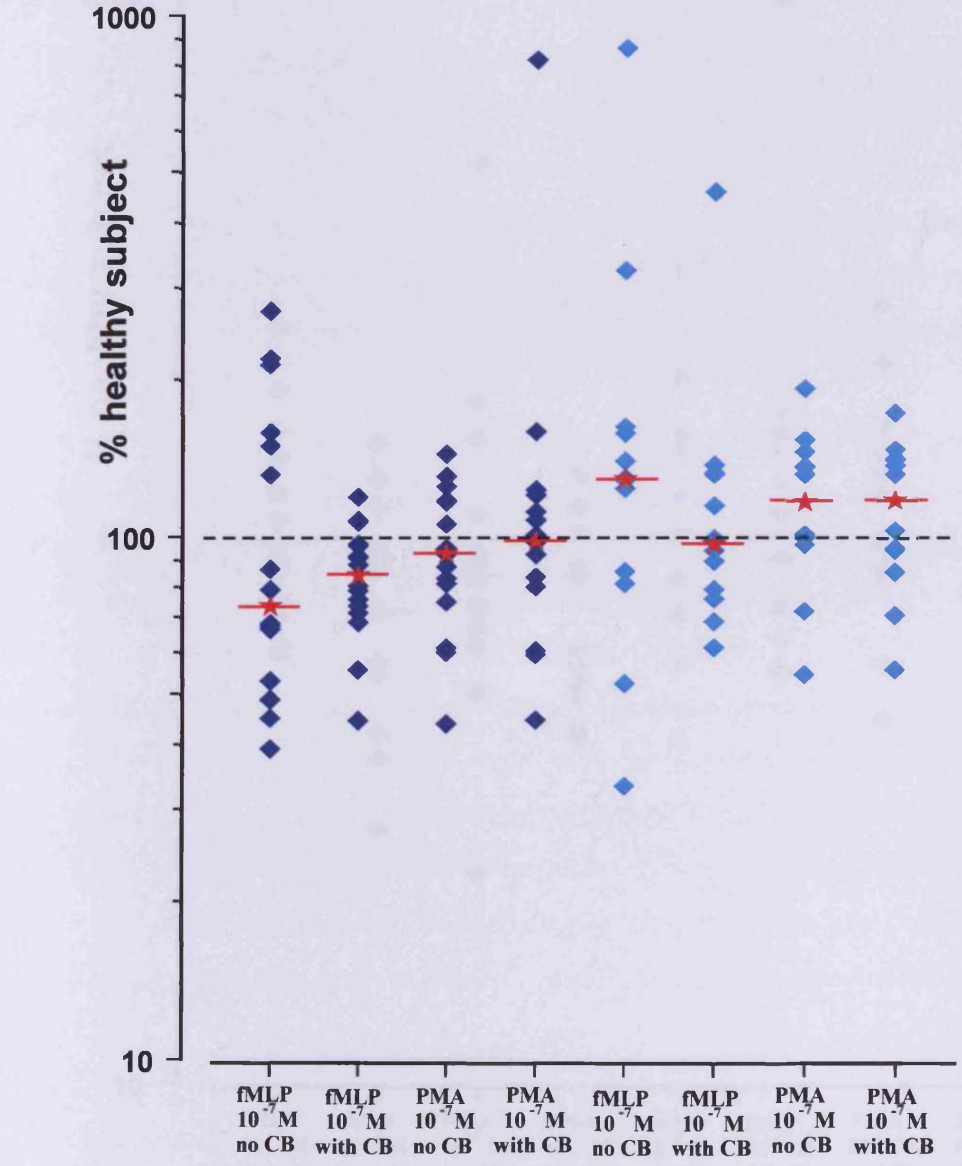
Arithmetic Mean ± S.E.M. n=12 Analysis by Wilcoxon signed rank test.

* p<0.05 Effect of CB, ** p<0.05 Effect of treatment,

* p<0.05 Patient with CF compared to healthy subject.

Figure 7.4a

Superoxide generation of neutrophils from patients with CF as a percentage of the matched healthy subject

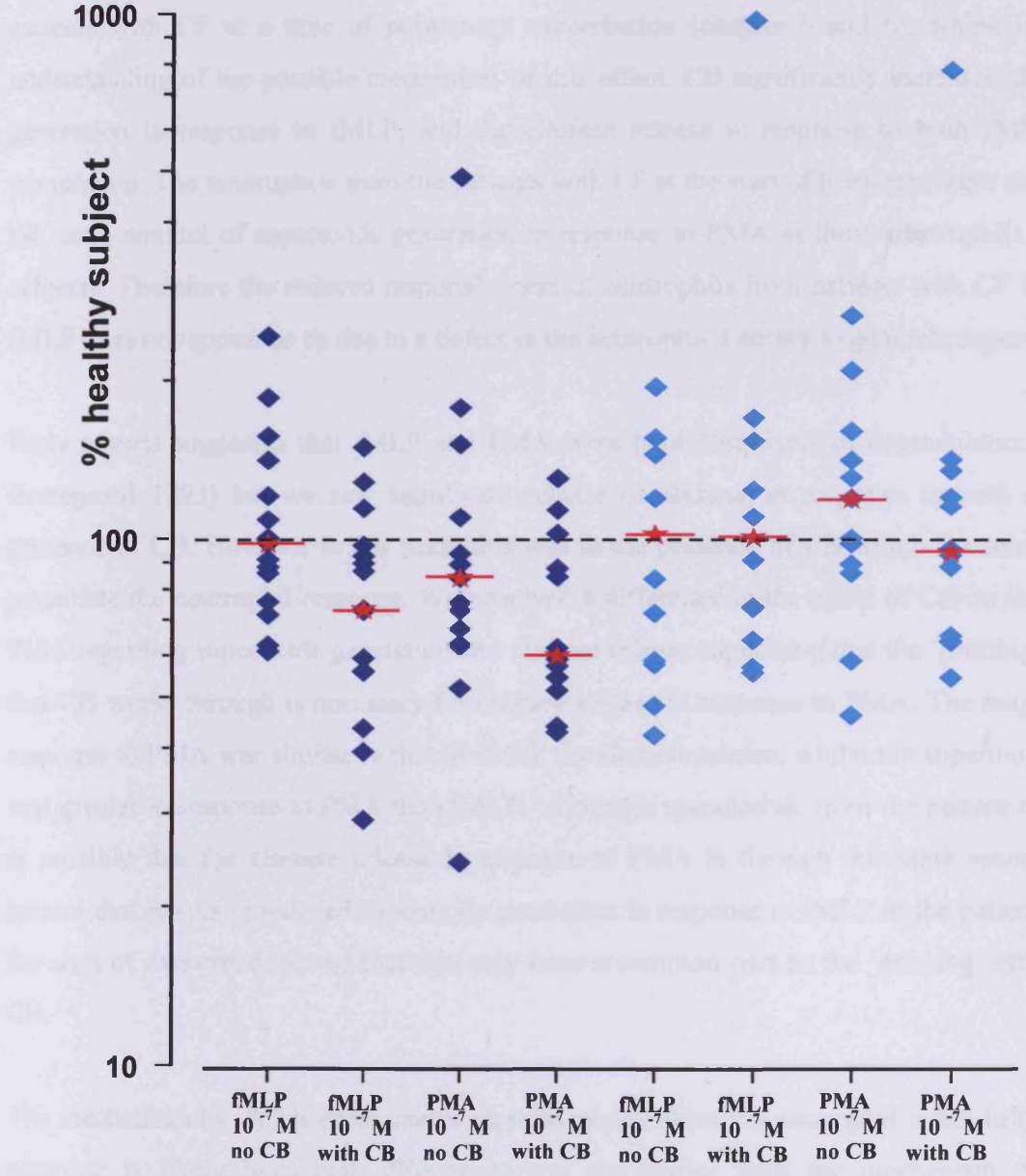


Median

Start of treatment, After treatment

Figure 7.4b

Elastase release of neutrophils from patients with CF as a percentage of the matched healthy subject



Median

Start of treatment, After treatment

Discussion

These results confirm the reduced superoxide generation and elastase release by neutrophils from patients with CF at a time of pulmonary exacerbation (chapter 5 and 6), whilst increasing our understanding of the possible mechanism of this effect. CB significantly increased the superoxide generation in response to fMLP, and the elastase release in response to both fMLP and PMA stimulation. The neutrophils from the patients with CF at the start of treatment were able to produce the same amount of superoxide generation in response to PMA as those neutrophils from healthy subjects. Therefore the reduced responsiveness of neutrophils from patients with CF in response to fMLP does not appear to be due to a defect in the neutrophil's ability to generate superoxide.

Early reports suggested that fMLP and PMA were poor stimulants of degranulation [Bjerrum & Borregaard 1993] but we saw significant release of elastase in response to both agents in the presence of CB. However in our study this was in the presence of CB which is commonly used to potentiate the neutrophil response. We observed a difference in the effect of CB on the response to PMA regarding superoxide generation and elastase release suggesting that the 'priming' mechanism that CB works through is necessary for elastase release in response to PMA. The magnitude of the response to PMA was similar to that of fMLP for elastase release, whilst the superoxide generation was greater in response to PMA than fMLP. Although speculative, from the pattern of response, it is possible that the elastase release in response to PMA is through the same second messenger system that results in reduced superoxide generation in response to fMLP in the patients with CF at the start of exacerbation, and that this may have a common part to the 'priming' effect shown by CB.

The mechanism by which PMA causes elastase release from the neutrophil is not fully understood, although is likely have both differences and similarities with the mechanism of superoxide generation. The difference seen between patients at the start and end of treatment for elastase release in response to PMA could be important. However, an understanding of the mechanism of action by which PMA acts with respect to elastase release is needed. It is possible that superoxide generation is dependent on the PKC pathway, but that elastase release is influenced more by the cytosolic Ca2+ level [Cabanis *et al* 1996]. Both fMLP and PMA promote phosphatidic acid (PA)

formation and increased PA formation induced by receptor agonists has been suggested to promote degranulation [Tou *et al* 2002].

It is well established that priming occurs *in vivo* in some diseases such as ARDS [Chollet-Martin *et al* 1992] blunt trauma [Krause *et al* 1988], and bacterial infection [Bass *et al* 1986]. Carletto *et al* [1996] showed a marked increase in the response to fMLP in exudate compared to blood neutrophils, although no change in the response to PMA, indicating that priming was induced by migration, but that it did not affect the superoxide generation in response to PMA.

However, we are seeing what could be considered the opposite effect of priming; desensitisation. This has been seen *in vitro* with relatively high doses of stimulus [Lee *et al* 1989], and appears to be stimulant specific in terms of fMLP and C5a [O'Flaherty *et al* 1979]. In addition heterologous desensitisation occurs, which is at least in part mediated by second messenger-activated kinases such as protein kinase C [Richardson *et al* 1995]. Receptor phosphorylation is required for desensitisation of immediate responses such as G protein activation as well as downstream responses such as calcium mobilization [Prossnitz *et al* 1997]. The phenomenon of reduced superoxide generation in response to fMLP, but not to PMA has been seen in other disease states. The superoxide generation in response to fMLP was reduced in septic patients using a whole blood technique, but showed a small increase in the superoxide generation in response to PMA [Pascual *et al* 1999]. After exposure of neutrophils to C5a in rats, the superoxide and H_2O_2 production in PMA activated cells was inhibited, indicating that exposure to C5a *in vitro* or *in vivo* can result in impairment of the respiratory burst in a different way to that observed in this study [Huber Lang *et al* 2002].

Alterations causing an impaired response to fMLP could occur at the receptor, the GTP-binding Gi protein, the PLC and the PIP^2 availability. In addition conditions that affect the membrane fluidity might influence the receptor G-coupling and abnormalities in this coupling decrease the O_2^- production. Increased membrane fluidity has been linked to reduced superoxide generation. Alteration of membrane fluidity can also affect the cell's use of extracellular Ca^{2+}. It has been shown that increased membrane fluidity is related to basal calcium pump activity, and that PMA activation is independent of the calcium concentration [Gaudry *et al* 1990, Di Virgilio *et al* 1984]

making this a possible explanation for the results in this study. PKC has a complex regulatory effect on neutrophil function, which interacts with the other intracellular pathways including Ca^{2+} concentrations and diacylglycerol activity, with multiple isoforms of the enzyme family responding differentially to the second messengers produced [Kent *et al* 1996].

A reduced superoxide generation in response to fMLP, but not in response to PMA was also seen in elderly patients with protein-energy malnutrition [Cederholm & Gyllenhammar 1999], patients with early onset peridontitis [Biasi *et al* 1999] and in aged rats [Alvarez *et al* 2001]. Age related changes in receptor-mediated effector functions occur, but this is not due to alteration of the receptor signal transduction mechanism, but an alteration of the post receptor signal transduction [Fulop 1989], and a similar mechanism may also occur in our patients who show other complications similar to aging such as increased rheumatoid arthritis and bone osteopenia [Ionescu *et al* 2002]. A reduced superoxide generation in response to zymosan was observed in smokers, who had an enhanced response to PMA. This was linked to reduced glutathione levels, indicative of oxidative stress [Sela *et al* 2002]. Although we observed no enhanced response to PMA, the role of oxidative stress should be considered, as it has been shown to occur in patients with CF [Rahman & MacNee 2000].

The β-adrenergic pathway which activates adenylate cyclase is one mechanism that can affect neutrophil function. Barnett *et al* [1997] showed that superoxide production in response to fMLP was significantly attenuated by blocking this by pre-treatment with both isoproterenol and forskolin. However, this pre-treatment did not significantly affect the elastase release making this unlikely to be the pathway affected in the neutrophils from patients with CF as we showed a similar difference in patients compared to healthy subjects for both superoxide generation and elastase release. Alternatively the activation of the phospholipase A_2 pathway leading to the arachidonic acid cascade could affect neutrophil functioning. The phospholipids predominantly produced by this pathway enhance the respiratory burst and increase degranulation [Muller *et al* 2001] therefore a fault in this pathway could result in the effect observed in this study. However little is known about the relative effects of PMA and fMLP stimulation so this is speculative.

In this study there was both inter-assay and inter-individual variation. The inter-assay variation was partly overcome by calculating the patient response as a percentage of the healthy subject response.

Despite this there was still considered to be wide inter-individual variation. We are not the only group to observe this. Fruhwirth *et al* [1998] detected deficient oxidative burst in a group of fourteen patients with CF, caused by abnormally high mean fluorescence intensity (MFI) of resting cells, but showed enhanced oxidative burst in another six patients.

Chapter 8

Cytokine receptors and adhesion molecules on neutrophils and their relationship to inflammation and clinical status in cystic fibrosis

Aim

To determine the expression of cell surface receptors on circulating neutrophils from patients and healthy subjects, and whether there are changes relate to the clinical status of the patients.

To determine the effect of stimulation with fMLP on the expression of L-selectin and CD11b.

To determine the myeloperoxidase (MPO) content of neutrophils from patients and healthy subjects.

Introduction

An early component of activation of neutrophils is the expression of various cell surface receptors which may be primarily involved in the adhesion process or regulation of function. Expression of both groups of these receptors both influence and are influenced by inflammation and neutrohil functioning. The number and affinity of such receptors has a marked influence on the reactivity of the cell and can change as a result of the activation state of the neutrophil. Therefore, changes in surface receptor number has the potential to influence superoxide generation and elastase release, and therefore may indicate or reflect a possible mechanism underlying the reduced responsiveness of neutrophils from patients with CF that we observed in the experiments discussed in the previous chapters.

There is increasing evidence suggesting that integrin-mediated signalling in leukocytes plays a role in the induction of pro-inflammatory cytokine production [Soler-Rodriguez *et al* 2000]. It has been shown that crosslinking of l-selectin and CD11b/CD18 molecules induce a rapid increase in Ca^{2+}, O_2^- generation, exocytosis of azurophilic granules and also increases tyrosine phosphorylation and activation of mitogen activated protein (MAP) kinase [Wize *et al* 1998]. L-selectin is the first

molecule by which neutrophils interact with the endothelium [Davey *et al* 2000]. It is cleaved and shed quickly after LPS stimulation in a dose-dependent manner [Soler-Rodriguez *et al* 2000]. IL-8, fMLP, PAF and some non-steroidal anti-inflammatory drugs are also known to induce L-selectin shedding [Hafezi-Moghadam *et al* 2001].

It is well established that stimulation of neutrophils results in the up-regulation of CD11b receptor expression and the reduction in L-selectin expression. Blockade of shedding causes enhanced activation of neutrophils, and shedding of L-selectin appears to be a physiologic mechanism to limit neutrophil recruitment during inflammation [Hafezi-Moghadam *et al* 2001]. L-selectin shedding has been shown to be reduced after migration, particularly after stimulation with fMLP [Mackarel *et al* 2000]. It also seems likely that the affinity of β_2-integrins are low in resting neutrophils and is suddenly increased upon activation [Anderson *et al* 2000].

Cell surface receptors for various pro-inflammatory cytokines are expressed on neutrophils and may be part of the regulatory pathways for the expression of adhesion molecules. In view of our finding that increased concentrations of IL-6 and TNFα occur in plasma in patients with CF and vary with clinical status we considered those receptors which might be involved in such changes.

IL-8 plays a key role in neutrophil chemotaxis and therefore IL-8 receptor expression could affect this process. Two IL-8 cell surface receptors occur on neutrophils; IL-8 RA (type 1 or CXCR-1) and IL-8RB (type II or CXCR-2) [Adams *et al* 2001]. They share 77% amino acid homology and are both G-protein linked receptors. In neutrophils the two receptors have been shown to be functionally different with respiratory burst and activation of PLD exclusively dependent on IL-8RA [Jones *et al* 1996]. It has been suggested that IL-8RB is primarily involved in neutrophil recruitment while IL8RA may have a more active role at the site of inflammation [Chuntharapai & Kim 1995] with the opposite being suggested in another study [Hammond *et al* 1995]. After ligand binding, these receptors are internalized and subsequently recycled and reappear on the cell surface within 60 min. [Mukaida *et al* 2003]. IL8-RA and IL-8RB are differently expressed after injury with selective expression of IL8RB after trauma, in which suppression appears to be linked to poor outcome [Quaid *et al* 1999, Adams *et al* 2001]. IL8RA deficient mice show an enhanced susceptibility to chronic infection, which is in agreement with studies in human disease [Mukaida 2003].

The sIL-6R has been shown to be raised in plasma of both patients with CF [Ionescu *et al* 2000] and COPD [Eid *et al* 2001]. The IL-6Rα is expressed by a limited number of cells including hepatocytes, neutrophils, and mononuclear cells, and its presence in the serum is due to it being shed by these cell types. IL-6 receptor shedding is activated by CRP [Jones *et al* 1999], and the sIL-6R mediated signalling is an important intermediary in the resolution of inflammation [Hurst *et al* 2001]. The released soluble form can bind IL-6 and protect it against enzyme inactivation [Marin 2001].

Receptors for TNFα are present on both neutrophils and monocytes, and their expression has been shown to be reduced in systemic inflammation [Rumalla *et al* 2002]. The effects of TNFα are mediated by two structurally related, but functionally distinct receptors, p55 (TNFR1) and p75 (TNFRII) that are co expressed on most cell types and can be released as soluble components capable of binding circulating TNFα [Vandenabeele *et al* 1995]. TNFR expression by neutrophils was shown to be reduced in patients with chronic heart failure [Rumalla 2002]. The circulating levels of TNFRs have been shown to be elevated in patients with chronic heart failure [Ferrari 1995] with CF [Ionescu *et al* 2002] and with COPD [Eid *et al* 2001]. It is also likely that TNFR1 plays a key role in LPS-induced lung neutrophil accumulation [Calkins *et al* 2001].

MPO synthesis occurs during myeloid differentiation in bone marrow and is completed within granulocytes prior to their entry into the circulation. The enzyme is stored within primary granules of neutrophils and monocytes and is released on activation causing degranulation [Zhang 2001]. MPO has been shown to be released from neutrophils on adherence, in particular to fibronectin, as well as on stimulation to both fMLP and PMA [Xu *et al* 2002]. MPO also plays a key role in the oxidative killing of bacteria [Oh-oka *et al* 2001].

In view of this evidence one of the aims of this study was to determine cell surface receptor density for L-selectin, CD11b, TNF R, IL-8 RA and RB, and IL-6 R on neutrophils from patients with CF and healthy subjects. The possible effect of the clinical status of the patients was investigated at the start and end of a course of antibiotics for an exacerbation of respiratory symptoms. The effect of fMLP stimulation on the receptor density for L-selectin and CD11b was determined for neutrophils

from non-CF healthy subjects and patients with CF at both time points. In addition, the intracellular content of MPO was also measured in all groups.

Method

Study Protocol

Patients and healthy subjects were recruited, and data collected as described in patient group C. Briefly patients with CF were studied at the start and end of antibiotic treatment for an exacerbation of respiratory symptoms. Matched healthy subjects were also studied at these times.

Basal levels of adhesion molecules L-selectin and CD11b, cytokine receptors TNF R, IL-8 RA & RB and IL-6 R and intracellular MPO content were all assessed by flow cytometry (2.2.11). Microbead controls were included to control for inter-assay variation. The effect of 20 min. fMLP (10^{-10} to 10^{-7} M) stimulation on the change in L-selectin and CD11b cell surface expression was also determined.

Circulating levels of CRP and hNEAPC were measured by ELISA (2.2.3 and 2.2.4 respectively) and the circulating neutrophil count recorded. Levels of IL-6, TNFα, IL6 SR and TNF RI &RII were also measured in plasma by ELISA (R&D systems Europe) according to the kit protocol.

Data from the FACS was analysed using the specialist software 'WinMDI'. Data was expressed as mean molecules of equivalent fluorescence (MEF) except where stated. Statistical analysis was by Mann Whitney U test on group data, and comparison between multiple doses of fMLP was by MANOVA on log transformed data. Values for patients were also calculated as a percentage of the matched healthy subject to allow for inter-assay variation.

Results

Clinical

The patients with CF had a mean age of 27 (S.D. 3.6) years and a FEV_1 of 52 (S.D. 22) % predicted. Healthy subjects had a mean age of 27 (4.7) years. The mean serum CRP of the patients was 18.193 (3.726 - 88.838) {Geometric Mean (95%CI)} μg/ml at the start of antibiotic treatment and reduced to 5.014 (1.085 - 23.157) (p=0.71) after treatment. The healthy subjects CRP was 1.332 (0.420 - 4.219) which was less than the patients at the start of antibiotic treatment (p=0.012) but not after treatment. hNEAPC also fell significantly with antibiotic treatment from 40.47 (23.4 - 69.8) ng/ml to 31.7 (17.3 - 58.1) (p=0.034), but was not different to the healthy subject value of 30.2 (18.7 - 48.6) ng/ml.

Surface expression of receptors, and intracellular MPO of neutrophils in basal state

Expression of CD11b was greater for neutrophils from patients at the start of treatment than for healthy subjects (p=0.008) and fell with treatment (p=0.04) (Figure 8.1). No significant differences were found between patients, healthy subjects or at the time points studied for sL-selectin, IL-8 A and B, TNFα and IL-6 receptors (Table 8.1). The intracellular MPO content increased significantly following treatment (p=0.024) (Figure 8.2), although it was not different to the healthy subject group at either time point. Receptor expression in patients with CF was also expressed as a percentage of the paired healthy subject (Figure 8.3). The receptor levels when calculated as a percentage of the paired healthy subject values were significantly correlated within subject for L-selectin, IL-8RA, IL-8RB, IL-6R, TNFR and MPO ($p<0.05$) but not for CD11b.

Clinical status and receptor expression related at the start of antibiotic treatment to the circulating neutrophil count and CRP (r=0.893, p=0.007). Expression of L-selectin was related to the circulating IL-6 receptor level (r=0.647, p=0.005 all patients; r=0.698 p=0.08; patients start; r=0.821, p=0.023 patients end) and to the TNF RI (r=0.572, p=0.011 all patients; r=0.905 p=0.002 patients start; r=0.238, p=0.570 patients end).

FMLP stimulation

Stimulation with fMLP reduced L-selectin expression in a dose related way, while increasing that of CD11b. The fluorescence from CD11b and L-selectin at different doses of fMLP is shown as dot plots of cells from one patient (Figure 8.4) and histograms of MEF of the patient and controls groups (Figure 8.5a and 8.5b). When quadrants were used to analyse the pattern of response, there was no difference between groups for the percentage cells in each quadrant.

There was a difference in the fMLP dose related reduction in L-selectin expression between patients at the start of treatment and healthy subjects ($p=0.021$) (Figure 8.6), however there was no significant in the change in MEF, or percentage maximum change. There was a difference in the fMLP dose related expression of CD11b for patients at the start of a pulmonary exacerbation compared to after treatment ($p=0.036$) and to healthy subjects ($p=0.021$)(Figure 8.7). When the change in cell surface expression, not the absolute values were used, there was no difference for either of the markers, i.e. the ability of the cells to responsd to fMLP by alteration of surface expression of L-selectin was equal in patients with CF and healthy subjects.

The surface expression in patients was calculated as a percentage of the healthy subject expression for each pair (Table 8.2).

Table 8.1

Surface receptors on basal neutrophils from patients with CF and healthy subjects

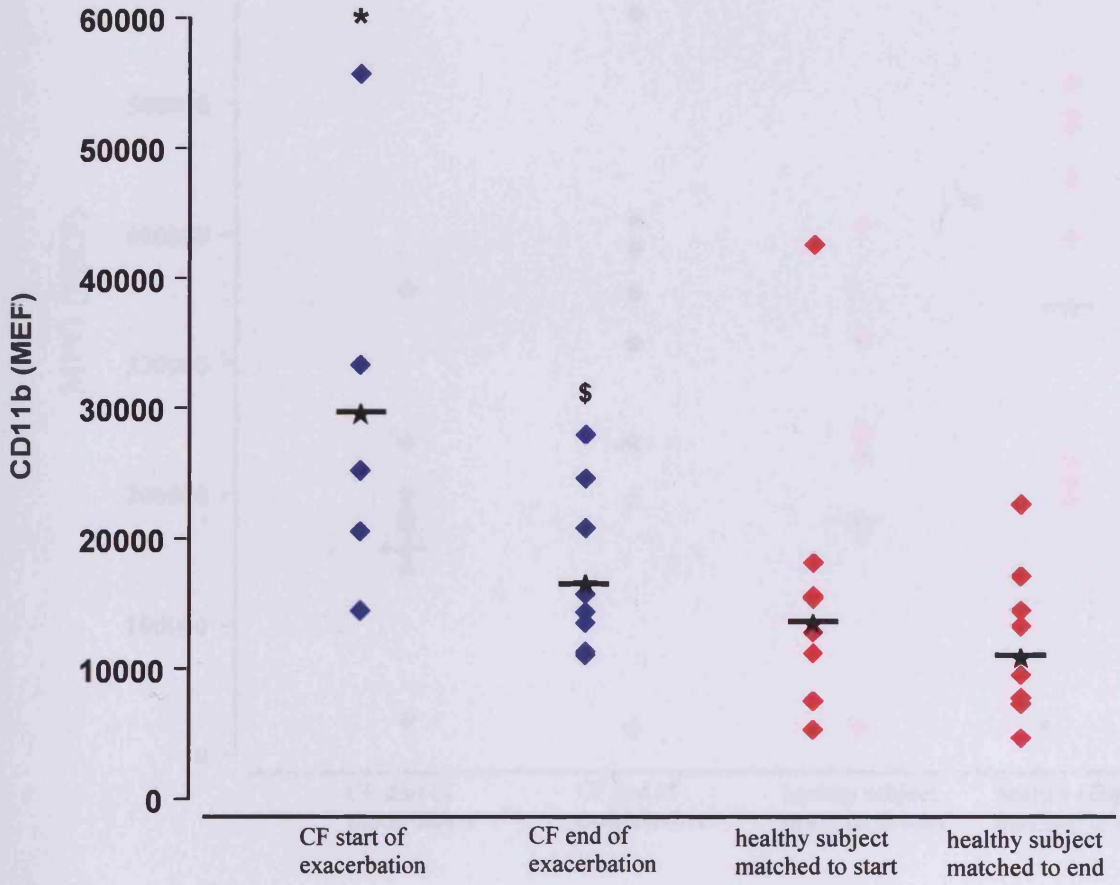
	CF Start of Treatment	CF End of treatment	Healthy Subject matched to start	Healthy subject matched to end
L-selectin (MEF)	32352 (20687 – 50582)	36241 (22177 – 59224)	37042 (22070 – 62173)	42277 (27068 – 66176)
CD11b (MEF)	29458* (17730 – 48955)	16459$ (12291 – 22034)	13406 (7991 – 22491)	10809 (7031 – 16866)
IL-8 RA (MEF)	28151 (24541 – 32285)	31362 (2682 – 35530)	30102 (26736 – 33892)	33783 (28074 – 40663)
IL-8 RB (MEF)	2478 (1288 – 4769)	2481 (1483 – 4150)	3512 (2598 – 4747)	2981 (2005 – 4430)
TNFα R (MEF)	20999 (6455 – 68328)	24338 (15184 – 39021)	15122 (7822 – 29235)	16192 (4626 – 56663)
IL-6 R (MEF)	25639 (12306 – 53420)	38788 (27033 – 55667)	35156 (20578 – 60062)	41324 (27214 – 62762)
MPO (MEF)	156856 (82832 – 297030)	236265 $ (99495 – 561048)	179019 (82376 – 389045)	342609 (240658 – 487753)
Serum CRP (μg/ml)	18.19* (3.73 – 88.84)	5.01$ (1.09 – 2.32)	1.33 (0.42 – 4.22)	1.72 (0.41 – 7.18)

Geometric Mean (95% Confidence Interval)

$ $p<0.05$ effect of treatment ; * $p<0.05$ patients compared to healthy subjects

Figure 8.1

CD11b expression on neutrophils from patients with CF and healthy subjects



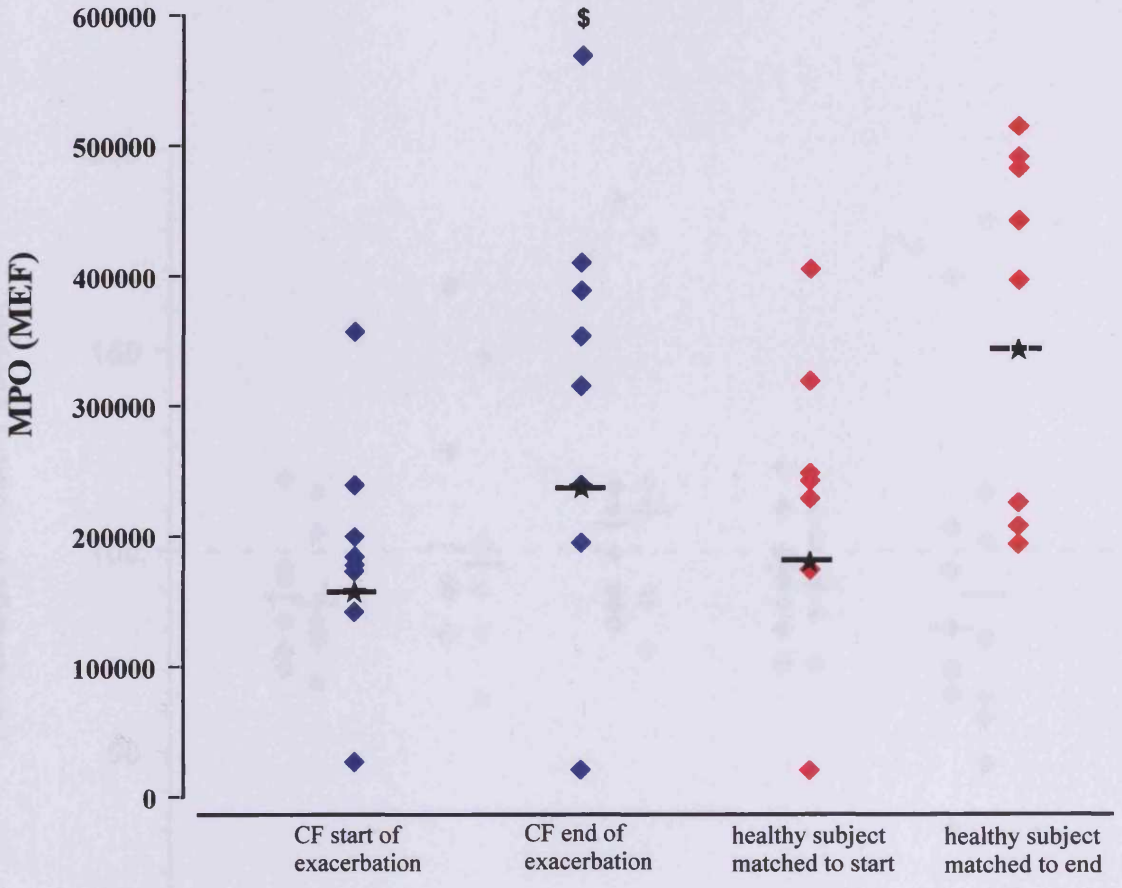
$ p=0.04 Effect of treatment in patients with CF (Paired t test on log transformed data)

* p=0.008 Patients compared to healthy subjects (Mann Whitney U test)

—★— Geometric mean

Figure 8.2

The intracellular MPO content of neutrophils from patients with CF and healthy subjects

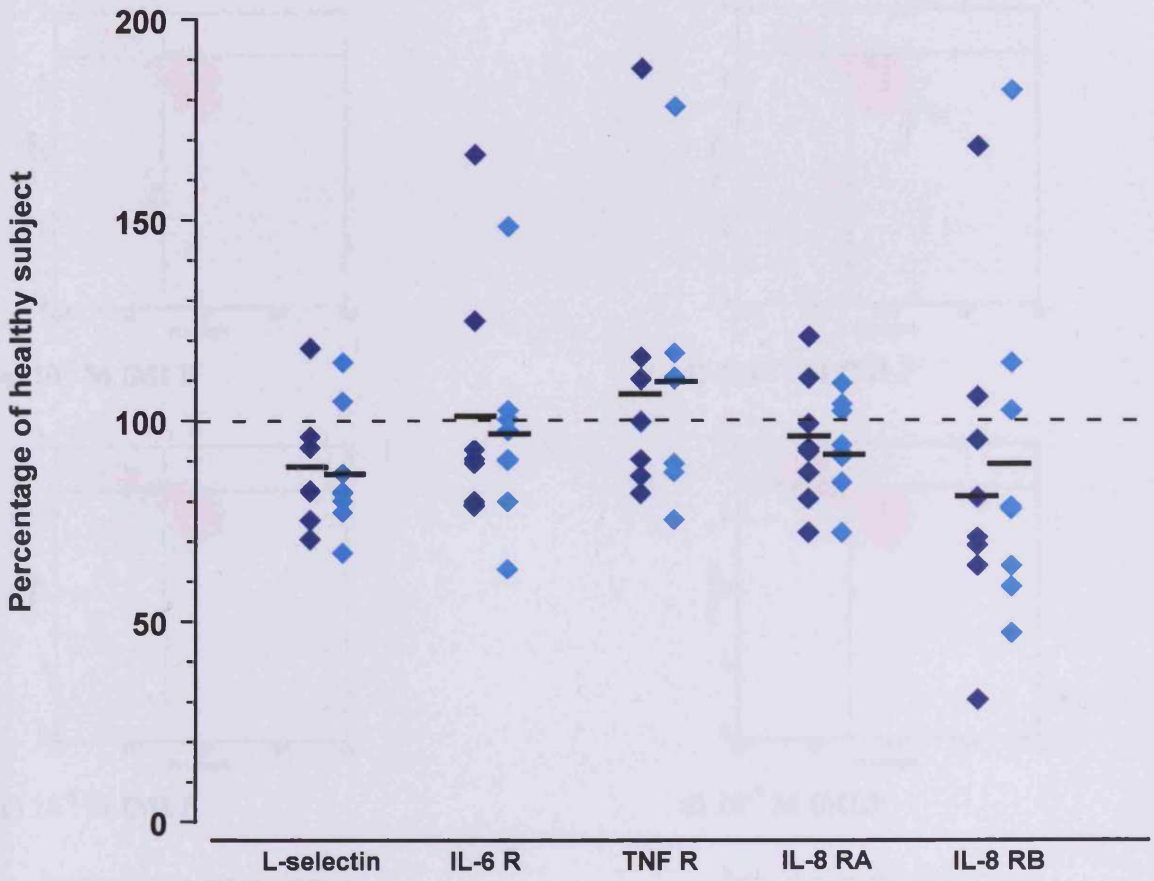


$ p=0.024 Significant effect of treatment in patients with CF (Paired t test on log transformed data)

—✱— Geometric mean

Figure 8.3

Neutrophil receptor expression of patients with CF expressed as a percentage of the matched healthy subject expression



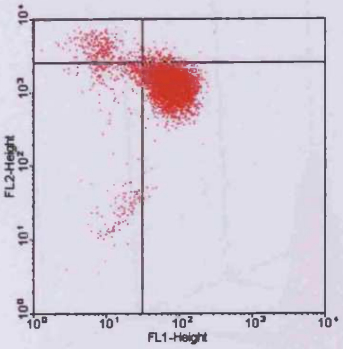
◆ Patients at start of antibiotic treatment, ◆ patients after antibiotic treatment

▬ Arithmetic mean of group

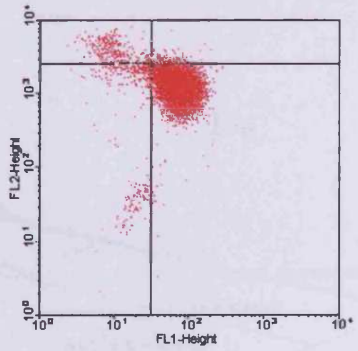
Figure 8.4

The fluorescence from CD11b and L-selectin at different doses of fMLP is shown with dot plots of cells from one patient as an example

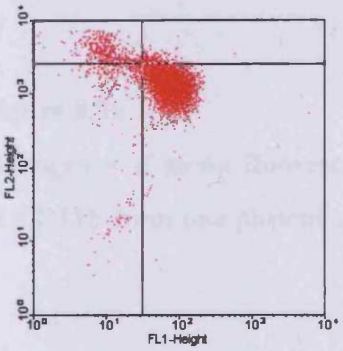
a) PBS



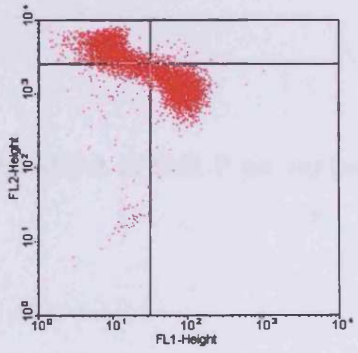
b) 10^{-10} M fMLP



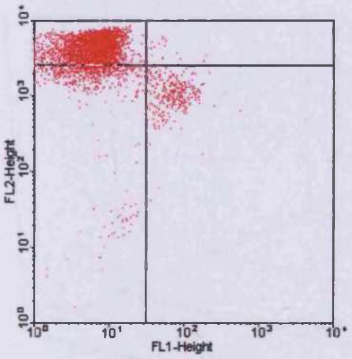
c) 10^{-9} M fMLP



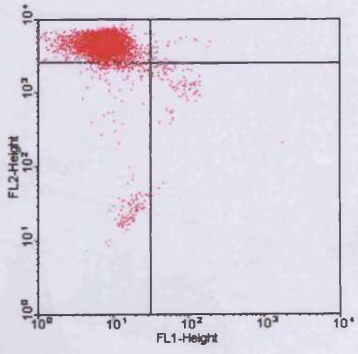
d) $3x10^{-9}$ M fMLP



c) 10^{-8} M fMLP



d) 10^{-7} M fMLP



FL-1: L-selectin FITC FL-2: CD11b PE

Gated for neutrophils from side scatter / forward scatter dot plot.

Figure 8.5a

Histogram of mean fluorescence (MEF) of showing the effect of fMLP on surface expression of L-selectin from one patient as an example.

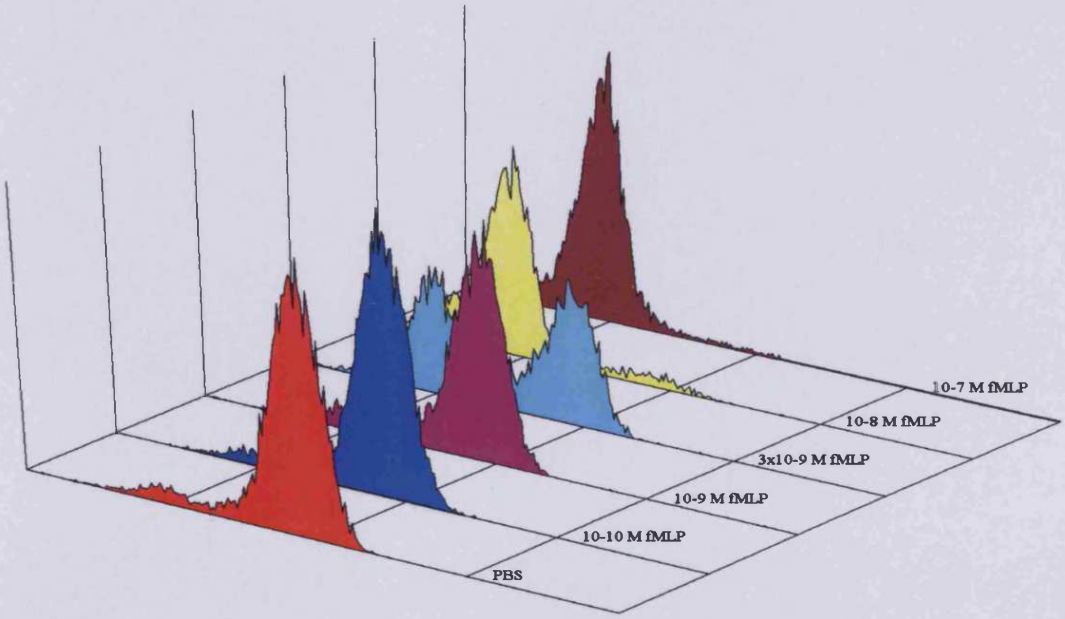


Figure 8.5a

Histogram of mean fluorescence (MEF) of showing the effect of fMLP on surface expression of CD11b from one patient as an example.

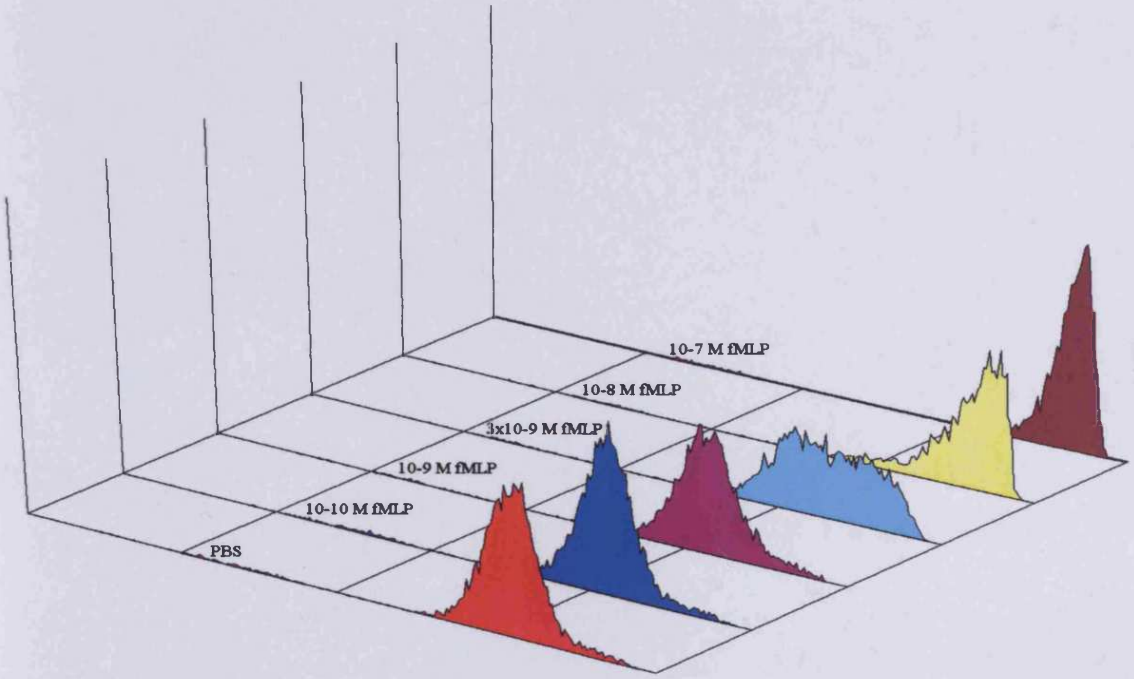
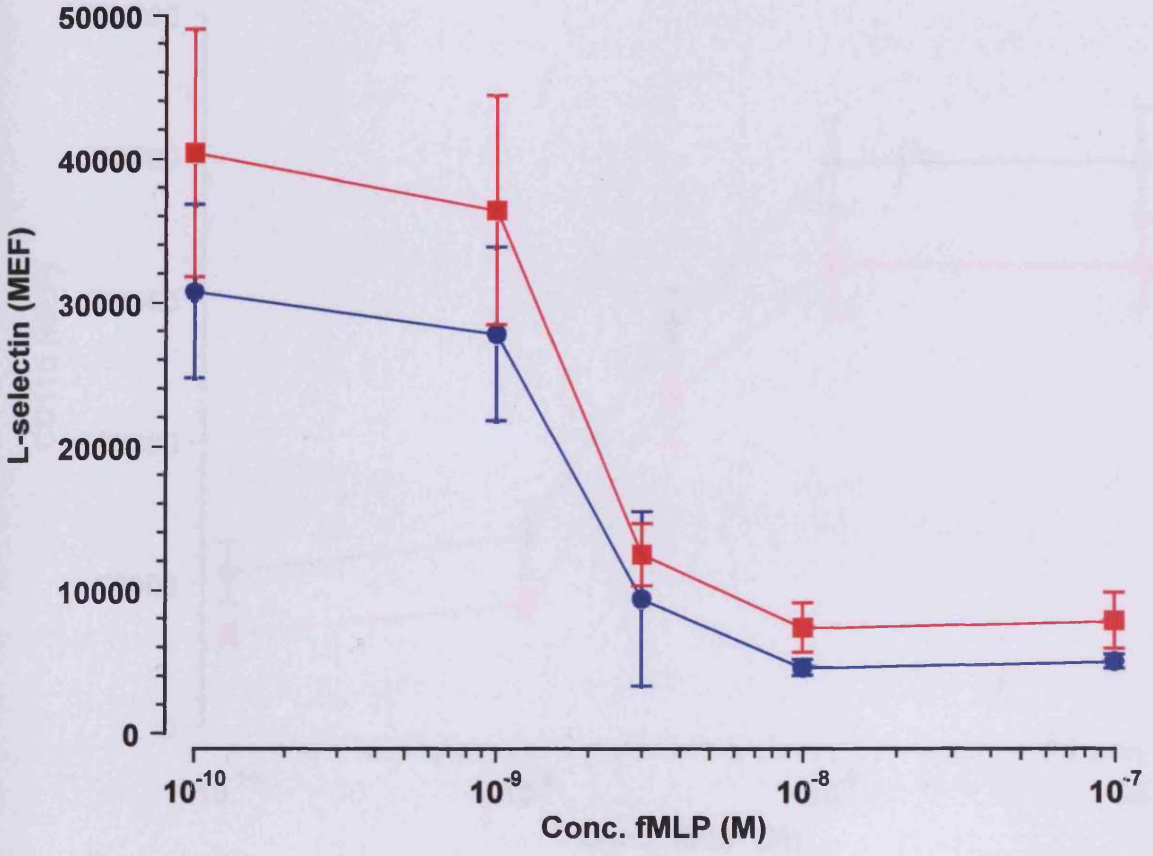


Figure 8.6

The effect of fMLP on L-selectin expression on neutrophils from patients with CF in exacerbation and healthy subjects



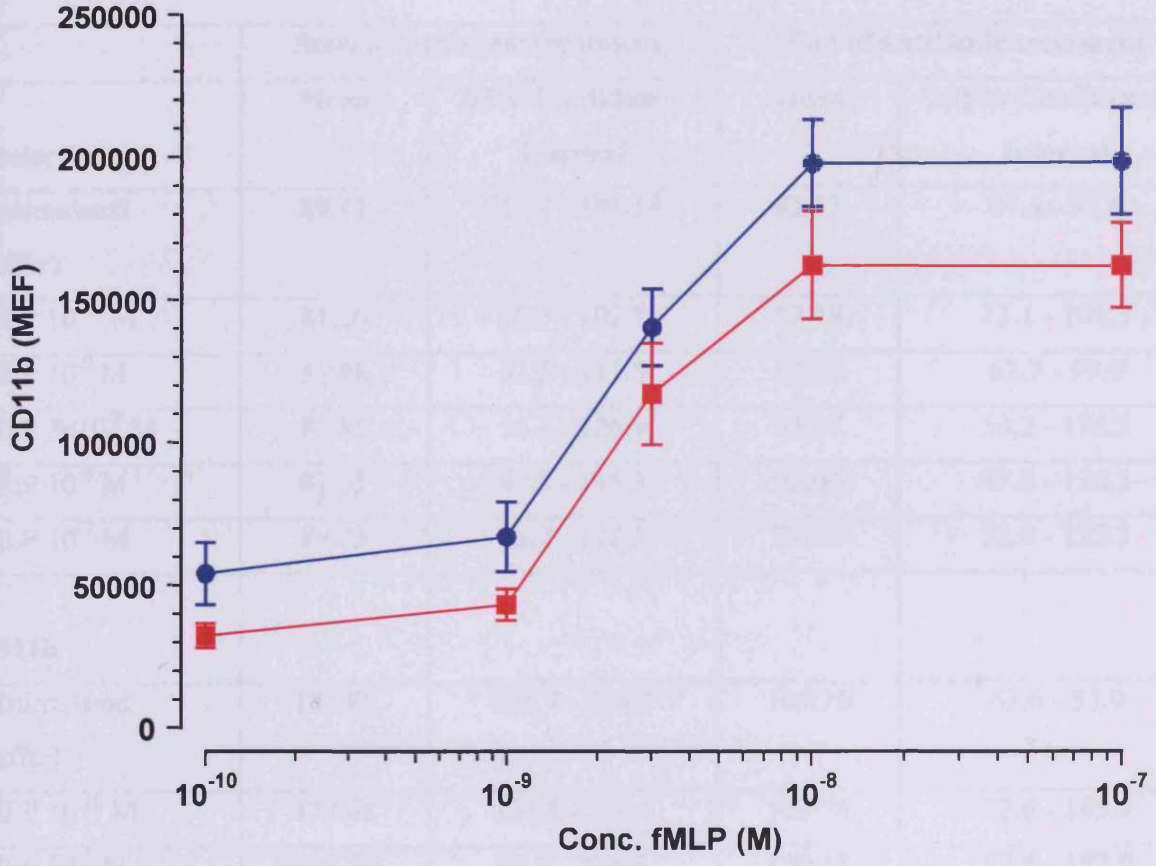
Arithmetic Mean ±S.E.M.

● CF in exacerbation (n=8), ■ Healthy subject (n=8),

Significance by MANOVA on log transformed data; p=0.076

Figure 8.7

The effect of fMLP on CD11b expression on neutrophils from patients with CF in exacerbation and healthy subjects



● CF in exacerbation (n=8), ■ Healthy subject (n=8),

Arithmetic mean ± S.E.M.

Significance by MANOVA on log transformed data; p=0.039

Table 8.2

Surface expression of L-selectin and CD11b in response to fMLP as a percentage of the matched healthy subject

	Start of antibiotic treatment		End of antibiotic treatment	
L-selectin	**Mean**	**95% Confidence Interval**	**Mean**	**95% Confidence Interval**
Unstimulated (buffer)	89.43	71.72 - 107.14	82.53	67.4 - 97.6
fMLP 10^{-10} M	81.26	60.3 - 102.3	87.18	73.1 - 101.3
fMLP 10^{-9} M	81.98	52.5 - 111.5	81.29	62.7 - 99.9
fMLP $3x10^{-9}$ M	81.85	56.8 - 106.9	93.37	50.2 - 136.5
fMLP 10^{-8} M	81.65	47.9 - 115.3	92.04	47.8 - 136.3
fMLP 10^{-7} M	89.23	56.2 - 122.3	77.23	32.0 - 122.5
CD11b				
Unstimulated (buffer)	184.85	105.4 - 264.2	109.76	63.6 - 55.9
fMLP 10^{-10} M	177.48	104.8 - 250.1	109.76	73.6 - 145.9
fMLP 10^{-9} M	150.74	74.7 - 226.8	120.18	57.4 - 182.9
fMLP $3x10^{-9}$ M	141.67	74.2 - 209.1	125.4	58.5 - 192.4
fMLP 10^{-8} M	134.38	84.5 - 129.9	142.87	64.9 - 220.8
fMLP10^{-7} M	126.51	95.9 - 157.1	113.27	71.9 - 154.7

Discussion

There was increased surface expression of CD11b in patients with CF at the time of an exacerbation of respiratory symptoms compared with both the post treatment state and healthy subjects. The up-regulation of surface CD11b, and reduction in L-selectin in response to fMLP was similar in both patients and healthy subjects showing that the neutrophils from patients with CF are behaving normally with regard to this aspect of function, though offset by the up-regulated basal state. The surface expression of the cytokine receptors was similar between patients with CF and healthy subjects and did not change with the clinical status of the patient. Intracellular MPO content increased significantly in patients' neutrophils following treatement with antibiotics, but was not different to healthy subjects at either time point.

We observed lower levels of sL-selectin in the circulation (Chapter 3) which is similar to the observation in other diseases such as ARDS [Donnelly *et al* 1994], trauma [Seekamp *et al* 2001] and SIRS [Ahmed 1996]. In contrast elevated plasma L-selectin was seen in sepsis, accompanied by a marked increase in shedding, and reduced delivery of neutrophils to the inflammatory site [Ahmed *et al* 1999]. This suggests that L-selectin may play an important role in the regulation of neutrophil migration and correct resolution of inflammation. Increased numbers of neutrophils enter the lungs during an infection, which would imply a high level of migration. If the circulating sL-selectin was a result of neutrophil shedding one theory would be that the neutrophils released from the bone marrow into the circulation at this time have less L-selectin to express and shed. It is unclear whether such small differences could be due to this. Other possibilities are that L-selectin involved in adhesion remains bound to the endothelial cells, or that migration into the lungs is not integrin dependent at this time, or that the major part of sL-selectin is lymphocyte, rather than neutrophil derived.

The increased expression of CD11b observed in unstimulated neutrophils from patients compared with healthy subjects is similar to that seen with low doses of stimuli such as fMLP, LPS and TNFα and other inflammatory mediators are likely to be present in the circulation of patients, which might prime or activate neutrophils to produce a similar effect. However, from our results reported in chapters 5 and 6, neutrophils from patients with CF do not appear to be primed or activated,

particularly in an exacerbation state where they produce less superoxide and elastase following fMLP stimulation *in vitro*.

Despite the increased CD11b and decreased L-selectin expression in the basal state, when stimulated with fMLP the patients' cells behaved as expected. The magnitude of the response to fMLP remained the same, though off-set by the basal increment indicating that the neutrophils were as responsive as those of healthy subjects in this aspect. Russell *et al* [1998] did not find a difference in basal levels of L-selectin or CD11b expression between patients and healthy subjects. However, they reported a reduced percentage change in the surface expression of L-selectin in response to fMLP in their patients. Analysing our data in this way did not show a difference between patients and healthy subjects or between subjects. Although there is a difference between this study and that of Russell *et al* [1998] it is possible that it is only reflecting a slight difference in technique, or patient population. The reduced responsiveness seen by Russell *et al* could be inteprted as in agreement with our observation of reduced responsiveness to fMLP stimulation of superoxde generation and elastase release (chapters 5&6).

Our findings could be intepreted as demonstrating normal function in respect of CD11b and L-selectin expression by neutrophils from patients with CF, both in the unstimulated and stimulated states. Indeed, the increased expression of CD11b and decreased expression of L-selectin at the time of increased respiratory symptoms is also appropriate to the understood physiology of this state. Since fMLP stimulation causes a reduced superoxide generation in neutrophils from patients in exacerbation, but produces the same changes in surface expression for L-selectin and CD11b as healthy subjects, this suggests that the difference may not be due to events occuring after the signal has been generated by the G-protein linked fMLP receptor and provides evidence for a difference in control of expression of surface receptors and responsiveness. Thus the underlying concept of a difference between neutrophils from patients with CF, particularly at a time of an exacerbation of respiratory symptoms, and healthy subjects remains. The basal expression of L-selectin was not significantly different when compared to controls, despite the mean value being lower. Maekawa [1998] reported substantial inter-individual variation in levels of L-selectin and CD11b cell surface expression. When values were calculated as a percentage of the paired healthy subject, the L-selectin expression was 88 % (SD) at start of antibiotic treatment. All but one of these percentage

values was below 100, suggesting that inter-assay variation may also have led to the lack of significance of this result.

No difference was found for the expression of IL-6 or TNF receptors on neutrophils between any of the groups studied. There is a lack of other studies reporting changes in these receptor numbers in disease states, which may reflect the lack of changes that occur. It has been shown that circulating IL-6, TNFα and their soluble receptors are raised in patients compared to healthy subjects in CF [Ionescu *et al* 2002] and COPD [Eid *et al* 2001]. Reduction in membrane TNFα receptor expression was associated with increased secretion of soluble TNF receptors by neutrophils and monocytes [Jablonska *et al* 1999]. TNF RI and RII are present on most cell types, and although neutrophils are known to shed their TNFα receptors [Dri *et al* 1999] it is not clear what proportion of the circulating soluble form of receptors is of neutrophil origin. The absence of differences in receptor density in this study suggests that changes in circulating soluble receptors are not primarily due to release from the cell surface of neutrophils or the receptors that are cleaved through activation may be re-expressed with no net loss of expression. It has been shown that the TNF receptors are shed (primarily the p75 R) or internalized (primarily p55 R) upon activation of neutrophils, but it is not clear whether they may later be re-expressed [Porteu & Hieblot 1994]. Equally, it is clear that increased circulating leveles of these immunoreactive cytokines and their soluble receptors do not lead to down-regulation of cell surface expression of their receptors by neutrophils. Both IL-6, TNFα and their soluble receptors have been implicated as modulators of CXC chemokines, which contribute to the regulation of neutrophil recruitment to the lungs. Absence of TNFRI but not TNFRII conferred a 53% reduction in lung eutrophil accumulation following systemic LPS administration in mice [Calkins *et al* 2001]. Both protein kinase C-δ (PKC-δ) and PI 3-kinase are associated with the TNFRI in response to TNFα, an association that requires engagement of β_2-integrins [Kilpatrick *et al* 2002]. However, it has been suggested that soluble TNF receptors down regulate the reactions medicated by TNFα by competing with the cellular TNF R for a ligand [Jablonska *et al* 1998]. Similarly, contrasting studies have reported IL-6SR to enhance the action of IL-6 [Jablonska *et al* 1998] and to suppress the recruitment of neutrophils to the lungs [Hurst *et al* 2001]. The relationship between surface expression of L-selectin and IL-6 R and TNF RI further support the involvement of these receptors in the migration process.

In this study we saw no change in the expression of either IL-8 RA or RB, although the IL-8 RB density was numerically less. IL-8 RB has been reported to be reduced in sepsis [Cummings *et al* 1999], HIV and in pneumonia following trauma [Adams *et al* 2001]. Soejima *et al* [1997] studied patients with chronic lower respiratory tract infection, and observed no difference in the IL-8 receptor expression on circulating neutrophils compared to healthy subjects, although there were significantly lower levels of IL-8RA on the BALF neutrophils from the patient group. Normal neutrophil CXC receptors are transiently internalized following in vitro stimulation by IL-8. Subsequently CXCR1 (IL-8RBA) is rapidly re-expressed on the cell surface, whereas CXCR2 (IL-8RB) is re-expressed at a considerably slower rate. Within 24 hours after severe injury there is a 50% loss of CXCR2 on the cell surface and complete desensitisation of the remaining receptors [Quaid *et al* 1999]. TNFα, hypoxia, GCSF and LPS can all down regulate CXCR2 [Cummings *et al* 1999]. Neutrophils from a mixed group of patients with lung disease, some of which had CF, at a time of acute exacerbation of respiratory symptoms were found to have less CXCR2 than healthy subjects, and when neutrophils from healthy subjects were stimulated with fMLP there was a selective down-regulation of the CXCR2 [Mackarel 2001]. Incubation of adhered neutrophils led to an increase in expression of CXCR1 and CXCR2 by 5 and 10 fold respectively, and this increase correlated well with the intracellular Ca^{2+} transient in response to IL-8 [Patel 2001] suggesting a link between receptor expression and chemokine responsiveness. Reduced migration induced by IL-8 of neutrophils from patients with CF was asociated with reduced binding of IL-8 to the cell surface [Dai *et al* 1994]. The small reduction we observed in IL-8 RB may be physiologically real and could be due to prior exposure to IL-8 as suggested by Dai *et al* [1994] and slower re-expression [Quaid *et al* 1999]. This would also support the reduction in sepsis [Cummings *et al* 1999], which is probably of a more severe nature, and therefore a greater and more statistically significant effect.

An unexpected finding was that there is less MPO present in the granules in neutrophils from patients when experiencing an exacerbation of respiratory symptoms. This may reflect some prior release or a less granular form of neutrophil at this time. The presence of granule proteins including elastase and MPO at raised levels in the circulation in patients with cystic fibrosis particularly at a time of an exacerbation suggests that some release occurs in the circulation (Chapter 3) [Koller

1996]. We also know from chapter 3 that there is a very large concentration of elastase in the lungs from measurements in sputum sol which could potentially move back into the circulation and could be the cause of the raised circulating level of elastase. The circulating concentrations of elastase are particularly high when the lungs most damaged prior to death [Hendry *et al* 1999]. The resultant products of lung degradation such as desmosine have been shown to be raised in the urine [Bruce *et al* 1985]. Similarly MPO has been shown to be raised in the sputum of patients with CF [Koller *et al* 1995]. MPO is present in the primary granules, and is produced at an early stage of neutrophil maturation and is divided with subsequent cell divisions. It is possible that an accelerated transit of neutrophils through the mitotic pool of the marrow could cause neutrophils to skip divisions resulting in the release of cells into the circulation with a higher MPO content [van Eeden *et al* 2000]. However, this would not fit with our data of reduced MPO at this time.

Experimentally the use of the calibration beads was not as effective at reducing day to day variation as was expected. During the period when samples were being collected, the FACS was serviced twice, and the flow cell replaced on one of these occasions. The compensation, voltage and threshold settings had to be altered on both these occasions, and it was not possible to get exactly the same patterns as previously. We have tried to compensate for this variation using patient values as a percentage of the matched healthy subject. This made the reduced L-selectin and increased CD11b on the neutrophils from patients with CF compared to controls much clearer.

Neutrophils from patients with CF have increased basal expression of CD11b which is suggestive of a prior exposure to an inflammatory stimulus. The small reduction in expression of L-section and IL-8RB, and the reduced MPO content is consistent with this. IL-8RA, IL-6R and TNFR were not altered and were remarkably similar to the values seen in healthy subjects. Expression of CD11b was up-regulated and L-selectin down-regulated by the same magnitude in both patients with CF and healthy subjects and was not affected by clinical state. The basal receptor changes shown in this study are most likely to be caused by low levels of inflammatory mediators. The fMLP stimulated response to fMLP indicates that these receptors are functioning normally with regard to alteration of surface adhesion receptors, and that this is not related to the mechanism of reduced superoxide generation and elastase release seen in chapters 5, 6 and 7.

Chapter 9

Neutrophil adhesion and the inflammatory response

Aim

To determine whether neutrophils from patients with CF were more adhesive than those from healthy subjects.

To determine where the inflammatory response affected adhesion of neutrophils

Introduction

The leukocyte delivery to an infected site is a precise and regulated process involving a series of adhesive interactions with the endothelium. Under physiological conditions leukocyte rolling is a prerequisite of firm adhesion, and firm adhesion a prerequisite of transmigration. Adhesion is an important phase in this migration. The initial contact of circulating neutrophils with the endothelial cells is transient, resulting in a rolling-and-release tumbleweed-like motion. Rolling is the first step in neutrophil recruitment and is mediated by selectins of which L-selectin is present on neutrophils. This binds to counterligands, the tetrasaccharide sial Lewisx (Sle), as well as other fucosylated and sulphated structures [Burg & Pillinger 2001]. The contribution of L-selectin mediated rolling prior to firm adherence is dependent on vessel size and the effect of flow [Eriksson *et al* 2001]. Following the initial attachment and rolling there is tight adhesion. After firm adhesion via β_2-integrins neutrophils must transmigrate either between or directly through endothelial cells to arrive at extravascular sites of infection [Burg & Pillinger 2001].

Adherence of neutrophils has important implications in lung disease. Neutrophils must deform to pass through the pulmonary capillary bed as they travel from the arterial to venous side of the pulmonary circulation. The biophysical properties (stiffness and deformability) of neutrophils are major determinants of the magnitude of neutrophil sequestration to an inflammatory site. Passive deformation induces an increase in the expression of CD11b/CD18 and an increase in neutrophil

adhesiveness [Anderson *et al* 2001]. However, an increase in CD11b/CD18 dependent adhesion does not necessarily translate into an increase in surface expression of the molecule [Vedder *et al* 1988].

From chapter 3 we know that low plasma sL-selectin concentration increased significantly following treatment, although sL-selectin levels were not significantly different from non-CF subject values at either time point. In patients with CF large numbers of neutrophils enter the lungs and are considered to be a major factor in the decline in lung function due to their release of toxic agents. Russell *et al* [1998] reported reduced shedding of L-selectin by neutrophils in response to fMLP and IL-8 in patients with CF, which suggests altered regulation of the inflammatory response in CF, although we did not see such alterations (Chapter 8).

Elevated levels of circulating soluble ICAM-1 has been shown to be predictive of multiple organ failure after severe trauma and sepsis [Whalen *et al* 2000]. Additionally, sICAM-1 is found to be elevated in acute and chronic inflammatory conditions as diverse as hepatitis, liver disease [Abdalla *et al* 2002] and heart disease [Haught *et al* 1996]. In rheumatoid arthritis elevated levels of sE-selectin and ICAM-1 have been observed, and were considered more relevant as markers of disease than sL-selectin [Bloom *et al* 1999].

Neutrophils are able to adhere through CD18 independent pathways as well as CD18 dependent pathways, and this is dependent on the nature of the stimulus [Burns *et al* 2001]. Despite this patients with Leukocyte Adhesion Deficiency (LAD) show a lack of extra vascular neutrophils [Horwitz *et al* 2001]. The lungs are particularly suitable for integrin independent adhesion due to the unique structure of the pulmonary capillary bed and the cell types present in the lungs. Neutrophil emigration in response to *E.coli*, LPS, *P.aeruginosa*, IgG complexes, IL-1 and PMA are thought to occur through adhesion pathways, whereas *Streptococcus pneumoniae*, Group B Streptococcus, *S.aureus*, hypoxia, C5a and HCl lead to emigration through pathways which cannot be inhibited through blockade of the CD18 adhesion pathway [Doerschuk *et al* 2000]. In a mouse model of gram-negative sepsis with *E.coli*, the time course of the neutrophil sequestration in the lungs paralleled the increase in lung micro vascular permeability, indicating a relationship between lung tissue neutrophil accumulation and increased vasopermeability [Gao *et al* 2001], but in CD18

blockade there is impaired clearance of *E.coli* but not S.aureus [Ramamoorthy *et al* 1997]. *In vitro* Mackarel *et al* [2000] have shown that neutrophil migration to fMLP is integrin dependent, whereas that to IL-8 and LTB4 is integrin-independent. It remains unclear what determines the selection of an adhesion pathway, and the molecules involved, although VLA-4, Galectin-3 and CD49 have been implicated in integrin independent adhesion [Birner *et al* 1999, Redger *et al* 2001, Sato *et al* 2002].

It has been shown in previous experiments that adherence is related to the expression of β_2 integrins and other CAM's on the neutrophil surface. Thom *et al* [1997] found that binding of neutrophils to nylon packed columns was directly related to CAM expression, therefore providing an accurate way to assess adherence *in vitro*. CAM expression and therefore neutrophil binding affinity is usually low, but may be up-regulated by a number of cellular signalling molecules thought to be present at higher concentrations in the blood of patients suffering from acute pulmonary inflammation. We had determined that there was greater CD11b present on neutrophils from patients with CF (chapter 8), and it therefore seemed relevant to determine whether there was also a functional effect. Adhesion in nylon columns has been reported to be integrin dependent [Thom *et al* 1997]. We therefore decided to determine adherence using nylon columns in patients with CF and healthy subjects. Adhesion was related to the clinical state of the patient. In addition plasma sL-selectin and IL-8 was measured in the same groups of subjects.

Method

Patient study

Venous blood was collected from 29 patients with CF (mean age 24) into Li Heparin, and 15 age matched healthy subjects were also studied. Serum was obtained for measurement of C Reactive Protein (CRP) by ELISA, and EDTA plasma for measurement of IL-8 and sL-selectin. Li Heparin anticoagulated blood from each subject was passed down nylon wool columns and the adherence measured. White cell count and differential measured before and after to determine adherence.

The 29 patients were split into 2 groups based on clinical symptoms. A first group who were in respiratory exacerbation (within 3 days of admittance to the hospital for *i.v.* antibiotic treatment) (n=18), and a second group of clinically stable patients on a routine clinic visit, or who had just completed a course of *i.v.* antibiotics (n=11).

Statistical analysis was by Mann Whitney U test on data. Normally distributed data is expressed as arithmetic mean (SD), non-normally distributed data as geometric mean (95%CI).

Results

Patient study

Patients recruited at the start of antibiotic treatment for a pulmonary exacerbation had a mean FEV1 of 46.7 (18.3), and those considered clinically stable an FEV1 of 56.9 (22.6) indicating a similar severity of lung disease. Circulating levels of CRP were greater in the patients at the start of treatment compared to clinically stable patients ($p=0.003$) and healthy subjects ($p<0.001$). The circulating sL-selectin followed a similar pattern to that observed previously in experiments described in Chapter 3 with patients at the start of antibiotic treatment having lower levels than healthy subjects ($p=0.015$). These data are all shown in Table 9.1. IL-8 and sL-selectin were related in patients at the start of treatment ($r=0.615$, $p=0.009$), and in the clinically stable group of patients ($r=0.673$, $p=0.023$).

Adhesion of neutrophils from patients with CF was much greater than in healthy subjects for both the % adherence (Figure 9.1) and the absolute number (Figure 9.2) of neutrophils adhered. The percentage neutrophils of the total cell population remained unchanged. Both the change in WCC and the change in neutrophil number were inversely related to the sL-selectin in clinically stable patents ($r=-0.720$, $p=0.008$; $r=-0.806$, $p=0.002$). However, sL-selectin levels were positively correlated to the change in lymphocyte number for patients at the start of treatment when the lymphocyte number was raised ($r=0.615$, $p=0.009$).

Subjects were split into two groups on the basis of circulating CRP, and the results shown in Table 9.2.

Table 9.1

sL-selectin levels, neutrophil adherence and CRP levels calculated in 2 patients groups and healthy subjects.

	CF Start of Treatment n=18	CF Clinically Stable n=11	Healthy subject n=15
Age (years)	25.6 ± 6.6	23.5 ± 5.4	26.6 ± 5.8
Plasma sL-selectin	1.14 * (0.648-1.641)	2.80 (0.771-4.831)	2.86 (1.565-4.160)
CRP (μg/ml)	30.37** (15.9-57.8)	1.50 ## (0.46-4.96)	1.22 (0.54 – 2.74)
IL-8 (ng/ml)	58.7 (37 -93)	126.6 (50 - 321)	139 (56 - 347)
White Cell count (10^6/ml)	11.31 ± 5.2	10.15 ± 4.9	7.9 ± 2.6
Neutrophil count (10^6/ml)	8.5 ± 5.0 **	7.4 ± 4.9	4.5 ± 1.6
Percentage neutrophil in WCC (%)	72.0 ± 13.3 **	68.5 ± 12.6 *	56.8 ± 6.2
White cells adhered (10^6/ml)	2.16 ± 1.8*	2.13 ± 1.3 *	1.23 ± 0.9
Neutrophils adhered (10^6/ml)	1.83 ± 1.7 **	1.61 ± 1.2 **	0.68 ± 0.5
Percentage of neutrophils adhered	23.66 ± 24 *	23.56 ± 12.7	15.62 ± 7.9

Mann Whitney U test:

Patients compared to healthy subjects: * ($p<0.05$); ** ($p<0.01$)

Difference between patient groups: # ($p<0.05$); ## ($p<0.01$)

Expressed as Arithmetic Mean as Mean ± SD or Geometric mean as mean (95% CI)

Table 9.2

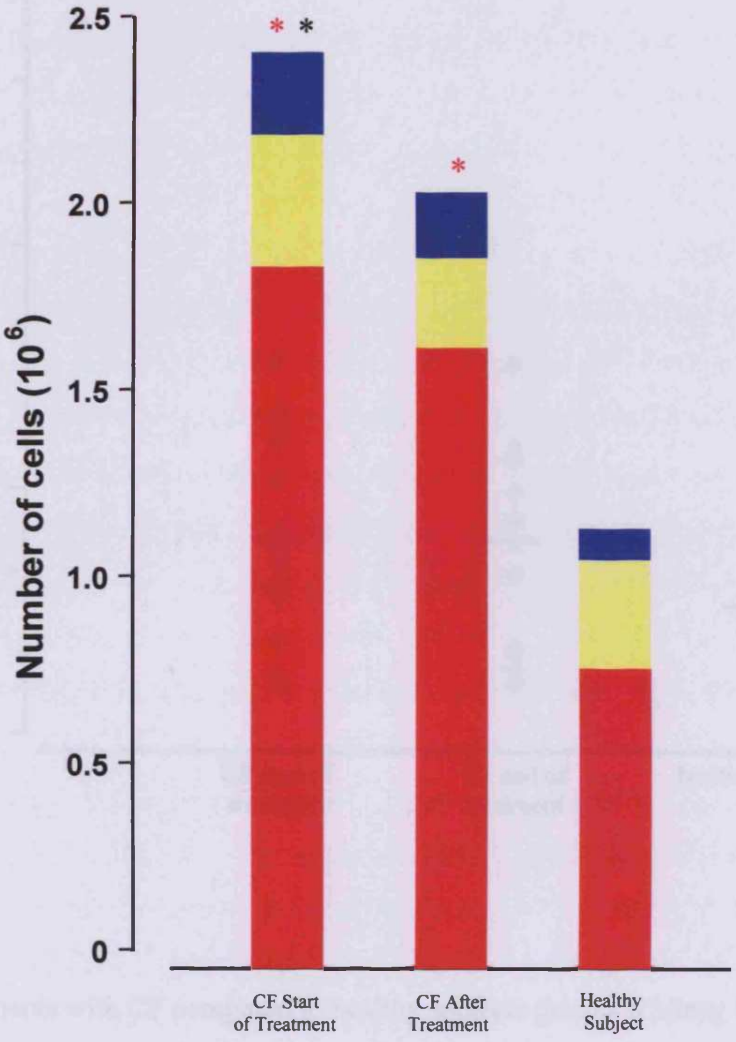
Relationship between CRP and adherence

	CRP > 10μg/ml n=18	CRP < 10 μg/ml n=11	Significance
sL-selectin	1.11 ± 0.96	2.71 ± 2.70	p=0.037
White cells adhered (10^6/ml)	2.52 ± 1.65	1.73 ± 0.90	ns
Neutrophils adhered (10^6/ml)	2.25 ± 1.57	1.05 ± 0.60	p=0.023
Lymphocytes adhered (10^6/ml)	0.271 ± 0.26	0.341 ± 0.38	ns
Percentage of neutrophils adhered	30.15 ± 20.1	20.73 ± 9.35	ns

Significance by Mann Whitney U test

Figure 9.1

The number of cells adhered when whole blood from patients with CF and healthy subjects was passed down a nylon column.



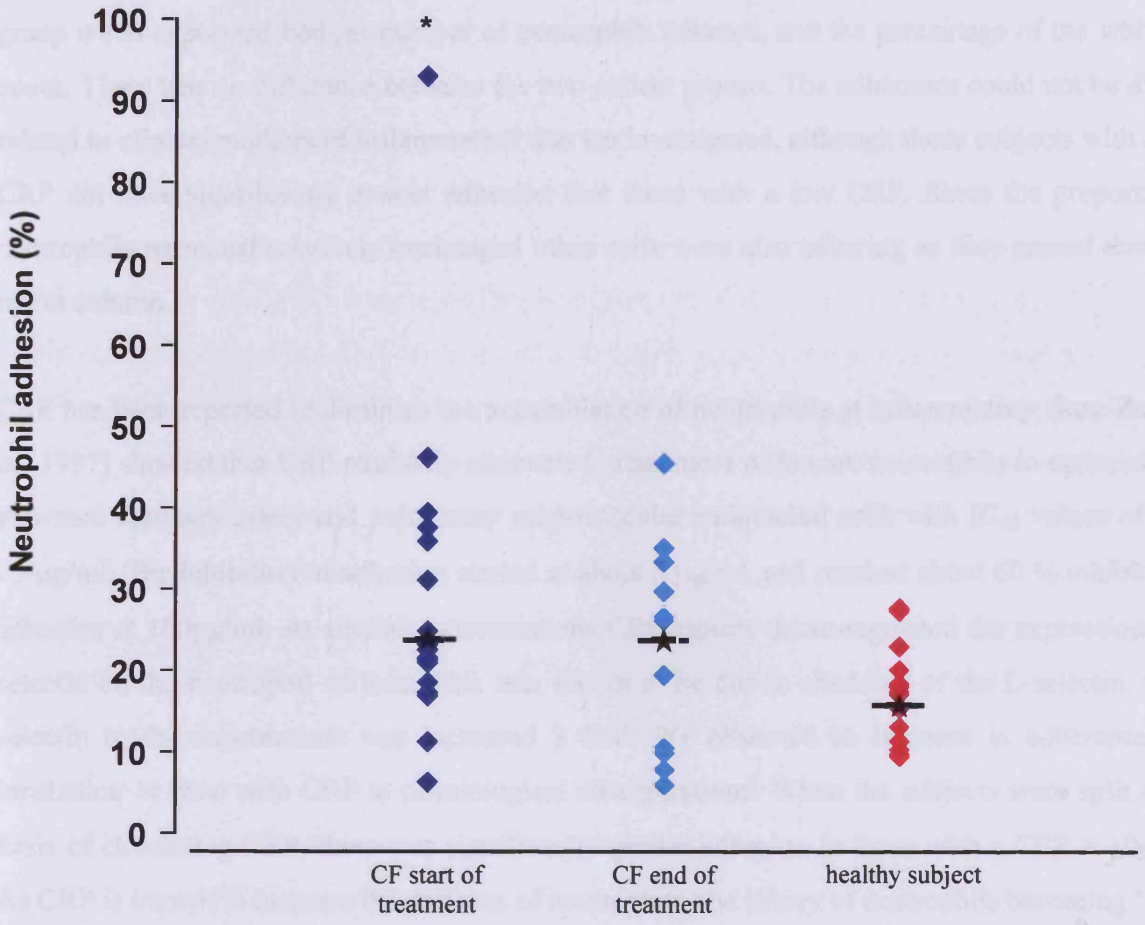
* p<0.05 for total white cells

* p<0.05 for neutrophils

■ Neutrophils; ■ Lymphocytes; ■ Monocytes

Figure 9.2

The percentage of neutrophils adhered in blood from patients with CF and healthy subjects



* $p<0.05$ Patients with CF compared to healthy subjects (Mann Whitney U test).

—✱— Geometric Mean

Discussion

There was greater adherence of neutrophils observed in the patients than in the healthy subject group when expressed both as number of neutrophils adhered, and the percentage of the white cell count. There was no difference between the two patient groups. The adherence could not be directly related to clinical markers of inflammation that we investigated, although those subjects with a high CRP did have significantly greater adhesion that those with a low CRP. Since the proportion of neutrophils remained relatively unchanged other cells were also adhering as they passed down the nylon column.

CRP has been reported to diminish the accumulation of neutrophils at inflammatory sites. Zouki *et al* [1997] showed that CRP markedly attenuated attachment of human neutrophils to cultured LPS-activated coronary artery and pulmonary microvascular endothelial cells with IC_{50} values of 20 to 22 μg/ml. The inhibition of adhesion started at about 5 μg/ml, and reached about 60 % inhibition of adhesion at 100μg/ml. At similar concentrations CRP rapidly down-regulated the expression of L-selectin on the neutrophil surface. This was shown to be due to shedding of the L-selectin, as sL-selectin in the supernatants was increased 3 fold. We observed an increase in adherence after incubation *in vitro* with CRP at physiological concentrations. When the subjects were split on the basis of circulating CRP, there was significantly greater adhesion in those with a CRP > 10μg/ml. As CRP is known to increase the stiffness of neutrophils, the theory of neutrophils becoming 'stuck' in the nylon wool, rather that a process involving L-selectin, is further supported. A pilot study indicated that adhesion could be increased by incubation of neutrophils from healthy subjects with inflammatory mediators such as CRP and IL-8 (data not shown). A well planned study would indicate whether the increase in adhesion we have shown in the neutrophils from patients with CF can be attributed to the presence of inflammatory mediators in the circulation.

The nylon column method used here is a passive system with neutrophils passing through the column with only gravitational force. Anecdotally, there were differences in the time it took the sample to pass through the column, but this was not recorded. With most samples taking about 5 to 10 min. to pass the 15mm, this is clearly much slower than the blood flow *in vivo*. Under flow conditions adherent neutrophils support the subsequent margination of other neutrophils, and this is

inhibited by L-selectin antibodies or the preactivation by fMLP. However under static conditions, no clustering of neutrophils occurs, and anti L-selectin antibodies, or fMLP do not affect adhesion [Kuijper 1997]. In our study the conditions could be considered to be static rather than under flow, and therefore the adhesion we have measured may well not be via L-selectin.

Integrin independent adhesion can occur through a mechanism which does not involve L-selectin [Doerschuk 2000]. Migration in a mouse model of *E.coli* sepsis is thought to occur through an adhesion mechanism related to vascular permeability, and related to the lack of neutrophil deformability particularly when travelling through the narrowed capillaries [Gao *et al* 2001]. Neutrophils are known to show stiffness after exposure to inflammatory agents such as LPS, IL-8 and fMLP [Ezurum *et al* 1992, Drost *et al* 2000]. It is possible that in this study we may simply be reflecting on the neutrophils becoming 'stuck' in the nylon wool as they are less deformable. This may still be important as it has implications for the process occurring *in vivo* in the narrow microvasculature of the lungs.

We measured sL-selectin and showed that the patients at the start of treatment for a pulmonary exacerbation had significantly lower levels than healthy subjects. This reflects both our results in chapter 3 and other published data [Muller *et al* 1998, Haught *et al* 1996, Gando *et al* 1999]. The final ELISA to measure sL-selectin developed did not prove ideal in quantifying sL-selectin levels in plasma and serum. Primarily this was due to lack of detection of the recombinant L-selectin standard in the assay and the use of a 'standard' control plasma sample instead. This may have been due to its recombinant nature, possible denaturation, or alternative protein folding during storage and use. In addition the goat polyclonal antibody was the only one available, and may not have been suitable for detection of recombinant sL-selectin protein. Despite these concerns about the validity of the sL-selectin assay due to the standard not being recognised, the same pattern of concentration was observed as had been described in chapter 3.

Elevated levels of ICAM-1 and sE-selectin have been shown to be raised in patients with CF at the start of antibiotic treatment for a pulmonary exacerbation compared to healthy subjects, and fall with treatment. The sE-selectin was also related to CRP ($p=0.69$, $p=0.0005$) [Bell MD thesis 1995]. This information combined with other literature [Bloom *et al* 1999] suggests that measurement of

sE-selectin may have provided useful information in this study. A number of studies have implicated sL-selectin to be more related to allergic diseases such as asthma [Fiscus *et al* 2001] and SLE [Font *et al* 2000]. Anti-IgE resulted in a marked inhibition of L-selectin density on lymphocytes accompanied by L-selectin shedding [Monteseirin *et al* 2001]. The T lymphocyte migration from the circulation into the lung could be partially blocked through L-selectin [Keramidans *et al* 2001]. The role of lymphocytes in production of sL-selectin also needs to be considered, as sL-selectin shows a positive correlation with virus load in HIV [Kourtis *et al* 2000].

In this study we have shown that adherence of both neutrophils and lymphocytes occurs when blood is passed through nylon wool columns. This adherence is greater in patients with CF than healthy subjects. The finding that inflammatory markers such as CRP and fMLP increase this adhesion, which is known to increase CD11b suggest an involvement of integrins, although we could not prove this. The effect of these markers on reducing deformability of neutrophils should be considered important, and has a role in the integrin independent adhesion thought to occur in the lung microvasculature. The relationship of sL-selectin to disease state and the adhesion of lymphocytes and neutrophils suggests that this selectin is involved in regulation of both cell types, and the proportion varies depending on the level of infection and inflammation.

Chapter 10

The effect of cystic fibrosis and chronic infection on the morphology of neutrophils and possible influence by colony stimulating factors

Aim

To determine whether there are changes in the morphology of neutrophils in patients with CF compared to healthy subjects, and the effect of pulmonary exacerbation.

To determine whether there is a relationship between the morphology, and the reactivity of neutrophil from patients with CF.

Introduction

Morphology

A continuous turnover and resupply of neutrophils to tissues is essential for the host defence system against infection to function effectively. Mature neutrophils arise from bone marrow stem cells following a process involving proliferation, commitment to differentiation along the granulocyte linage, and terminal maturation [Ogawa & Matsunga 1999]. Neutrophils are formed with a single horseshoe shaped nucleus (band form) and the nucleus becomes increasingly lobed as it matures. Mature human neutrophil nuclei typically consist of three or four large heterochromatic lobes joined by thin DNA-containing filaments [Sanchez & Wangh 1997].

The function of the mature neutrophil is dependent on its granules, each with its characteristic content of proteins. During granulopoieses azurophil and MPO negative granules sequentially appear before secretory vesicles, which indicate the terminal step of maturation [Borregaard 1997]. Neutrophils when in the early stages of maturation and differentiation in the bone marrow are CD11b/CD16 negative. There is a continuous increase in CD11b/CD16 along the later part of granulocyte maturation pathway. Metamyelocytes, band forms, and segmented neutrophils express

similar levels of CD11b, but can be distinguished by differential expression of CD16 and different FSC according to Lund-Johansen & Terstappen [1993], but it is not clear whether this splits band form and mature neutrophils apart. Another difference is the volume of band cells (415 fl) has been shown to be greater than mature neutrophils (360 fl) [Linderkamp *et al* 1998].

To provide the high requirement of neutrophils during infection, marrow maturation time is shortened and might be insufficient [Zimmermann 1999CCM]. Myeloperoxidase (MPO) is present in the primary granules, and is produced at an early stage of neutrophil proliferation and is divided with subsequent cell divisions. It is possible that an accelerated transit of neutrophils through the mitotic pool of the marrow could cause neutrophils to skip divisions resulting in the release of cells into the circulation with a higher MPO content [Bainton 1992]. High levels of G-CSF (300μg/day) halve the transit time of neutrophils from the myelocyte stage to entering the blood (from 6 to 3 days) [Cebon & Leyton 1994]. Repeated injections of G-CSF shows a relative increase in immature cells, and decreased percentage of maturing cells [Kerrigan *et al* 1989]. This shift is attributable to accelerated maturation and release of cells from the marrow and the stimulated proliferation of their precursors.

L-selectin has a crucial role in the initial attachment of circulating neutrophils to vascular endothelium during the initiation of neutrophils recruitment into a systemic inflammatory site. L-selectin is low in the mitotic pool, increases as they mature in the post mitotic pool of bone marrow, and is constitutively expressed on circulating neutrophils. Those cells released from the bone marrow by inflammatory stimuli such as endotoxin express higher levels of L-selectin than their circulating counterpart, and they progressively loose this L-selectin as they age in the circulation. Glucocorticoids also induce the release of neutrophils from the bone marrow, but recent studies have established that, in contrast to inflammatory stimuli, this release is associated with a decrease in L-selectin expression [Nakagawa *et al* 1999].

The potent granulopoietic effects of G-CSF have been reproduced in humans and have led to its widespread clinical application in the setting of chemotherapy induced neutropenia and bone marrow transplantation [Crawford 1991, Sheridan *et al* 1989]. In deficient humans G-CSF withdrawal results in a dramatic loss of neutrophils mainly through apoptosis but it is unclear

whether this is due to accelerated apoptosis or a result of the normal cellular ageing process [Molineux 2002]. Several studies indicate that G-CSF may increase with neutropenia, but the increases have generally been noted with neutrophil counts of less than 0.2 x 10^9/l, at which point fever and inflammation predictably occur in most clinical circumstances [Cebon & Layton 1994]. Thus it is not yet clear if milder degrees of neutropenia, in the range of 0.2 - 1.0 x 10^9/l, are associated with elevations of G-CSF, suggesting a feedback relationship. G-CSF knockout mice were more susceptible to infection and had a higher mortality rate. The literature suggests G-CSF is necessary not only for the maintenance of normal neutrophil levels, but also for the development of neutrophillia with infections. Circulating G-CSF levels rise promptly as an acute-phase response to infection [Kawakami 1990, Cheers 1988].

GM-CSF has less impact on differentiation of bone marrow than GCSF, and greater effects on neutrophil functioning. GM-CSF is best known for its ability to prime neutrophils [Dang 1999]. GM-CSF also enhances the function on neutrophils including prolonging survival, enhancing phagocytosis, increasing antibody-dependent cell-mediated cytotoxicity of neutrophil and induce the synthesis and release of a number of cytokines, including IL-1 and TNFα from monocytes. GM-CSF has been shown to maintain the viability of neutrophils in culture, preventing them from undergoing apoptosis (programmed cell death) [Brach *et al* 1992]. GM-CSF causes an increase in increases β2 integrin molecules and fMLP receptor number for prolonged periods [Novella *et al* 1997], decreases l-selectin and Fcγ RIII and increases lactoferrin and hNE, and IL-8 *in vivo* in healthy volunteers [van Pelt 1996] making it a stronger activator of mature neutrophils but with a less pronounced effect on the production and maturation of neutrophil precursors. The physiological role *in vivo* is less clear, as GM -/- mice show no perturbation of major haematopoietic populations in marrow or blood. Surprisingly there was an increases of pulmonary alveolar proteinosis. The BALF contained markedly raised levels of SP-A, -B, and –C. This would suggest a defect of surfactant catabolism of clearance in the absence of GM-CSF, and it may be postulated that interactions of GM-CSF with alveolar macrophages are crucial to mediate pulmonary surfactant homeostasis within the airspaces [Luisetti 1997]. The results suggest that neutrophils recruited into inflammatory lesions by chemotactic factors will nonetheless die by apoptosis unless activated by certain 'trophic hormones' such as GM-CSF. GM-CSF is mainly produced by activated T

lymphocytes and monocytes. IL-6 inhibits apoptosis of early myelopoietic cells [Yonisch-Ronach 1991]. IL-1B protects neutrophils from apoptosis [Bossu 2001].

In summary, increased circulating concentrations of G-CSF would be expected to cause an increase in neutrophils, particularly band forms, whereas an increased circulating concentration of GM-CSF would be expected to result in less apoptosis of neutrophils, potentially with more mature neutrophils and possible priming of circulating neutrophils making them more reactive to stimulation. The aim of this study was to investigate the effect of pulmonary infection in patients with CF on the morphology of neutrophils, and compare this to healthy subjects, and to determine whether nuclear morphology was related to the previously observed reduced responsiveness of neutrophils from patients with CF at the time of a pulmonary exacerbation.

Methods

G-CSF and GM-CSF were measured by ELISA (R&D Systems) according to the manufacturers protocol. Details are included in Appendix 3d and 3e. Patients with CF (n=12) were studied on presentation of a respiratory exacerbation and again at the end of a course of *i.v.* antibiotics, and healthy subjects (n=12) matched to the start of treatment (Study Group B).

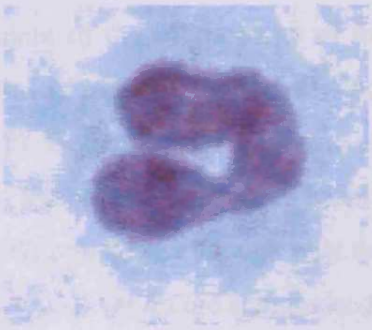
Absolute neutrophil count was determined in whole blood (EDTA) using a Coulter Counter. Blood smears, stained with Leishman's, were used to determine the age of circulating neutrophils by assessment of band forms and nuclear lobes. Neutrophils were assessed as band, 2, 3, 4, and 5 lobed microscopically from whole blood smears (500 cells/smear) (Figure 10.1).

The band percentage of neutrophils was compared between blood smears and cytospins of separated neutrophils (Table 10.1) and was not different. It was therfore relevant to relate the percentage band cells to the responsiveness of isolated neutrophils. Band counts were determined for subjects where superoxide generation and elastase had been measured as described in other sections of this thesis (chapters 7 and 11) for a total of 28 patients (n=7 patients at start of treatment (B), n=6 after treatment (B), n=15 clinically stable (E)) and 7 healthy subjects (B). Incomplete band data was available for study group B.

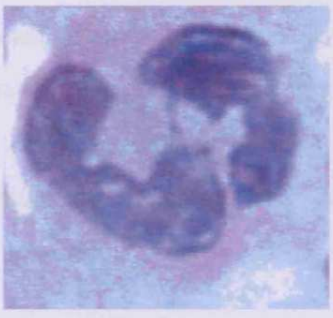
Statistical analysis was by Wilcoxon signed rank for comparisons of patients before and after treatment, and Mann Whitney U test for comparisons between patients and healthy subjects.

Figure 10.1 **Morphology of neutrophils**

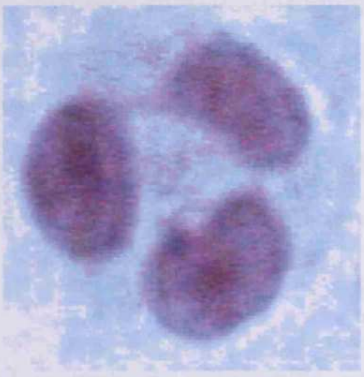
Band form



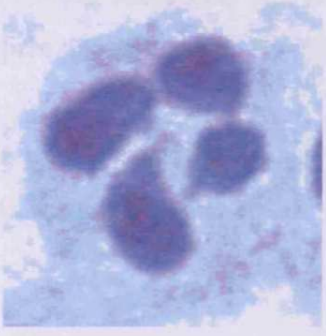
2 lobed



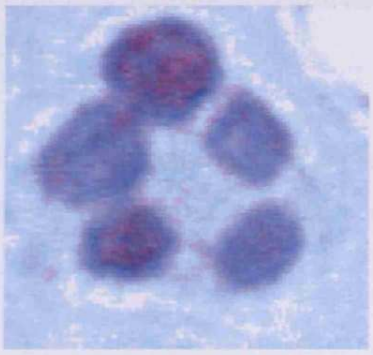
3 lobed



4 lobed



5 lobed



Results

The number of circulating neutrophils was raised in patients with CF compared to healthy subjects at the start of antibiotic treatment ($p=0.013$) but was not signficantly reduced by treatment in this group. Based on assessment of the morphology of the neutrophils the nuclear lobe number was skewed towards band form in patients, particularly those at the start of treatment for a pulmonary exacerbation (Figure 10.2). Both the percentage and absolute number of band cells was greater in the patients at the start of treatment compared to healthy subjects ($p=0.001$, $p<0.001$) and remained so after treatment ($p=0.037$, $p=0.021$), although only the percentage band cells showed a significant fall ($p=0.006$) (Figure 10.3). All values are summarised in Table 10.2.

Circulating levels of G-CSF decreased with antibiotic treatment ($p=0.023$) (Figure 10.4), remaining greater than in healthy subjects before and after treatment ($p=0.001$, $p=0.008$). GM-CSF increased with antibiotic treatment in patients with CF ($p=0.017$) but was not significantly different to levels in healthy subjects (Figure 10.5). These values are also summarised in Table 10.2 in which circulating levels of CRP have been included to provide further confirmation of a pulmonary exacerbation.

The percentage of band cells was inversely related to both the superoxide generation and the elastase release in response to 10^{-8} M fMLP in a group of 28 patients at different clincial states ($r= -0.469$ $p=0.012$, $r= -0.409$, $p=0.030$) but not for healthy subjects. When split into groups on the basis of clinical state, only the group of patients after treatment remained significantly correlated with superoxide generation ($r=-829$, $p=0.042$) (Figure 10.6).

Morphology was also determined for the neutrophils studied in chapter 8. The percentage band cells was related to MPO ($r= 0.690$, $p=0.058$) and surface expression of L-selectin ($r=0.762$, $p=0.028$).

Table 10.1

Comparison of band counts performed on whole blood smears, buffy coat smears and cytospin preparations of iolated neutrophils

Sample	Total cells counted	Band %	2 lobe %	3 lobe %	4 lobe %	5 lobe %	Blood neutrophil count (10^9/l)	Band number (10^9/l)
1 smear	735	43.5	41.5	13.7	1.2		7.9	3.436
1 buff coat	768	44	45	11	0.5			3.476
1 cytospin	588	47.5	39.6	11.7	1.1	0.2		3.737
2 smear	585	25	48	24.5	2.5		3.5	0.875
2 buff coat	672	28.4	53.6	16	1.6			0.994
2 cytospin	495	27.2	45.5	24.4	2.6			0.952
3 smear	569	44.3	43.9	10.4	1.4		4.8	2.126
3 buff coat	706	56.7	35.4	6.9	0.9			2.722
3 cytospin	615	53	33.9	11.5	1.5			2.544
4 smear	51	16.2	45.5	30.3	7.3	0.7	2.7	0.437
4 buff coat	524	24.2	50.8	19.8	5.0	0.2		0.653
4 cytospin	552	29.5	44.2	23.5	2.5	0.2		0.797

Subjects 1 and 3 are patients with CF and subjects 2 and 4 are healthy.

Table 10.2

Circulating levels of cytokines and neutrophils

	CF Patient Start n=12	CF Patient End n=12	Control n=12
Circulating neutrophil count (10^9/l)	5.25* (3.47 – 7.95)	3.67 (2.43 – 5.54)	3.43 (2.72 – 4.33)
Circulating band count (10^9/l)	2.69* (1.32 – 5.50)	1.44*$ (0.76 - 2.73)	0.89 (0.65 – 1.12)
Band Cells (%)	51* (36 – 73)	39*$ (29 – 53)	25 (21 – 29)
G-CSF (pg/ml)	54.9* (30.1 – 100.2)	32.8*$ (22.8 – 47.2)	25.6 (20.1 – 32.7)
GM-CSF# (pg/ml)	0.62 (0.34 – 1.13)	1.32$ (1.09 – 1.59)	0.88 (0.54 – 1.40)
Circulating CRP (μg/ml)	26.67* (5.63 – 126.4)	7.33*$ (1.67 – 32.22)	1.12 (0.38 – 3.26)

GM (95%CI)

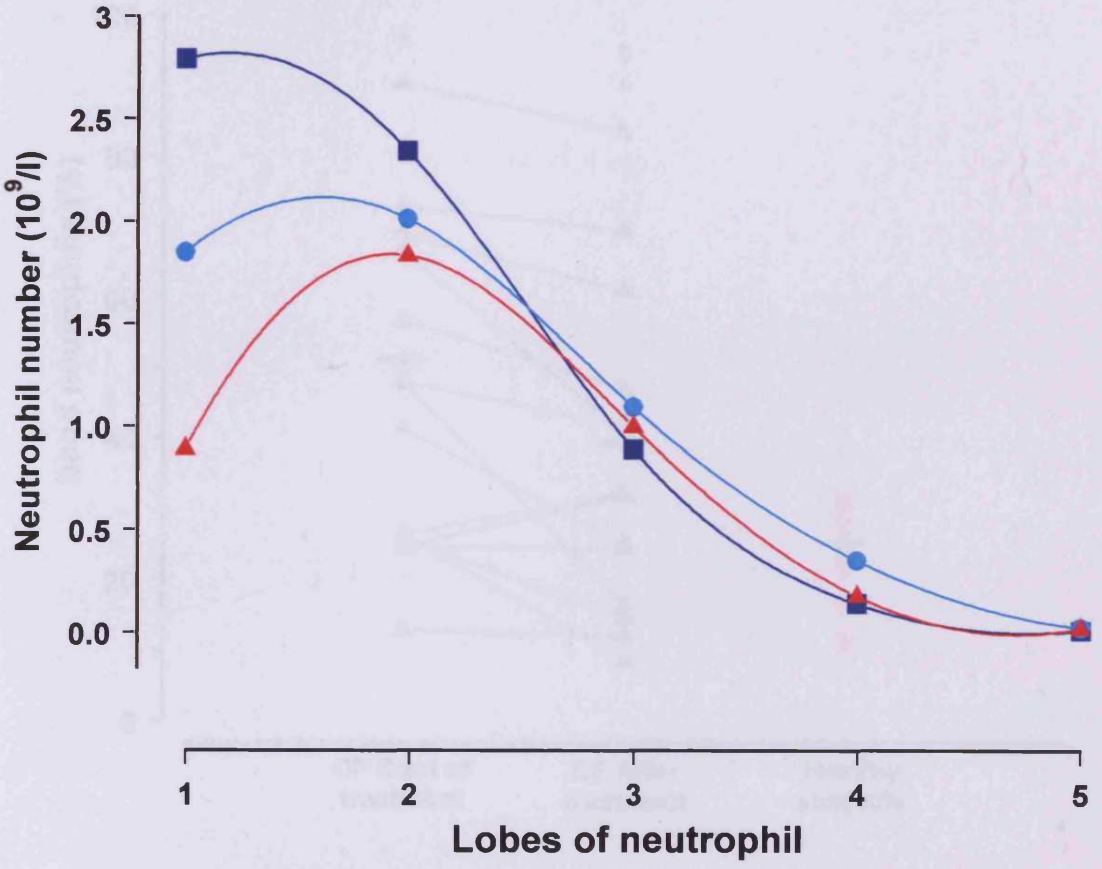
\# Only n=8 in each of the groups for this marker

$ Significant effect of treatment ($p<0.05$) by Wilcoxon signed rank test.

* Signifcantly different to controls ($p<0.05$) Mann-Whitney U test

Figure 10.2

The effect of infection on nuclear lobe number of circulating neutrophils

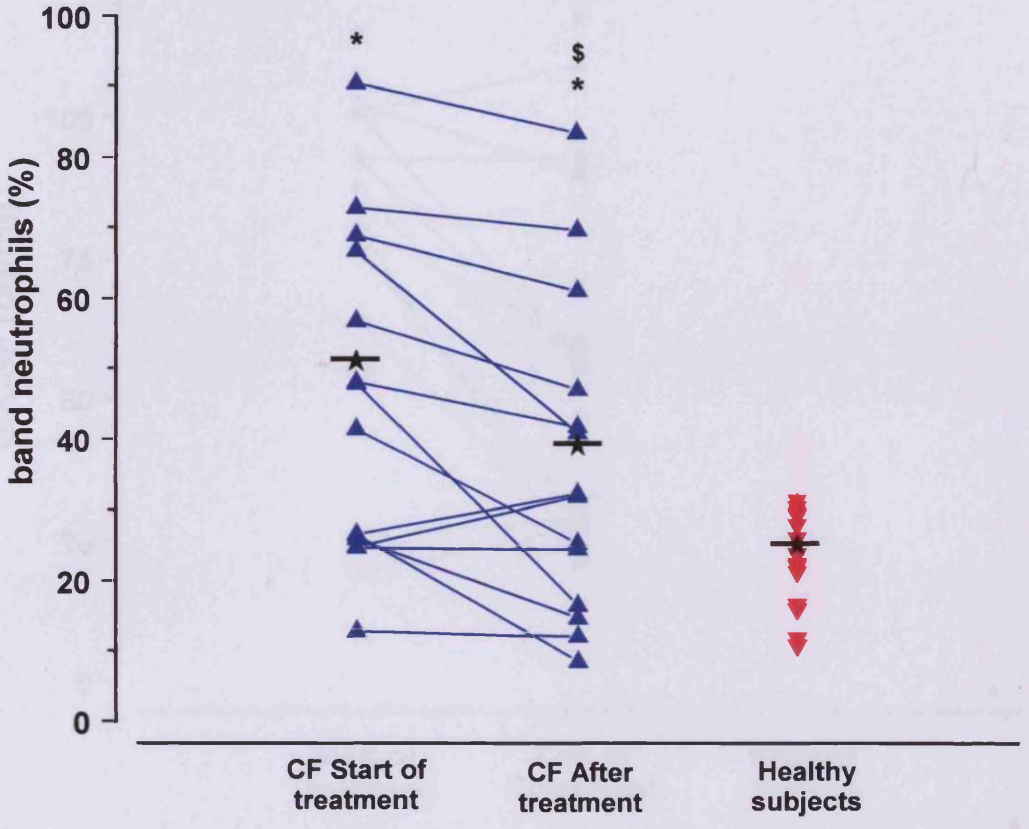


■ CF in exacerbation, ● CF after treatment, ◆ Healthy subject

Arithmetic mean

Figure 10.3

The effect of infection on morphology of circulating neutrophils expressed as a percentage of band neutrophils.



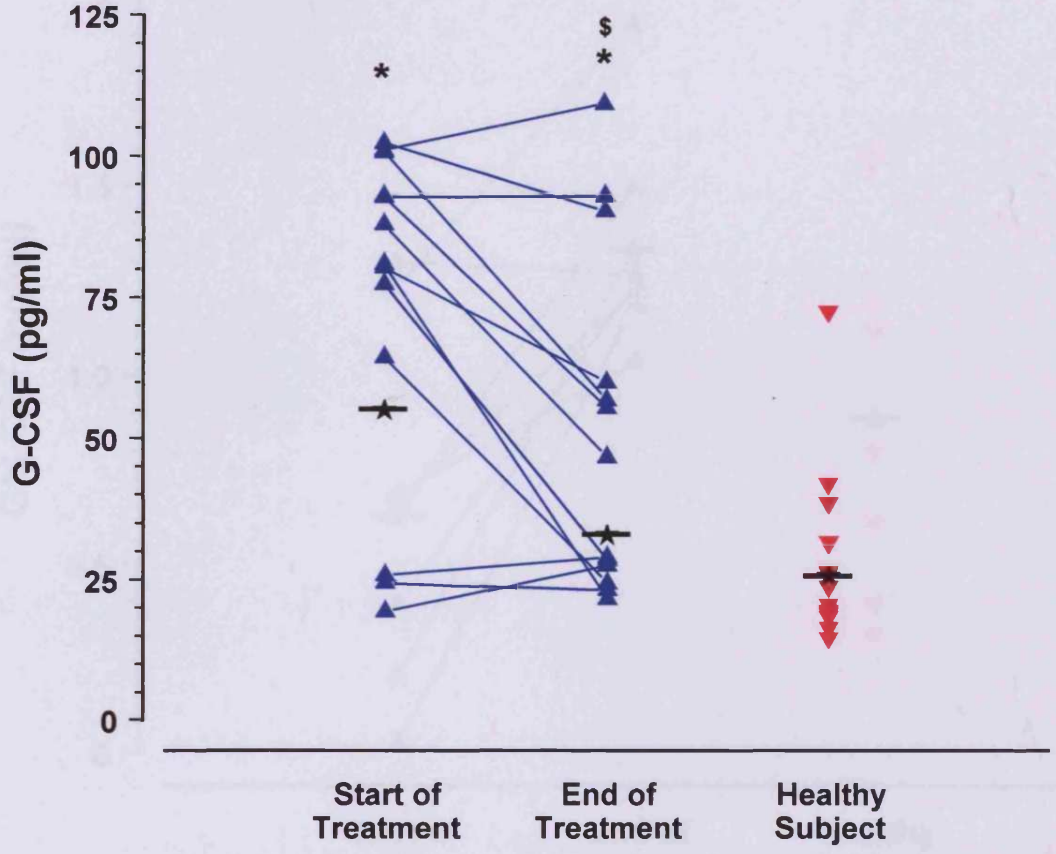
* p<0.05 Patients with CF compared to healthy subjects

$ p=0.006 Effect of antibiotic treatment

—✱— Geometric mean

Figure 10.4

Circulating levels of G-CSF in patients with CF and healthy subjects



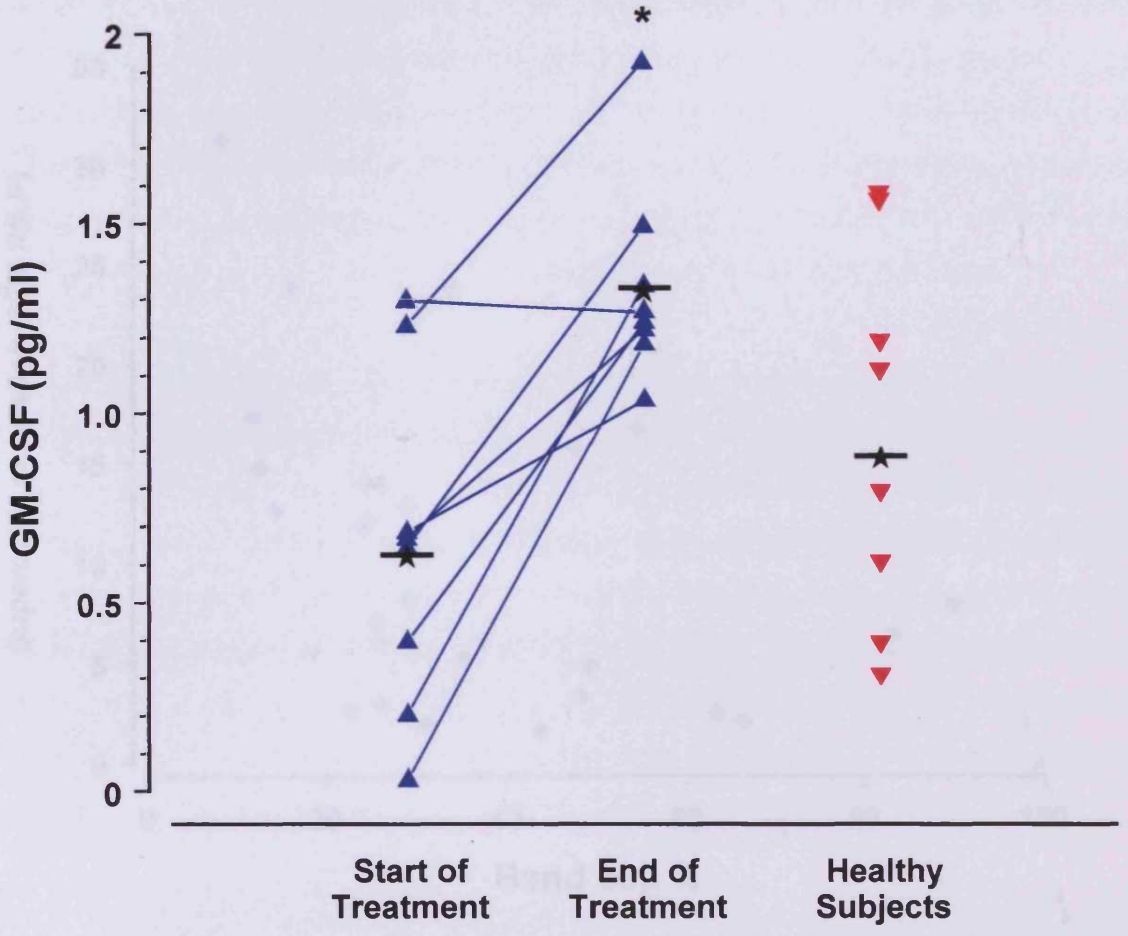
* p<0.01 Patients with CF compared to healthy subjects

$ p=0.023 Effect of antibiotic treatment

—✱— Geometric Mean

Figure 10.5

Circulating levels of GM-CSF in patients with CF and healthy subjects

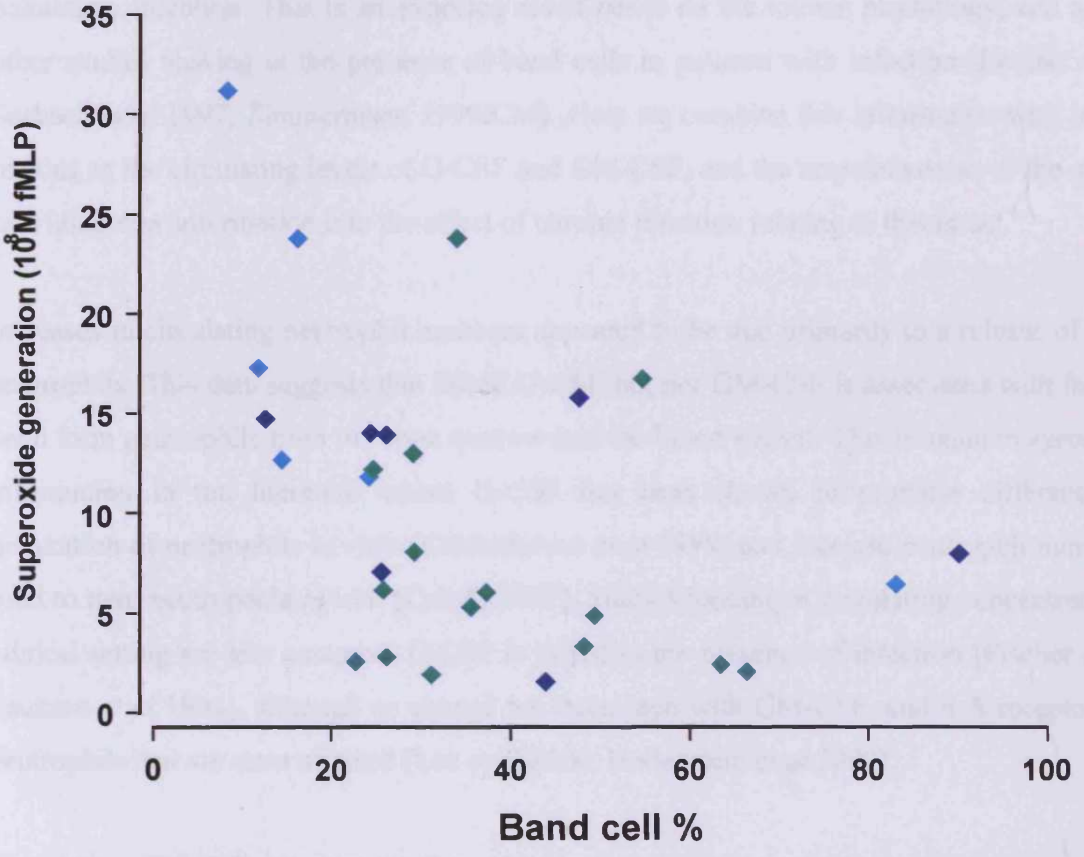


$ p=0.017 Effect of antibiotic treatment

—✱— Geometric Mean

Figure 10.6

The relationship between the percentage of band cells and the superoxide generation in response to fMLP.



◆ Start of Treatment (n=7), ◆ After Treatment (n=6) (r=-0.829, p=0.042),

◆ Clinically Stable (n=15)

Combined spearman correlation of all patient groups (r= -0.469, p=0.012)

Discussion

In this study we have found that there are increased numbers of band form of neutrophils in patients with CF and that this was most marked at the start of antibiotic treatment for an exacerbation of pulmonary infection. This is an expected result based on the known physiology, and agrees with other studies looking at the presence of band cells in patients with infection [Lembo *et al* 1991, Seebach *et al* 1997, Zimmermann 1999ICM]. Here we combine this information with information relating to the circulating levels of G-CSF and GM-CSF, and the responsiveness of the neutrophils, providing new information into the effect of chronic infection relating to this issue.

Increases in circulating neutrophil numbers appeared to be due primarily to a release of band form neutrophils. This data suggests that raised G-CSF but not GM-CSF is associated with the influx of band form neutrophils from the bone marrow into the blood stream. This is again in agreement with information in the literature where G-CSF has been shown to promote differentiation and maturation of neutrophils *in vitro* [Caldenhoven *et al* 1998] and increase neutrophil numbers when used to treat neutropenia *in vivo* [Carulli 1997]. Studies looking at circulating concentrations in the clinical setting are less common. G-CSF is raised in the presence of infection [Fischer *et al* 2002, Pauksen *et al* 1994], although no change has been seen with GM-CSF, and it is receptor levels on neutrophils that are most affected [Lee *et al* 2000, Hollenstein *et al* 2000].

Circulating GM-CSF concentration was lower in patients in exacerbation, rising towards control levels with treatment. GM-CSF primes neutrophils [Dang *et al* 1999] in a dose dependent way. Priming occurs at the range measured here in the circulation. The measurement of 1pg/ml (a mid range point in our measures) is equivalent to 22 pmol/l. Significant priming has been seen with 100 to 300 pmol/l [Weisbart 1987, McColl *et al* 1990]. It is possible that the reactivity of neutrophils is controlled by the presence of GM-CSF in the circulation of healthy subjects, therefore if this priming effect is removed, the neutrophils will be less responsive, which is what we see in neutrophils from patients in exacerbation.

When combined with the superoxide generation results, these data suggest that band cells produced less superoxide than more mature neutrophils in response to fMLP, which acts via cell surface

receptors. This agrees with the report that neutrophils from patients after severe trauma showed cellular dysfunctions including chemotactic migration phagocytosis and bacterial killing. Decreased leukotrienes generation from neutrophils after multiple trauma strongly correlated with the occurrence of immature neutrophils (band cells) [Koller *et al* 2001]. Contrastingly Ogura *et al* [1999] showed a significant increase in priming and the fMLP stimulated oxidative burst which was accompanied by reduced apoptosis, but this research did not look at morphology. Increased superoxide generation in trauma patients was also reported in other studies [Tunaka *et al* 1991, Shaked *et al* 1994].

The patient group were taking a number of drugs including antibiotics, and the possibility of one or more of these drugs affecting band percentage cannot be ruled out as antipsychotic drugs have been shown to increase the percentage of band forms [Delieu *et al* 2001].

An *in vitro* study showed CD11b, CD35, GM-CSF receptor, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and $gp91^{phox}$ to all increase with granulopoiesis accompanied by fMLP stimulated superoxide generation, which increases considerably in the last day of differentiation [Pedruzzi *et al* 2002]. If cells are released early as band forms, it is possible that such proteins have not quite reached their full number, and therefore could explain the reduction in function particularly regarding the priming potential of GM-CSF and the role of the 'phox' proteins in superoxide generation.

Erduran *et al* [1997] showed a positive correlation between serum IL-1 and GM-CSF levels in acute infections, however this did not occur in patients with repeat infections, even though levels of CRP was similar. It would be expected that CF patients would have more in common with the 'repeaters' and they do have a similar small increase in concentrations of GM-CSF. The granule contents of MPO and lactoferrin have been shown to be reduced during the first week of bacterial meningitis, pneumonia and bacterial infection [Hansen *et al* 1976, Venge *et al* 1978]. In glycogen storage disease type I patients experience recurrent or chronic bacterial infections as a consequence of abnormalities in myeloid maturation and of functional defects in circulating neutrophils [Scherer *et al* 2001]. The effects of abnormalities in myeloid maturation in Glucose Storage Disease Type Ib are more severe than the effects seen here, but is more evidence to suggest possibility of myeloid

maturation affecting functionality in CF. Such an effect may simply be the result of continual and long term chronic bacterial infection.

There was less MPO at the start of an exacerbation when the numbers of band cells is highest. If van Eeden & Hogg [2000] are correct in their hypothesis, that neutrophils which have had accelerated release undergo less divisions, and therefore contain more granules, then either MPO has been released whilst in the circulation, or the chronic inflammation in CF is different to that observed in chronic cigarette smoking with regard to the phenotypic characteristics of circulating neutrophils. However, the treated CF patients do have slightly raised MPO compared with healthy subjects, and a higher number of band cells, so at this time the hypothesis would fit. However, L-selectin surface receptor numbers have been reported to be higher on band cells [Lawrence *et al* 1996]. We found the L-selectin receptor number to be lower on neutrophils from patients with CF. The theory that the reduced MPO is due to prior release into the circulation would therefore fit better with the data obtained here.

Smith *et al* [1989] found that neutrophils from patients with rheumatoid arthritis were less chemotactically responsive, yet more metabolically active with an increased ability to produce LTB_4. There was a significant inverse correlation between the cells ability to produce LTB_4 and their chemotactic potential at 20nM fMLP. This group speculated that in RA, normal peripheral blood neutrophils have become 'processed' by previous exposure to either circulating rheumatoid factors, immune complexes, or other chemotactially-responsive, yet more metabolically active, cells. Another theory is that of compartmentalization of activated neutrophils to the site of infections such as the lung which has been supported by greater chemiluminescence in pre pulmonary neutrophils than in post pulmonary neutrophils. But the capacity for exocytosis is similar in the two compartments [Zimmermann 1999 CCM].

The determination of band cells is labour intensive, and the reproducibility is highly dependent of the technique and training of the examiner. There can be large differences between the percentages published by different laboratories. A large study looking at band cells in patients gave a mean of 13.2 % where CRP $<$ 1mg/dl and 24.3% where CRP $\geq$ 1mg/dl [Seebach *et al* 1997]. These values

are slightly higher than obtained in this study, but since all counts were done by the same operator, the comparison between patients and healthy subjects shows real differences.

In patients recovering from antineoplastic chemotherapy, there is a sudden large increase in leukocytes, predominantly neutrophils. Abnormal morphology is common in such neutrophils, with 33% being reported as hypolobulated (without rh-GCSF), and the presence of hypogranular cytoplasm. Hypolobulated nuclear changes included circular nucleus and convoluted 'corkscrew' shapes [Kerrigan *et al* 1989]. Examples given had been observed in the samples of patients with CF in this study, although not specifically investigated. Neutrophils from patients with rapidly progressive periodonititis had a higher percentage of the area of the cell profile occupied by azurophil lysosomes greater absolute numbers of azurophil lysosomes per cell and increased levels of β-glucuronidase than healthy subjects [Pippin *et al* 2000].

Separation of neutrophil precursors can be carried out either by density centrifugation on a Percoll gradient, where increasing density is associated with maturity, or by multidimensional flow cytometry [Cowland & Borregaard 1999 JIM]. It would have been useful in this study to be able to separate band from segmented neutrophils and determine their responsiveness separately. Experiments using centrifugation through Percoll gradient were not found to select the band neutrophils from peripheral blood. Although separate layers of neutrophils with different densities were obtained, the percentage band was equal in all fractions [data not shown]. There was no obvious selection of a separate neutrophil population based on side and forward scatter in the FACS analysis (Chapter 8) although this study did not look specifically at this issue. There are no reports of a method to single out a specific surface marker that would split band from segmented neutrophils, and further investigation was not carried out.

We therefore conclude that patients with CF experience increased numbers of band form neutrophils particularly at a time of pulmonary exacerbation, and that this release is likely to be under hormonal control by G-CSF, exaggerated by the presence of infection. The increased band form is likely to be a result of reduced maturation time, and is probably associated with reduced functional proteins which have the potential to affect the responsiveness of these neutrophils, which could be further exacerbated by reduced levels of GM-CSF. Although this study does not prove

these speculative links, the findings fit well with the known physiology and support the results described in previous chapters of this thesis.

Chapter 11

Metabolic effects on neutrophil superoxide generation and elastase release

Aim

To determine whether *in vivo* blood glucose level affects superoxide generation and elastase release in response to fMLP by neutrophils *in vitro*.

To determine whether the circulating lipid levels (Cholesterol, LDL, HDL and triglycerides) affects superoxide generation and elastase release in response to fMLP by neutrophils *in vitro*.

To determine whether the circulating fatty acid levels affects superoxide generation and elastase release in response to fMLP by neutrophils *in vitro*.

Introduction

The chronic pulmonary infection in CF is linked to weight loss and cachexia [Elborn *et al* 1993CS, Bell *et al* 1996, Ionescu *et al* 1998]. A number of studies have shown that patients with CF are poorly nourished and as a consequence dietary supplementation forms a major part of the management strategy for such patients [Shale 2003]. Despite nutritional supplements, some patients continue to lose weight, as the lung function deteriorates. Patients with CF, particularly adults, tend to have a lower body mass index (BMI) than healthy non-CF age and sex matched subjects. Moreover, a sub group of patients preferentially lose fat free mass (FFM), including skeletal muscle and bone mass [Bolton in press, Ionescu *et al* 2001]. The relationships between chronic pulmonary infection and inflammation, systemic inflammation and the systemic complications of CF are poorly understood. We therefore investigated the relationship between altered metabolism and nutritional status and neutrophil function as an index of the inflammatory response. Neutrophil responsiveness to fMLP was determined in relationship to circulating glucose, lipids and fatty acid concentrations.

Glucose

Impaired glucose tolerance and diabetes mellitus occurs with a greater incidence in patients with CF than in the non-CF population [Lanng *et al* 1995]. Glucose intolerance may be evident in children who are asymptomatic and have a normal fasting blood glucose [Solomon *et al* 2003]. The presence of glucose intolerance in patients with CF results in a faster decline in FEV1 % than in those patients with a normal glucose tolerance test [Milla *et al* 2000]. There remains some debate about the pathophysiology of CF related diabetes, which can be differentiated from Type I and Type II forms by the characteristics of insulinopenia and the rarity of ketacidosis [Lanng 2001]. The insulinopenia probably reflects reduced insulin secretion due to loss of beta cells secondary to fibrocystic changes in the exocrine pancreas. Other possible factors could include altered hormonal relationships within the islets, pancreatic-duodenal interactions, hepatic impairment, intermediary metabolic factors [Hardin 1999D] and the action of autoantibodies [Jensen *et al* 2001, Nousia-Arvanitakis *et al* 2000]. The WHO diagnostic criteria for diabetes are a fasting plasma glucose <7.0, and during a standard oral glucose tolerance test the 2 hour plasma glucose is <7.8 mmol/l for healthy subjects, 7.8 to 11.0 mmol/l indicates impaired glucose tolerance, and ≥ 11.1 mmol/l suggests diabetes mellitus [Reineur *et al* 2002].

Abnormalities of neutrophil function have been reported in diabetes mellitus. In Type I (insulin dependent) non-CF related diabetes neutrophils were reported to have a reduced responsiveness to PMA compared with healthy non-diabetic subjects [Marhoffer *et al* 1993]. Neutrophils from patients with type II (non-insulin dependent diabetes) were reported to have impaired phagocytosis which was related to an elevated intracellular ($Ca^{++}{}_i$) [Alexlewicz *et al* 1995].

Neutrophils must increase their metabolic rate in order to sustain the respiratory burst. The duration and extent of the respiratory burst may vary according to the state of sensitivity and responsiveness of receptor transduction and effector mechanisms of the cells [Carletto *et al* 1996]. The neutrophil respiratory burst can be suppressed by hypoglycaemia, an effect which is more pronounced in non-diabetic subjects [Thomson *et al* 1997]. Neutrophils can use glutamine as well as glucose as an energy substrate, but this only occurs when the availability of glucose is limited such as in severe infection. Glutamine utilisation by neutrophils enables a post-operative increase in phagocytosis and

reactive oxygen production to occur confirming neutrophil use of glutamine as an energy substrate [Furukawa *et al* 2000ANI].

Complex Lipids

Biological membranes are sheet-like structures composed of protein and lipid molecules held together by non-covalent interactions. The major classes of membrane lipids are phospholipids, glycolipids and cholesterol. Fatty acids are contained within phosphoglycerides. Membrane fluidity is controlled by fatty acid composition and cholesterol content. The degree of fluidity partly depends on fatty acid chain length and the extent to which their constituent fatty acids are unsaturated [Stryer 1981]. The membrane fluidity is determined mainly by membrane lipid composition and the molar ratio of cholesterol/phospholipid is considered to be a main determinant of lipid fluidity of biological membranes [Shinitzky *et al* 1974]. Fluidity of membranes has an influence on the speed of movement of intracellular components, such as exocytosis of granules in neutrophils [Gallin & Seligmann 1984]. In rats an increased cholesterol:phospholipid ratio results in a decreased membrane fluidity and impairment of phagocytosis [Lichtenstein *et al* 1987].

LDL are low-density lipoproteins and HDL high-density lipoproteins. The main lipids in HDL are phosphatidylcholine on the particle surface and cholesterol esters in the core with apoA1 the major apolipoprotein. HDL actively participates in the transport of cholesterol in plasma. In acute phase responses the acute phase reactant, serum amyloid A, becomes an apolipoprotein that binds specifically to HDL. This association displaces apoA1 from HDL and possibly modifies HDL metabolism. In plasma HDL is often associated with the acute phase protein serum amyloid A [Jastrand 1990].

Serum levels lipoprotein, particularly LDL, have been reported to be associated with altered neutrophil function with increased superoxide generation in hyperlipoproteinaemic subjects. Bonneau *et al* [1997] reported that LDL at physiological concentrations interacts with neutrophils either directly by its own stimulating effects or indirectly by modification of the response to fMLP causing increased calcium mobilization and superoxide generation. *In vitro* LDL was able to produce a large increase in phosphoinositides, an important regulator in neutrophil activation [Catz

et al 1998]. Oxidised LDL has been shown to evoke an increase in $Ca^{++}{}_i$ through the phospholipase pathway and through phospholipase C-dependent Ca^{2+} influx [Van Tits *et al* 2000]. HDL has been demonstrated to enhance migration and phagocytosis of human neutrophilic granulocytes *in vitro* [Jarstrand *et al* 1990] suggesting that diminished plasma HDL level or a relative lowering of this lipoprotein might cause dysfunction in neutrophils. Jarstrand *et al* [1990] also showed that both HDL from patients with sepsis and normal subjects increased the chemotactic and phagocytic functions of neutrophils, however these responses were significantly reduced when increased concentrations of serum amyloid A, and acute phase protein, were present. Differences in the stimulated respiratory burst were seen after neutrophils were incubated in lipid emulsions with different triglyceride structure, with the medium chain triglycerides able to induce superoxide generation [Wanten *et al* 1999]. Both the medium- and long-chain triglycerides induced faster and therefore greater production of oxygen radicals [Kruimel *et al* 2000]. This effect may in part be due to an effect on cellular membrane fluidity [Wanten *et al* 2001].

Most patients with CF will malabsorb dietary fats if pancreatic treatment is inadequate to address pancreatic insufficiency. However, many patients with apparently adequate enzyme replacement continue to experience a degree of steatorrhea with 10-20% of the dietary fat being malabsorped. This is probably due to defective intestinal uptake of long-chain fatty acids [Kalivianakis *et al* 1999] although it has also been shown that the products of fatty acid digestion were not selectively malabsorped [Murphy 1997]. It is also thought that there is futile cycling of fatty acids between fat stores and the liver in CF [Grunfeld & Feingold 1992], and therefore increased lipolysis that occurs in patients with CF may not be due to malabsorption. NEFA levels have also been shown to be raised at a time of pulmonary exacerbation, and fall with antibiotic treatment adding more evidence for abnormal fat balance [Bell MD Thesis 1995].

Plasma lipids may be altered in CF due to potential malabsorption and to catabolic intermediary metabolism [Kaliviankis *et al* 1999]. Lower levels of plasma lipids were found in an adult CF patient group. Cholesterol, LDL, HDL and Apo B were all significantly lower in patients with CF than healthy subjects ($p<0.001$), however triglycerides were not significantly different between patients and healthy subjects [Ionescu *et al* 1998].

Fatty Acids

Fatty acids carry out many functions that are necessary for normal physiological function and good health. They may be saturated, monosaturated or polyunsaturated (PUFA). In fatty acid nomenclature the first number denotes the number of carbon atoms in the acyl chain of the molecule. This number is followed by a colon, and then the number of unsaturated bonds then the letter 'n' and the number of carbon atoms from the methyl end to the first double bond. See Table 11.1 for the more common and physiologically relevant fatty acids. Mammalian cells lack the delta-12 and delta-15 desaturase enzymes for insertion of a double bond at the n-6 or the n-3 position. Hence linoleic acid (n-6) and alpha-linolenic acid (n-3) are essential fatty acids required in the diet while arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be synthesized from these two precursors. AA is the main substrate for synthesis of the eicosanoid mediators, which are primarily pro-inflammatory, produced by the cyclo-oxygenase, lipoxygenase, and cytochrome p450 pathways. A high intake of its precursor, linoleic acid, may lead to increased inflammation. Humans evolved on a diet where the balance between n-6 and n-3 PUFA was close to 1:1. Today the ratio of these lipids is usually 14:1, and nutritional formulas have ratios from 7:1 to 50:1, thus both healthy subjects and patients with CF are exposed to a relatively large quantity of n-6 compared with n-3 PUFAs with the potential for a pro-inflammatory state [Zaloga 1999].

The biological activity of PUFA on neutrophils is highly dependent on their structure, with the number of methylene-interrupted double bonds, the carbon chain length and the presence of the carboxyl group all crucial. PUFAs affect many cell signalling pathways via mechanisms including integration into cell membranes where they affect membrane fluidity, ion channel flow, cell surface receptor function, generation of cell messengers and activation such as cyclic adenosine monophosphate (cAMP) calcium and modulate the production of prostaglandins, leukotrienes, cytokines and protein kinases (such as protein kinase C) and phosphorylation of intracellular proteins [Zaloga 1999].

Table 11.1

Common PUFA in humans

Name	Carbon chain	double bond position	Abbreviation / alternative name
Palmitic acid	16:0		
Stearic acid	18:0		
Oleic acid	18:1		
Linoleic acid	18:2	n-6	
Alpha-linolenic acid	18:3	n-3	Linolenic acid
Gamma-linolenic acid	18:3	n-6	GLA
di-homo-gamma linolenic acid	20:3	n-6	DGLA
Eicosatrienoic acid	20:3	n-9	ETA
Arachidonic Acid	20:4	n-6	AA
Eicosapentaenoic acid	20:5	n-3	EPA
Dohexaenoic acid	22:6	n-3	DHA

Large intakes of dietary linoleic acid increase the quantity of AA in our cell membranes. The pro-inflammatory products of AA are formed by the enzyme 5-lipoxygenase [Keicher *et al* 1995] through the cyclo-oxygenase and cytochrome p450 pathways. Upon activation AA is converted to pro-inflammatory eicosanoids (2 series) and leukotrienes (4 series). These products are potent activators of granulocyte function such as chemotaxis and degranulation. The best known derivative affecting neutrophils is LTB_4, which has been found in significant quantities in sputum and BALF from patients with CF [Sampson *et al* 1990, Konstan *et al* 1993].

Recently attention has been given to the interactions between n-3 and n-6 PUFA resulting in alterations in the profile of eicosanoids produced by various cell types, including neutrophils, and

possible anti inflammatory roles of the n-3 PUFA. Dietary modification of fatty acid balance by supplementation has been investigated in a number of studies in a variety of diseases [Horrocks *et al* 1999]. Moderate improvement was reported in inflammatory disorders including duodenal ulcer [Manjari *et al* 1998] and rheumatoid arthritis [Simopoulos 2002] after dietary supplementation with fish oil. This anti inflammatory effect appears to be primarily due to the inhibitory effects of EPA and DHA on the formation of the lipid mediators of inflammation, which are derived predominantly from cellular phospholipids and on the cellular-function of inflammatory cells. Fish oils (containing EPA and DHA) cause diminished production of pro-inflammatory eicosanoids, as well as cytokines (IL-1b, IL-6 and TNFα) and reduced expression of leukocyte adhesion molecules (VCAM-1, ICAM-1 and E-selectin) resulting in a lower recruitment of phagocytes to the sites of acute inflammation [Schneider 2001]. Dietary fish oil also decreases superoxide generation by human neutrophils without involvement of the cyclo-oxygenase pathway and without altering neutrophil lysosomal enzyme release [Luostarinen *et al* 1996]. There are a number of contradictions in the literature and the reduction in inflammation may be influenced by the presence of γ-linolenic acid (GLA), a pre-cursor of AA, which is often found in PUFA supplemented diets, where it can increase serum AA. If EPA is added to such diets, the pro inflammatory AA metabolites are reduced as well as the serum AA levels [Barham *et al* 2000]. The physiological explanation is that a high dietary ratio of linoleic to linolenic acid causes depletion of the longer chain n-3 fatty acids including DHA by competing for the enzymes necessary for desaturation and elongation in the liver [Horrocks 1999]. Hence a diet high in linoleic acid will shift the fatty acid composition of the body to a potentially pro-inflammatory state.

The AA content of neutrophils showed a positive relationship with superoxide generation in response to fMLP and PMA [Carletto *et al* 1996] and a reduction in superoxide generation following n-3 PUFA supplementation [Varming *et al* 1995]. Dietary n-3 supplementation inhibited neutrophil chemotaxis as well as augmentation of depressed neutrophil chemotaxis to LTB4 and fMLP [Sperling *et al* 1998]. Addition of EPA significantly inhibited human neutrophil and monocyte adhesion to endothelial cells in vitro by inhibiting endothelial adhesion receptor expression [Sethi *et al* 2002]. The palmitic acid content of neutrophils was inversely related to superoxide generation [Carletto *et al* 1996]. Changes in the fatty acid content of neutrophils is not only dependent on diet. Neutrophils from exudate had greater palmitic and linoleic acid, and less

stearic and arachidonic acid than circulating cells – changes that occur in the same patient with migration [Carletto *et al* 1996]. In pregnancy compared to the non pregnant state there was a lower response to fMLP stimulated superoxide generation in pregnancy compared to non pregnant women. This decrease was related to a decrease in serum and neutrophil AA content. Incubation of neutrophils with PUFA rich serum showed an increase in superoxide generation by increasing linoleic and AA content [Crocker *et al* 1999]. EPA may act to stabilise membrane fluidity, and result in an inhibition of phagocytosis and chemotaxis, although it is likely that this is through a different mechanism to the changes in superoxide generation [Spika *et al* 1996].

Essential fatty acid deficiency occurs in CF in all ages [Lloyd-Still 1996], but its pathogenesis remains unclear. Some of the hypotheses proposed have included malnutrition, fat metabolism, and abnormal membrane lipid turnover [Freedman *et al* 2000]. Although fatty acid deficiency might be related to protein-energy malnutrition, it has been demonstrated in well nourished young patients with CF [Roulet 1997], suggesting it may be related to a specific defect in fatty acid metabolism. Despite this the fatty acid status can be improved by dietary supplementation [Steinkamp *et al* 2000]. In addition DHA regulates membrane fluidity possibly through structural changes in the membrane lipid bylayer [Spector 1999]. Dietary supplementation with n-3 PUFA significantly decreased the production of IL-1β, TNF and IL-6 [Meydani 1992] which could potentially be due to increase in DHA. Its actions include the formation of free radicals in response to oxidative stress, and the prevention of apoptosis [Spector 1999].

Freedman *et al* [1999] reported a profound membrane lipid imbalance in cftr -/- mice characterized by an increase in phospholipid-bound AA and a decrease in phospholipid-bound DHA. This was most pronounced in the organs clinically affected by CF (pancreas, lungs and ileum) and represents the first disease where phospholipid-bound AA levels have been shown to be elevated. Such findings could be explained by either a defect in DHA biosynthesis, or an increase in membrane bound AA giving rise to an increase in the AA to DHA ratio and predisposing the cell to an enhanced inflammatory response. Analysis of plasma levels of fatty acids show there is a defect in fatty acid metabolism in patients with CF independent of their nutritional status [Roulet 1997]. Freedman *et al* [2000] went on to show that adding AA to the cell membrane inhibited apical membrane chloride channels in both CF and normal cells suggesting that increased AA may worsen

the chloride channel defect observed in CF. Increased levels of AA occurs in the airway mucus of patients with CF, compared with healthy subjects, chronic bronchitis infected and *P.aeruginosa* infected patients without CF [Gilljam 1986]. This suggests elevated AA levels are not secondary to lung infection or inflammation, but may be the result of a primary defect associated with carriage of the gene could result in increased inflammation [Freedman *et al* 2000]. Further supportive evidence is that fatty acids are incorporated into cell lipids by an active process involving chloride channels and the AA content of membrane phospholipids was greater in ΔF508 cells, which also had a reduced uptake of linoleic acid [Bhura-bandali 2000]. DHA may also play a role here, as it modulates the function of delayed rectifier potassium channels as well as carrier medicated transport [Spector 1999].

Patients with CF are known to have a higher incidence of glucose intolerance, and patients with diabetes have reduced superoxide generation. We therefore hypothesised that there may be a link between glucose intolerance and neutrophil responsiveness. In addition knowledge of altered metabolism, and reduced cholesterol and PUFA in patients with CF, and the link between cholesterol/phospholipid membrane content and superoxide generation led us to investigate whether a link existed in patients with CF. In addition, we hypothesised that an altered AA:DHA ratio would affect the responsiveness of neutrophils.

Method

Patients were recruited as described (2.3.2, Study Group E).

Glucose challenge

To investigate the effect of glucose, the 120 min. time point of a standard glucose oral tolerance test was chosen for two reasons. Firstly this was when circulating neutrophils would have been subjected to a possible raised glucose level for the longest period. Secondly, the patients with CF have a slower rise in blood glucose after challenge, often not peaking until 60 min. compared to healthy subjects, possibly due to slowed rate of absorption. Patients were later grouped into those defined as glucose intolerant, and glucose tolerant (normal) using the WHO criteria. None of the patients studied had diabetes mellitus at the time of the study.

Circulating measures

Blood glucose was measured as described (2.3.14) and serum fatty acids were carried out by Gas Chromatography as described (2.3.13). Blood lipids were measured using the standard spectrophotometric method by the Biochemistry department in Llandough Hospital.

Neutrophil function

Neutrophils were separated as described (2.3.7) and superoxide generation (2.3.8) and elastase release (2.3.5) measured in response to fMLP stimulation.

Results

Clinical measures

Patients had a mean age of 21 (S.D. 6) years, and a mean FEV_1 of 68 (S.D. 19) % predicted. The group were not obviously undernourished with a mean BMI of 22.0 (S.D. 3.4) kg/m^2, and a moderate inflammatory status was indicated by a CRP of 5.38 (95%C.I. 1.97 - 14.72) μg/ml.

Glucose

Of the 15 patients studied, 7 were glucose intolerant. Glucose concentrations at the two time points are shown in Figure 11.1.

There was no significant difference between the superoxide generation by neutrophils at time zero and 120 min. and no significant difference between the normal and glucose intolerant groups of CF patients (Figure 11.2a). There was no significant difference between the elastase release by neutrophils when the same groups were compared (Figure 11.2b). The range of data is shown in Table 11.1.

Table 11.2

Superoxide generation and elastase release by neutrophils from patients with CF pre- and 2 hours post-glucose challenge

	fMLP					PMA
	10^{-9}M	10^{-8}M	$3x10^{-8}$M	10^{-7}M	$3x10^{-7}$M	10^{-7}M
Superoxide generation						
Glucose normal						
Fasting (n=8)	0.24 (0.2 - 0.3)	4.63 (2.0-11.6)	12.13 (7.2-20.5)	16.56 (11.1-24.7)	16.52 (9.7-28.0)	29.88 (10.5-52.0)
2 hour post glucose challenge	0.18 (0.1-0.3)	3.22 (1.4-6.9)	8.61 (4.8-15.3)	12.3 (7.8-19.6)	13.4 (7.4-24.2)	20.8 (1.4-3.4)
Glucose intolerant						
Fasting (n-7)	0.31 (0.2 - 0.5)	4.96 (2.2-11.4)	11.32 (5.9-21.4)	14.09 (8.6-23.2)	17.78 (10.7-29.5)	23.33 (20.9-42.6)
2 hour post glucose challenge	0.36 (0.3-0.4)	4.98 (2.1-12.1)	7.40 (2.0-27.4)	14.24 (9.1-22.4)	16.52 (10.1-27.1)	21.26 (11.7-38.4)
Elastase release						
Glucose normal						
Fasting (n=8)	7 (1-45)	174 (66-461)	374 (200-704)	459 (264-800)	541 (276-1065)	501 (351-718)
2 hour post glucose challenge	8 (2-39)	122 (44-340)	257 (123-538)	356 (176-722)	318 (101-997)	350 (196-626)
Glucose intolerant						
Fasting (n=7)	13 (2-102)	241 (136-429)	429 (282-653)	471 (319-694)	630 (391-1016)	376 (158-893)
2 hour post glucose challenge	46 (6-341)	212 (104-432)	312 (113-862)	483 (344-680)	392 (145-1060)	384 (172-861)

Figure 11.1

Blood glucose levels in patients with CF included in this study when fasting and after glucose challenge

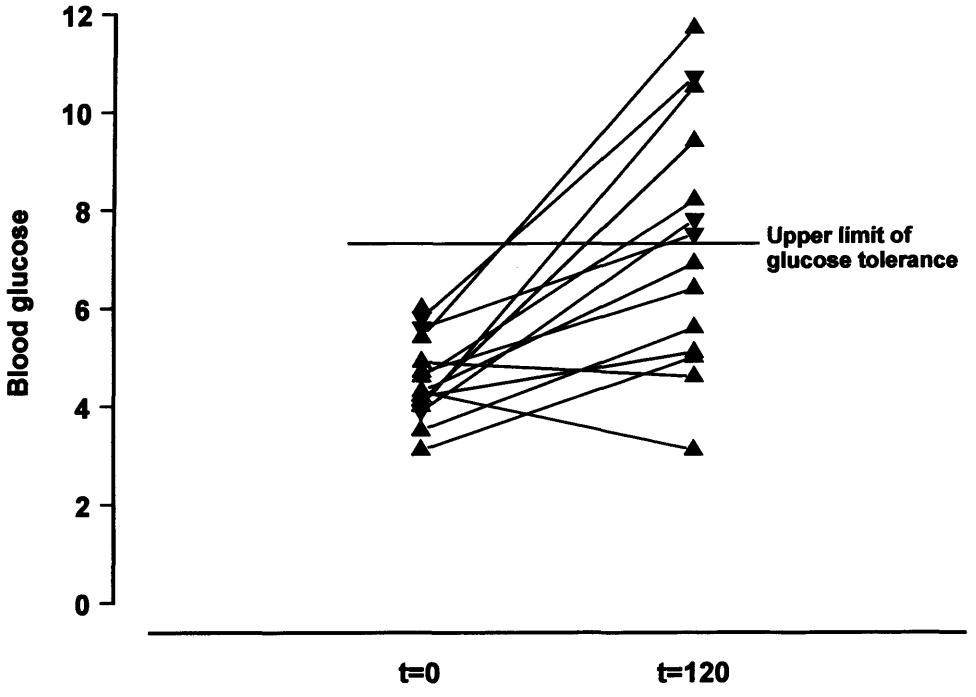
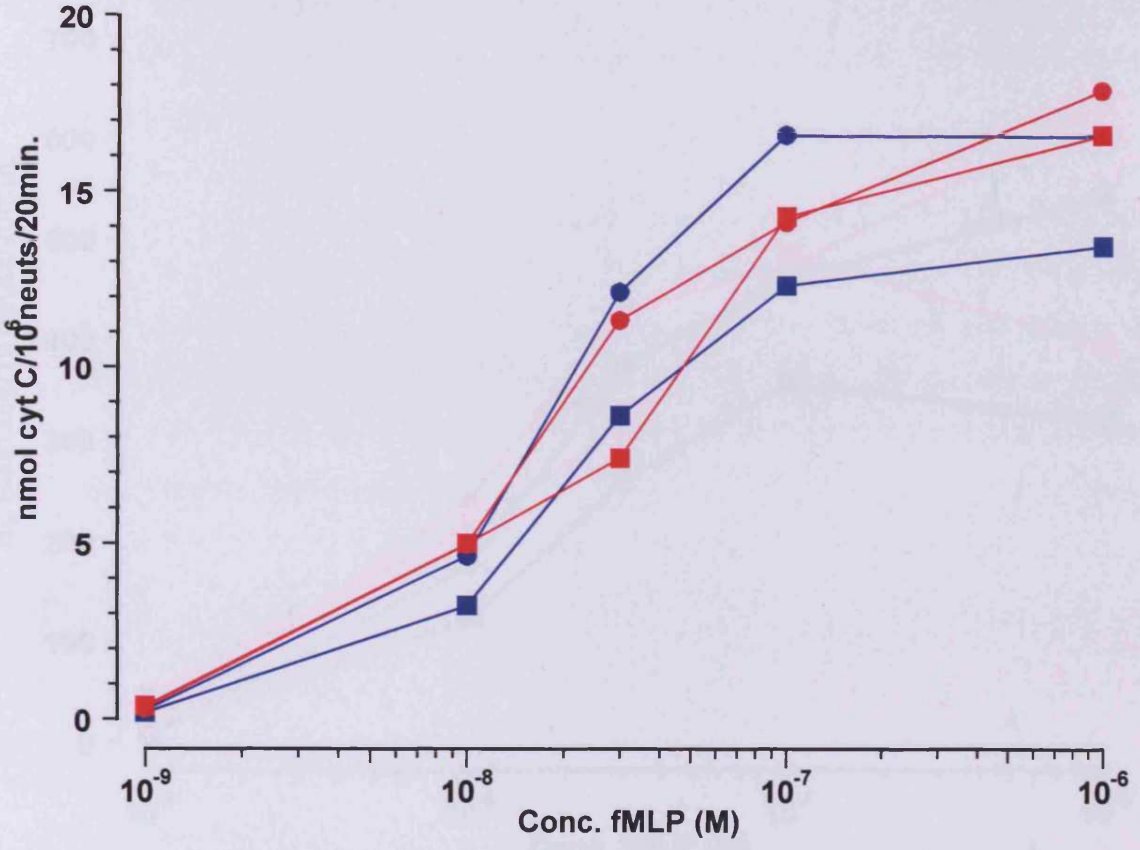


Figure 11.2a

Superoxide generation in patients with CF who had normal and intolerant response to glucose when fasting and after glucose challenge



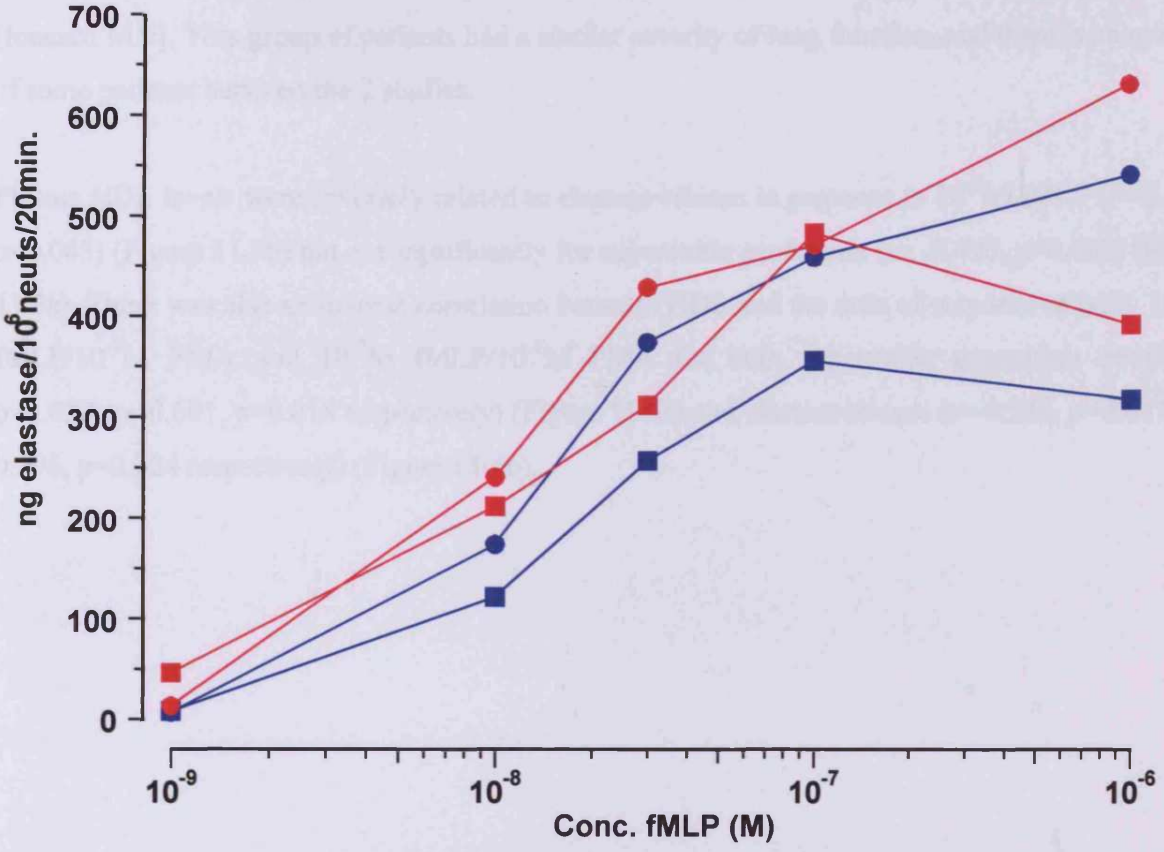
Values geometric mean (95% confidence Intervals in shown in Table 11.1)

CF normal glucose tolerance; (n=8) ● fasting ■ 2 hours post glucose challenge

CF glucose intolerant; (n=7) ● fasting ■ 2 hours post glucose challenge

Figure 11.2b

Neutrophil elastase in patients with CF who had normal and intolerant response to glucose when fasting and after glucose challenge



Values geometric mean (95% confidence Intervals in shown in Table 11.1)

CF normal glucose tolerance; (n=8) ● fasting ■ 2 hours post glucose challenge

CF glucose intolerant; (n=7) ● fasting ■ 2 hours post glucose challenge

Complex Lipids

Cholesterol in this group of patients was 3.38 (3.53 – 3.98) mmol/l, of which LDL was 2.41 mmol/l and HDL was 1.07. Triglycerides were 1.17 mmol/l. Lipids measured in a larger group of patients with CF and healthy subjects for comparison are shown in Table 11.3. reproduced with permission [Ionescu MD]. This group of patients had a similar severity of lung function, and there is an overlap of some patients between the 2 studies.

Plasma HDL levels were inversely related to elastase release in response to 10^{-8}M fMLP (r=-0.529, p=0.043) (Figure 11.3b) but not significantly for superoxide generation (r= -0.493, p=0.062) (Figure 11.3a). There was also an inverse correlation between HDL and the ratio of response of both 10^{-8}M fMLP/10^{-7}M PMA and 10^{-7}M fMLP/10^{-7}M PMA for both superoxide generation (r=-0.574, p=0.032; r=-0.601, p=0.018 respectively) (Figure 11.4a) and elastase release (r=-0.538, p=0.047; r=-0.596, p=0.024 respectively) (Figure 11.4b).

Table 11.3

Lipid concentrations in patients with CF and healthy subjects

	CF Start of treatment n=14	CF After treatment n=14	CF 4 weeks after treatment n=14	Healthy subjects n=25
Cholesterol (mmol/l)	3.98 * (3.56 – 4.46)	4.16 * # (3.79 – 4.58)	3.98 * (3.58 – 4.44)	4.75 (4.36 – 5.18)
LDL (mmol/l)	2.13 * (1.84 – 2.47)	2.25 * # (1.97 – 2.57)	2.02 * (1.69 – 2.41)	2.69 (2.43 – 2.98)
HDL mmol/l	1.30 * (1.12 – 1.51)	1.34 * (1.19 – 1.51)	1.39 * (1.26 – 1.53)	1.59 (1.47 – 1.72)
Triglycerides mmol/l	1.05 (0.95 – 1.17)	1.10 (0.98 – 1.23)	1.09 (0.98 – 1.20)	1.05 (0.92 – 1.20)
Apo B	41.9 * (37.2 – 47.3)	42.4 * (37.6 – 48.0)	40.2 * (35.2 – 45.9)	51.4 (47.7 – 55.3)

Geometric Mean (95% Confidence Interval)

* $p<0.001$ compared to healthy subjects (Mann Whitney U test)

\# $p<0.01$ start compared to end of treatment (Wilcoxon signed rank)

Figure 11.3a

The relationship between superoxide generation in response to 10^{-8}M fMLP and plasma HDL

Spearman correlation: r=-0.493, p=0.062 (fasting); n=15

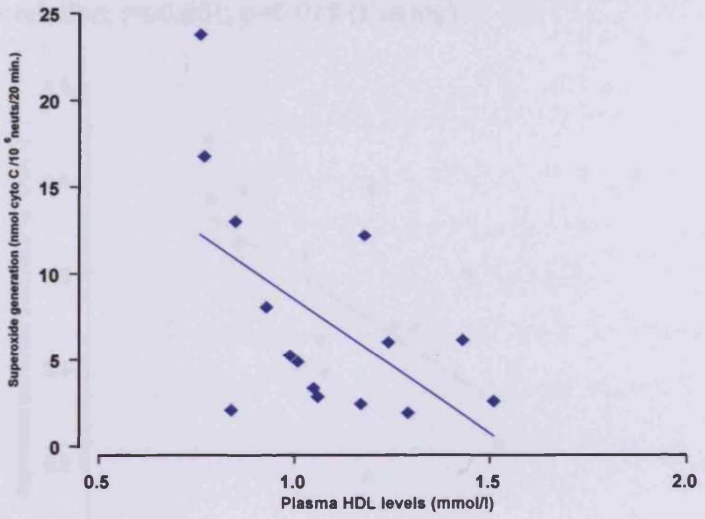


Figure 11.3b

The relationship between elastase release in response to 10^{-8}M fMLP and plasma HDL

Spearman correlation: r=-0.529 (p=0.043) (fasting); n=15

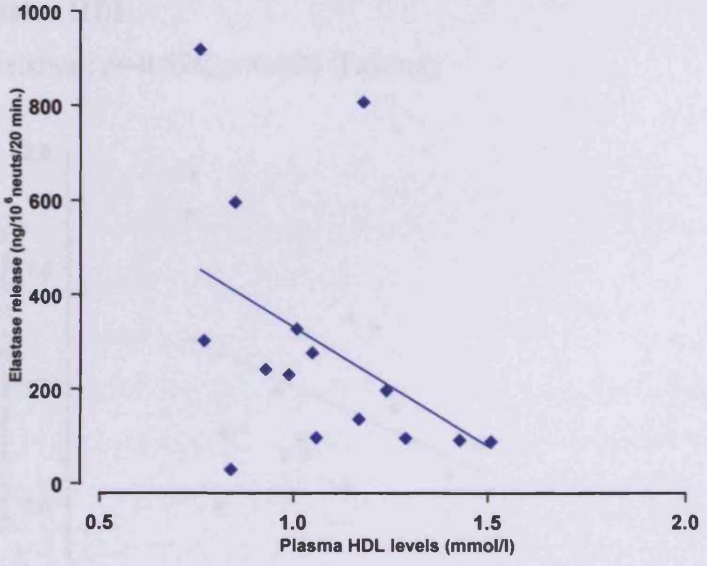


Figure 11.4a

Correlation of superoxide generation ratio of the response to 10^{-7}M fMLP and 10^{-7} M PMA and the relation to plasma HDL

Spearman correlation; r=-0.601, p=0.018 (Fasting)

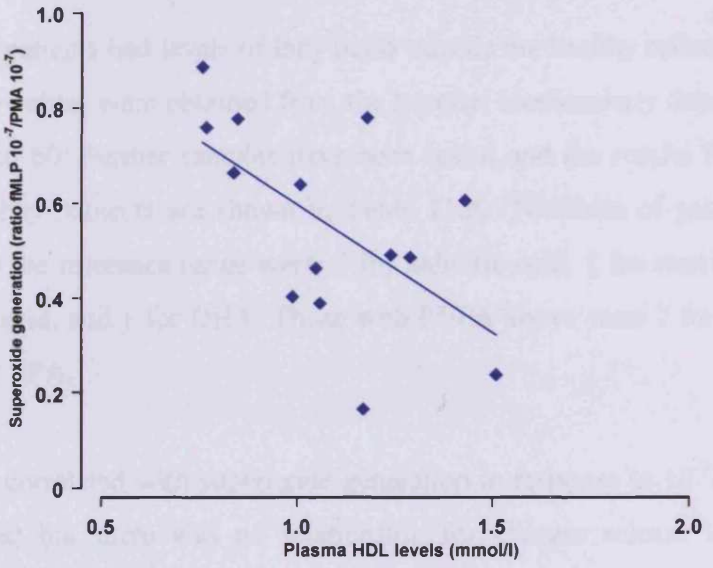
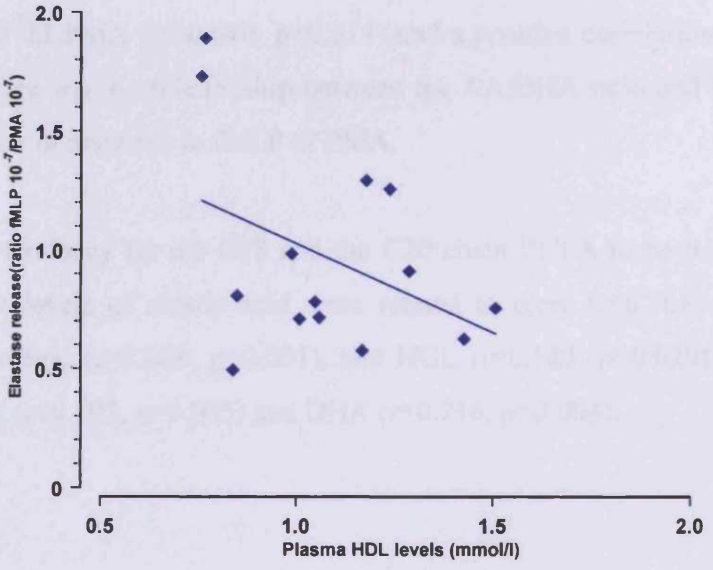


Figure 11.4b

Correlation of elastase release ratio of the response to 10^{-7}M fMLP and 10^{-7} M PMA and the relation to plasma HDL

Spearman correlation; r=-0.596, p=0.024 (Fasting)



Fatty Acids

Fatty acids concentrations were measured in the fasting sample, and therefore this has been used for all statistics including those involving neutrophil function.

A number of patients had levels of fatty acids outside the healthy reference range (Figure 11.5). The normal range values were obtained from the hospital biochemistry department and represent an age range of 18 to 60. Further samples have been tested and the results for patients with CF and age matched healthy subjects are shown in Table 11.3. Numbers of patients with concentrations of PUFA below the reference range were; 3 for palmitic acid, 1 for stearic acid, 3 for linoleic acid, 5 for linolenic acid, and 1 for DHA. Those with PUFA above were 2 for oleic acid, 7 for HGL, 2 for AA and 1 for EPA.

Plasma EPA correlated with superoxide generation in response to 10^{-7}M PMA, ($r=0.636$, $p=0.015$) (Figure 11.6a) but there was no relationship for elastase release in response to 10^{-7}M PMA ($r=0.266$, $p=0.358$) (Figure 11.6b). However, the ratio of response to 10^{-8}M fMLP and 10^{-7}M PMA was inversely related for both superoxide generation ($r= -0.752$, $p=0.02$) (Figure 11.7a) and elastase release ($r=-0.581$, $p=0.029$) (Figure 11.7b).

There were inverse relationships with AA and ratio of superoxide generation in response to 10^{-8}M fMLP and 10^{-7}M PMA ($r=-0.640$, $p=0.014$) and a positive correlation with 10^{-7}M PMA ($r=0.547$, $p=0.043$). There was no relationship between the AA:DHA ratio and the superoxide generation or elastase release in response to fMLP or PMA.

There was a tendency for the C18 and the C20 chain PUFA to be positively correlated with each other. Higher levels of stearic acid were related to oleic ($r=0.701$, $p=0.005$), linoleic ($r=0.754$, $p=0.002$) linolenic ($r=0.803$, $p=0.001$), and HGL ($r=0.583$, $p=0.029$). Similarly EPA levels were related to AA ($r=0.702$, $p=0.005$) and DHA ($r=0.716$, $p=0.004$).

Table 11.4

Levels of Fatty acids in patients with CF and healthy subjects

	CF In Exacerbation n=24	CF after antibiotic treatment n=21	CF Clinically Stable n=37	Healthy subjects (age matched) n=34	Biochem.Dept Adult normal range
Palmitic Acid (16:0)	39.13$ (33.57 - 45.74)	46.43 (40.24 - 52.35)	41.66$ (36.78 – 46.78)	48.99 (43.84 – 54.74)	29.2 – 71.2 mg/dl
Stearic Acid (18:0)	11.56$ (10.06 – 13.29)	14.98* (12.76 – 17.61)	13.38$ (12.13 – 14.75)	15.87 (14.42 – 17.46)	8.4 – 24.6 mg/dl
Oleic Acid (18:1)	42.14 (36.07 – 49.22)	47.84 (41.52 – 55.12)	48.22 (42.60 – 54.60)	43.70 (34.87 – 54.76)	24.8 – 66.1 mg/dl
Linoleic Acid (18:2) {n-6}	30.43$ (22.29 – 41.54)	33.78$ (22.75 – 50.16)	36.78$ (29.99 – 45.10)	63.49 (56.95 – 70.78)	38.9 – 99.5 mg/dl
Linolenic Acid (18:3){n-3}	0.86$ (0.53 – 1.42)	0.98 (0.56 – 1.71)	0.68$ (0.48 – 0.97)	1.49 (1.21 – 1.85)	0.5 – 3.6 mg/dl
ETA (20:3){n-6}	2.85 (2.38 – 3.41)	3.42 (2.78 – 4.20)	3.89 (2.65 – 5.70)	3.31 (2.94 – 3.72)	0.9 – 10.9 mg/dl
AA (20:4){n-6}	9.41 (7.83 – 11.31)	10.09 (8.28 – 12.31)	9.30 (7.14 – 12.11)	10.86 (9.35 – 12.19)	7.6 – 23.1 mg/dl
EPA (20:5) {n-3}	0.85 (0.57 – 1.26)	1.15 (0.83 – 1.58)	1.05 (0.72 – 1.52)	1.02 (0.76 – 1.37)	0.5 – 3.4 mg/dl
DHA (22:6){n-3}	0.54$ (0.40 – 0.73)	0.74$ (0.54 – 0.99)	1.15 (0.80 – 1.66)	1.58 (1.13 – 2.23)	2.3 – 8.6 mg/dl

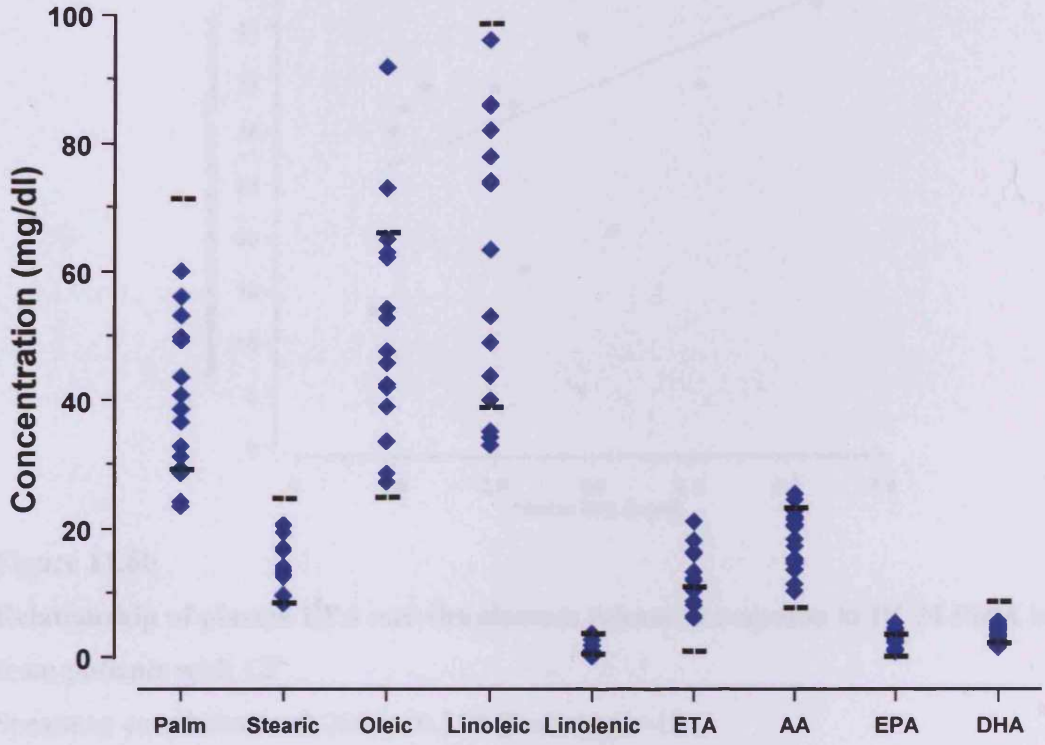
Geometric mean (95% Confidence Interval)

Significant difference ($p<0.05$): * CF exacerbation vs. antibiotic treatment;

$ CF vs. healthy subjects

Figure 11.5

Fatty Acid concentrations in patients with CF



Biochemistry reference range shown in black.

Figure 11.6a

Relationship of plasma EPA and the superoxide generation in response to 10^{-7}M PMA in neutrophils from patients with CF

Spearman correlation: r=0.636, p=0.015 (Fasting); n=15

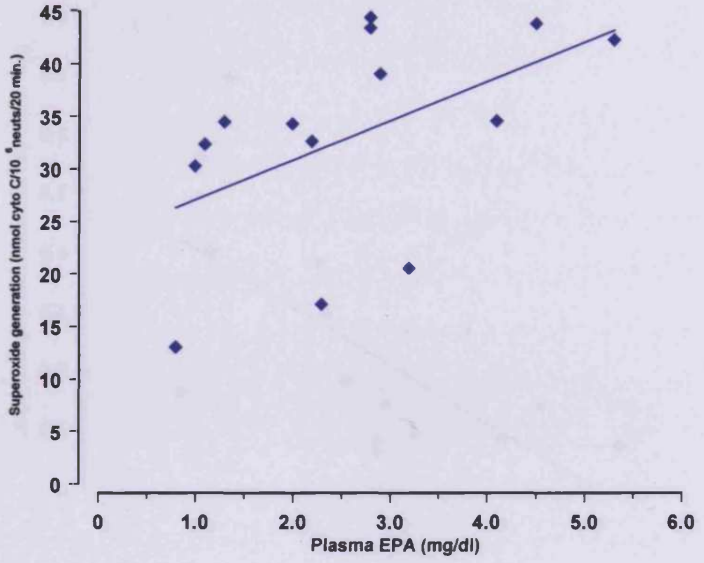


Figure 11.6b

Relationship of plasma EPA and the elastase release in response to 10^{-7}M PMA in neutrophils from patients with CF

Spearman correlation: r=0.266, p=0.358 (Fasting); n=15

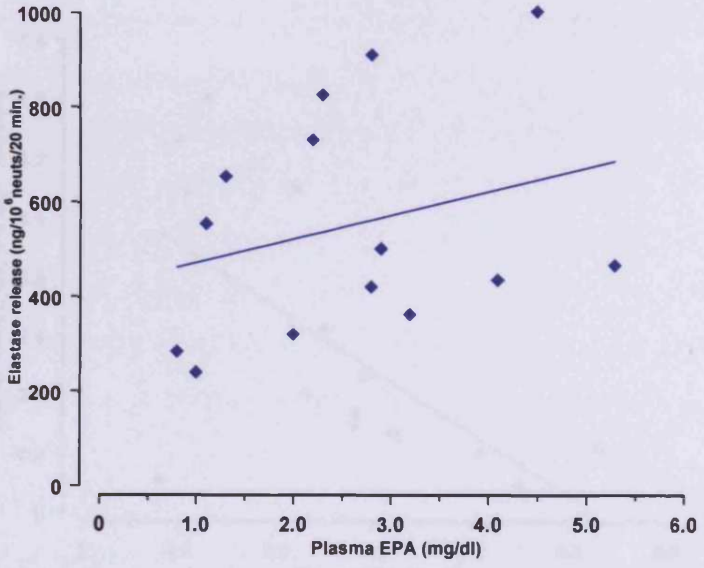


Figure 11.7a

Relationship of plasma EPA and the ratio of the superoxide generation in response to 10^{-8}M fMLP/ 10^{-7}M PMA by neutrophils from patients with CF

Spearman correlation: r= -0.752, p=0.002 (Fasting); n=14

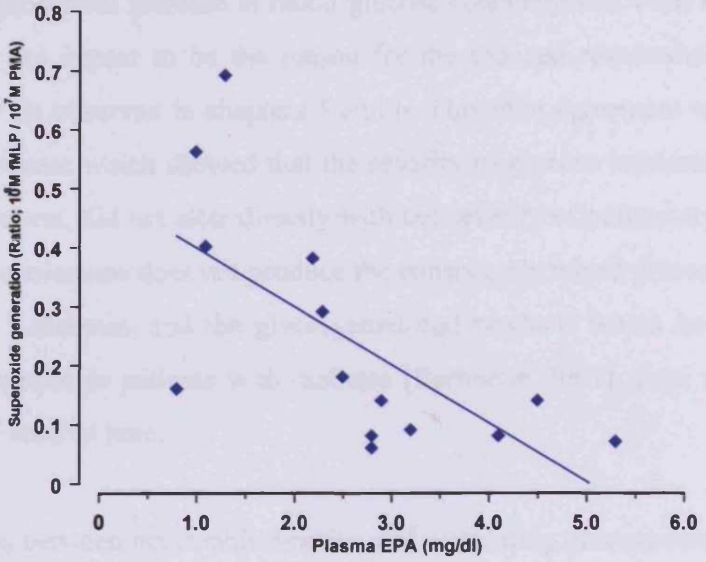
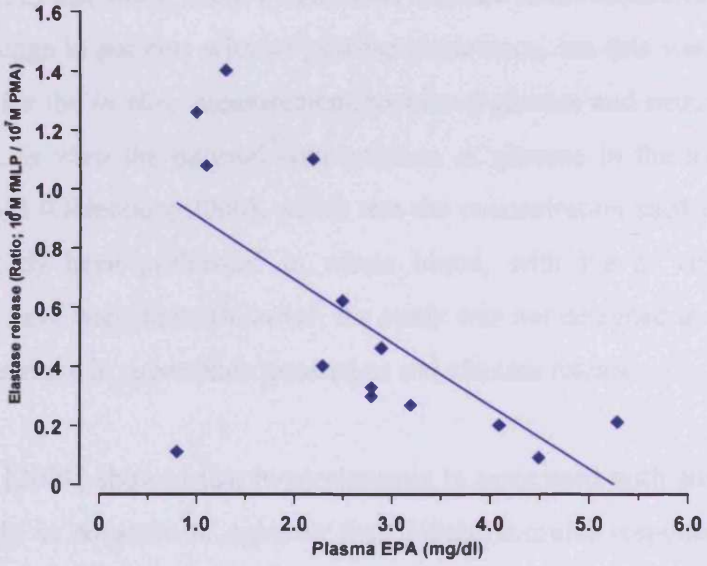


Figure 11.7b

Relationship of plasma EPA and the ratio of the elastase release in response to 10^{-7}M fMLP/ 10^{-7}M PMA by neutrophils from patients with CF

Spearman correlation: r= -0.581, p=0.029 (Fasting); n=14



Discussion

Glucose

The effect of a short term increase in blood glucose concentration, even in the presence of glucose intolerance, did not appear to be the reason for the reduced responsiveness of neutrophils from patients with CF as observed in chapters 5 and 6. This is in agreement with other research carried out in the Department which showed that the severity of glucose intolerance, as demonstrated by a glucose tolerance test, did not alter directly with the severity of pulmonary infection [Ansari 1998]. Impaired glucose tolerance does not produce the consistently raised glucose concentrations as found in typical Type 1 diabetes, and the glycosylated end products which have been shown to reduce superoxide generation in patients with diabetes [Berhneim 2001], does not occur in the group of patients with CF studied here.

The relationships between neutrophil function and circulating glucose concentrations are unclear. It has been shown that fasting increases superoxide generation, and reverts to pre-fasting values with refeeding [Walrand *et al* 2001]. Contrastingly fasting has been shown to reduce superoxide generation in healthy subjects [Dandona *et al* 2001]. Another study showed that glucose challenge resulted in an increased generation of reactive oxygen species by neutrophils in healthy subjects [Mohanty 2000]. In this study, there was a small increase in the superoxide generation from fasting to glucose challenge in patients without glucose intolerance, but this was not significant. However the buffer used for the *in vitro* measurement contained glucose and neutrophils are able to take up glucose rapidly. *In vitro* the optimal concentration of glucose in the incubation buffer has been shown to be 5mM [Oldenborg 2000], which was the concentration used in this study. It is possible that had the study been performed in whole blood, with the *in vivo* glucose concentration, differences may have been seen. However, the study was not designed to address this issue but that of a sustained decrease in superoxide generation and elastase release.

McManus *et al* [2001] showed that hyperglycemia is associated with sustained decreases in PMN function but only in response to agonists that initiate stimulus-response coupling via G-protein-coupled receptors. This agonist-selective reduction in PMN responsiveness may contribute to the

compromised host defence associated with sustained hyperglycemia in diabetes. In healthy subjects reactive oxygen species fell after 24 hours fasting, and the level of $p47^{(phox)}$ subunit, an index of NADPH oxidase, the enzyme converting molecular oxygen to superoxide, also fell in parallel [Dandona 2001]. These studies indicate that both too much and too little oxygen can lead to a reduced superoxide generation, indicating that for optimal superoxide generation the metabolic state is important and reactivity is tightly controlled. The effect of glucose on enzyme release has been less well studied with the functioning of the receptor coupling being the most likely place for effects of the fMLP activated response [McMauns 2001]. Reduced MPO release was seen in patients with diabetes in parallel to reduced superoxide generation [Sato *et al* 1992] suggesting coupling of these secretory functions as we have seen with superoxide and elastase.

Complex Lipids

Reduced cholesterol has been reported in patients with CF as well as reduced fatty acids particularly linoleic and AA [Benandeslam *et al* 1998], but a low cholesterol was not related to other markers of disease such as BMI, glucose intolerance, CRP or pulmonary function [Figueroa *et al* 2002]. The reduced cholesterol reported in other studies agrees with the findings reported here.

The inverse relationship of HDL with superoxide generation and elastase release is the opposite to what might be expected. Patients with CF have a reduced HDL compared to healthy subjects, but reduced neutrophil responsiveness and therefore a positive relationship would be expected. Similarly, we would expect increased fluidity with increased HDL [Jarstrand *et al* 1990] reflected in a greater ratio of fMLP to PMA response. Again the relationship was unexpectedly inversely related to the neutrophil responsiveness. The observation that high levels of serum amyloid A, an acute phase protein, may reduce the HDL induced responses [Jaastrand *et al* 1990] may partly explain the effects. We have shown that CRP is raised in patients with CF even at times of clinical stability (Chapter 3), and therefore it would be likely that amyloid serum A, another acute phase protein, would also be raised, and could affect the response. However, serum amyloid A has been shown to reduce fMLP stimulated MPO release at concentrations higher than 10μg/ml [Gatt *et al* 1998] and to reduce chemotaxis when in complex with HDL [Jastrand *et al* 1990].

The LDL induction of PKC translocation to the membrane may be the mechanism by which LDL is able to increase the oxidative burst [Lara *et al* 1997] or by the induction of $gp91^{PHOX}$ expression [Ruechshloss *et al* 2001], although we were unable to see a direct relationship with LDL and superoxide generation. Plasma LDL was lower in patients with CF, and could be a factor in reduced superoxide generation although it does not play a major role.

Levels of LDL and Triglycerides were not significantly different in the patients with CF than healthy subjects, and no relationship was seen with these lipids and the responsiveness of neutrophils was seen in this study. Hypertriglyceridemia has been reported to occur in 16% of patients with CF [Figueroa 2002]. A delayed superoxide generation may not have been detected in this assay system, and therefore the effects shown by Van tits *et al* [2000] would not have been evident.

An age related decrease in free cholesterol, together with an increase in the phospholipid content of neutrophils was seen in rats [Yuli *et al* 1982]. The increased molar ratio of cholesterol/phospholipid that occurred with age that was associated with increased membrane fluidity and a reduction in the fMLP stimulated, but not the PMA stimulated superoxide generation [Yuli *et al* 1982]. Supporting this, Alverez et al [1995] showed that NADPH oxidase activity in macrophages from rats did not vary with age, however there was a decreased membrane fluidity in older animals due to an increase of the molar ratio for cholesterol/phospholipid in the membranes [Alvarez *et al* 1993] and a reduced superoxide generation in response to fMLP [Alvarez *et al* 2001]. Similarly a modest age-related decline in superoxide generation has been seen in human neutrophils [Scott *et al* 1990], no associated decline in fMLP receptor number has been shown [Lippschitz *et al* 1991] and an increase in membrane fluidity [Perskin *et al* 1992]. We did not determine the membrane cholesterol/phospolipid content of the neutrophils in our study, but this explanation would fit well with the observations in this study.

Fatty Acids

Our study shows that some of the fatty acids and neutrophil function are related. More research is needed in order to determine optimum proportion of n-3 and n-6 PUFA for efficient neutrophil

functioning. The relationship of lipid and fatty acid levels in plasma to levels in the neutrophil membrane is not known and needs to be determined.

The concentrations of fatty acids were expected to be altered in patients with CF based on other studies [Freedman *et al* 2000, Roulet *et al* 1997]. However, the changes we observed were not large and only linoleic and DHA were below the healthy reference range. The ratios of n-3 to n-6 fatty acids are probably more important when relating this to neutrophil functioning, particularly the role of AA, DHA and EPA. However we saw no relationships between the plasma AA:DHA ratio and the reponsiveness of neutrophils in this study. Since we do not have data on the ratio of AA:DHA in the neutrophil membrane we are unable to draw conclusions regarding the importance of this in neutrophil functioning.

Fatty acids are able to influence functioning of neutrophils as shown by Bellavite *et al* [1995] where palmitic and linoleic acid were inversely correlated with SO generation in response to fMLP, and AA correlated positively with SO generation. AA is also able to stimulate SO generation through a kinase-insensitive mechanism [Arita *et al* 2001]. In contrast Akamatsu *et al* [2001] showed no effect of palmitic acid on the opsonized zymosan stimulated SO generation, but did see reduction in H_2O_2 generation. The mode of stimulation of SO generation by PUFAS differed significantly depending on the number of double bonds and the carbon chain length, however there was no appreciable difference between n-3 and n-6 PUFAS in the mode of activation of SO generation by neutrophils [Arita *et al* 2001]. PUFA also alter the transcription of genes involved in inflammatory pathways [Simopoulos 1996]. Guarini *et al* [1998)] compared superoxide generation and adhesion of neutrophils from healthy volunteers with dietary fish oil or soy phosphatidylcholine supplementation. A slight increase in superoxide generation in response to fMLP was seen with the soy phosphatidylcholine. Both dietary supplements were effective at producing changes in the PUFA content of the neutrophils. Similarly Healy *et al* [2000] found no change in superoxide generation or chemotaxis after dietary fish oil. However, these studies were carried out in healthy individuals, where neutrophil functioning is likely to be optimum, and the effects of supplementation may be different when deficiency is present.

Clinical trials in subjects with CF have shown that supplementation with PUFAs results in improvements in intermediate biomarkers of the disease. Dietary changes in PUFA intake can result in changes in membrane phospholipids in CF subjects [Henderson *et al* 1994]. Kurlandsky *et al* [1994] reported that leukotriene B4 decreased, but there was no clinical improvement. Lawrence and Sorell [1994] reported that supplementation with EPA restored neutrophil LTB4 receptors to normal in cystic fibrosis patients. Christophe *et al* [1994] reported that vital capacity was increased in patients with CF supplemented with 1.5g borage oil/d (contained 330mg g-linolenic acid) compared with baseline.

This study only gives a small insight into the possible effects that lipids and fatty acids may have on neutrophil function, particularly in patients with CF. How plasma values relate to those in neutrophils requires further study. The control of neutrophil function is clearly a fine balance of factors increasing and decreasing responsiveness. This pilot study provides sufficient information to highlight the need for a larger study looking in detail at the relationship between the various lipids and fatty acids, regulation of their incorporation into neutrophils and how this affects functioning and in what aspect.

Chapter 12

Final summary and conclusions

This project was based on a series of studies relating to neutrophil function in patients with CF and chronic lung infection with *P.aeruginosa*. The objective was to explore elements of altered function which could be linked to the failure of neutrophils to eradicate bacterial infection in the lungs. This involved relating function to the possession of the gene and to secondary changes related to chronic infection and clinical status. From this we hoped to improve our understanding of how these various measurements relate *in vivo* to the lung disease in cystic fibrosis.

The key findings were as follows:

- The circulating neutrophil count was greater in patients with CF in exacerbation and when clinically stable than healthy subjects. Antibiotic treatment of an exacerbation significantly reduced the neutrophil count.
- The circulating concentration of CRP and IL-6 was greater in all patients with CF than healthy subjects and both mediators were significantly reduced by antibiotic treatment of an exacerbation. CRP and IL-6 were significantly related to each other.
- hNEAPC was raised in patients with CF in exacerbation and clinically stable patients compared to healthy subjects, and reduced significantly with antibiotic treatment. Concentrations of hNE were 5800 times greater in the sputum sol than the circulation.
- Phagocytosis and intracellular killing of *P.aeruginosa* was similar in patients and healthy subjects and was unaffected by clinical status.
- Sputum sol reduced the ability of neutrophils from patients with CF to kill *P.aeruginosa* and was more pronounced at higher concentrations of sputum sol.
- Superoxide generation and release of elastase by circulating neutrophils in response to fMLP *in vitro* was reduced in patients with an exacerbation of respiratory symptoms.
- Treatment of an exacerbation with antibiotics specific for *P.aeruginosa* restored the responsiveness of the neutrophils to fMLP, to that seen in healthy subjects.

- Superoxide generation was not reduced in response to PMA and was unaffected by the clinical status of the patients, suggesting an alteration in the fMLP receptor or signalling in the neutrophils during an exacerbation.
- There was no difference in the down-regulation of L-selectin or up-regulation of CD11b in response to fMLP, suggesting no alteration in number or function of the fMLP receptors.
- There was increased adherence to nylon columns by neutrophils from patients with CF.
- There was an increased number of band form neutrophils in patients with CF, and this was related to both the superoxide generation and elastase release in patents with CF, particularly at a time of pulmonary exacerbation.
- Superoxide generation and elastase release, in response to fMLP, was related to circulating lipids and fatty acids which have the potential to affect membrane fluidity and therefore function.

The finding that phagocytosis and intracellular killing of *P.aeruginosa* was the same in neutrophils from patients and healthy subjects in the absence of sputum sol indicates that intrinsic functioning was sufficient to prevent occurrence of infection under normal conditions. This may reflect the recognised lack of systemic spread of lung infection in the clinical setting. However, the reduced killing in the presence of sputum sol by neutrophils from patients with CF, but not healthy subjects, suggests that there may be a underlying defect in function which becomes apparent when the neutrophils are in contact with bacterial inhibiting products.

These series of studies indicate altered secretory function in neutrophils from patients with CF at the time of an exacerbation of their respiratory symptoms. Treatment of the bacterial infection with antibiotics returned neutrophil secretory function to that of healthy subjects within 14 days. There was evidence in patients in a clinically stable state of a lesser but similar impairment of neutrophil function. The key features of this impairment were a reduction in the response to fMLP in terms of superoxide generation and elastase release. Superoxide generation in particular is thought to play a key role in bacterial killing, and therefore we could expect a reduction in superoxide generation to reflect reduced bacterial killing. This did occur in the presence of sputum sol. However, the reduced superoxide generation seen at a time of exacerbation returned to the healthy subject level with

antibiotic treatment, but the reduced killing in the presence of sputum sol was independent of the clinical state of the patient.

Several studies in this project demonstrated the reproducibility of this reduced secretory function in neutrophils in response to fMLP during an exacerbation of their respiratory symptoms. This reduced effect was not seen with PMA which does not act through receptors but stimulates intracellular pathways directly. This effect also did not affect the up-regulation of CD11b and down-regulation of L-selectin in response to fMLP. This would suggest that the mechanism of reduced secretory function is due to an alteration somewhere downstream of the G-Protein stimulation or surface receptor regulation, but upstream of the intracellular site of action of PMA.

The increased adherence in patients with CF did not provide any additional information relating to the secretory defect or mechanism, but may have implications *in vivo*. There is clear evidence for a constant and excessive movement of neutrophils into the lungs resulting in the excessively high concentrations of neutrophil derived proteases such as elastase. Increased adherence even in the absence of a chemotactic signal, and in patients after treatment of an exacerbation would increase such neutrophil derived lung degradation.

The control of neutrophil function *in vivo* is clearly very complex and it is difficult to develop a complete overview view of the way in which the many control mechanisms interact when studying different aspects *in vitro*. Here we have shown that there is functional defect in superoxide generation and elastase release in patients with CF. Extrapolation of this to the reduction in killing in the presence of sputum sol provides a mechanism which may contribute to the persistent infection in the lungs of patients with CF. It remains unclear if this is related to the possession of the CF gene. Such effects may be related to the intracellular pH or to the fatty acid metabolism and distribution, which may affect membrane fluidity. The effect of chronic infection appears to have more relevance, as the functional defect was both altered by treatment of an exacerbation and was related to the number of band form of neutrophils, the number and percentage of which were higher in the exacerbation state. Evidence would also support desensitisation of the response by the continued presence of inflammatory markers at some point in the signalling pathway between that for regulation of surface receptor expression and that for release of superoxide generation.

These findings also indicate disease specific effects relating to CFTR which may enhance the maintenance of bacterial infection in the respiratory tract in CF and possibly have a role in the early acquisition of lung infection, as well as bacterial related factors capable of evading the host defence processes once infection is established.

Future Research

This study could be considered to create more questions than it answers. There are several areas where further research would increase our understanding of how alterations in neutrophil function contribute to the clinical manifestations in CF. Firstly, identification of the factor(s) in sputum sol responsible for the impairment of *P.aeruginosa* killing, and how such factors affect the neutrophil. Is this specific to CF and could it be reduced as a potential *in vivo* treatment ? Secondly more information about the relation of lipids, fatty acids and neutrophil functioning may also highlight possible *in vivo* treatments. In this case there is already interest in modulation of inflammation by dietary supplementation. Further information regarding the mechanism of the benefit of PUFA supplementation already seen in RA would not only be beneficial to CF, but also to these other inflammatory diseases.

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Appendix 1a **Materials**

ELISA plates (Immulon IV) were obtained from Dynex Technologies (Daux Road, Billinghurst, West Sussex). Dynex Technologies is now Thermo Labsystems, Acton Court, Ashford Road, Ashford, Middlesex, TW15 1XB.

Neutrophil elastase standard was obtained from Calbiochem-Novabiochem (Beeston, Nottingham).

CRP standard was obtained from Protein Reference Unit (Sheffield).

Mouse anti-human neutrophil elastase (MO752, NP57), anti-human alpha 1 antitrypsin (A0012), anti-human MPO FITC conjugate (F0714), Flurospheres (K0110), anti-human CD11b PE conjugate (R0841) and anti-mouse IgG FITC conjugate (F0479) were obtained from Dako (16, Manor Courtyard, Hugenden Avenue, High Wycome, Bucks).

Sheep anti-human elastase (AHP051) and Goat anti-human α_1-anti trypsin peroxidase conjugate were obtained from Serotec (22, Bankside, Station approach, Kiddlington, Oxford).

HBSS -phenol red (x10), HBSS -Ca -Mg -phenol red (x10), BSA 7.5% solution, HEPES (1M), Bicarbonate solution was obtained sterile from Life Technologies (3, Fountain Drive, Inchinnan Business Park, Paisley, Scotland).

Leishman's Staining solution was obtained from Merk (Hunter Boulevard, Magna Park, Lutterworth).

Anti-goat IgG-alkaline phosphatase conjugate (A4062), Sigma-104 phosphatase substrate, Tetra-methylbenzidine (T8768), BSA (A7284), Alpha 1 antitrypsin (A6150), Diethanolamine (D2286), fMLP (F3506), cytochalasin B (C6762), cytochrome C (C2506), Sodium Diatrizoate (S4506) and cellular microspheres (C1557) were obtained from Sigma (Fancy Road, Poole, Dorset).

Ficoll-Paque was obtained from Pharmacia Biotech (23, Grosvenor Road, St Albans, Herts)

Buffer salts (AR) were obtained from Fisher Scientific (Bishop Meadow Road, Loughborough, Leicestershire).

IL-6 and TNFα high sensitivity ELISA kits, and sL-selectin ELISA kit were obtained from R&D Systems Europe (4-10 The quadrant, Barton Lane, Abingdon). Anti-human L-selectin FITC conjugate (BBA33), Anti-human IL-8 RA FITC conjugate (FAB330F) and anti-human IL-8 RB PE conjugate (FAB331P) were also obtained from R&D systems Europe.

Caltag Fix & Perm (GAS-004) and anti-human TNF receptor p75 (CD120b) were obtained from Bradsure Biologicals (67A Brook Street, Shepshed, Loughborough, Leistershire LE12 9RF).

FACS Brand Lysing solution (349202) was obtained from Becton Dickinson (Between Towns Road, Cowley, Oxford, OX4 3LY).

Anti-human IL-6 receptor (BMS135) was obtained from Bio Whittaker (Bio Whittaker House, 1 Ashville Way, Wokingham, Berkshire RG412PL).

Nutrient agar plates were made by dissolving 28g of nutrient agar in 1l, boiled with steady stirring, then autoclaved. This was allowed to partly cool, then poured onto petri dishes (quantities sufficient for 30 plates). 24 hours were needed for the plates to set and dry sufficient for use, and could be kept for up to 2 weeks.

Appendix 1b **Equipment**

Bio Rad Microtitre plate reader 3550 and specific PC based software (Microman) (Bio Rad Laboratories Ltd., Bio Rad House, Maylands Avenue, Hemel Hempstead, Herts. HP2 7TD).

FACScan, Beckman spctrophometer DU 640 and Beckman centrifuge GS-15R (Beckman Coulter UK Ltd., Kingsmead Business Park, London Road, High Wycombe, Bucks. HP111JU).

Titertek Plate Shaker (Flow Laboratories).

Plate Washer (Dynex Technologies, Daux Road, Billinghurst, West Sussex).

Cytospin 3 (Shandon Scientific)

Appendix 1c **ELISA Buffer contents**

Bicarbonate coating buffer (BCB) (pH to 9.6)

Na_2CO_3	0.015 M	1.59g/l
$NaHCO_3$	0.0349 M	2.93g/l

Phosphate buffered saline (PBS) (pH to 7.4)

NaCl	0.137 M	8g/l
Na_2HPO_4	0.00809 M	1.149g/l
$NaHPO_4$	0.00158 M	0.247g/l
NaN_3	0.1496 M	0.2g/l

Wash buffer

PBS		
Tween 20	0.1%	1ml/l
BSA	0.1%	

Appendix 2a **Protein Assay protocol**

Prepare Standard Tubes by diluting the Protein Standard Solution with water to a volume of 1.0ml in appropriately labelled test tubes.

Label a test tube Bland and add 1.0 ml water.

Add sample to appropriately labelled test tube and dilute to 1.0ml with water.

Add 1.0ml Lowry Reagent Solution to Standard, Blank and Sample tubes. Mix Well

Allow solutions to stand at room temperature for 20 minutes.

With rapid and immediate mixing, add 0.5ml Folin & Ciocalteu's Phenol Reagent Working Solution to each tube.

Allow colour to develop for 30 minutes.

Transfer solutions to curvets and measure the absorbance of the Standards and Sample tubes vs. the Blank at a wavelength between 500 and 800 nm. Complete readings within 30 minutes.

Plot the absorbance values of the Standards vs. their corresponding protein concentrations to prepare a calibration curve.

Determine the protein concentration of the Sample tube from the calibration curve. Multiply the result by the appropriate factor to obtain the protein concentration in the original sample.

Appendix 3a **IL-6 ELISA kit Protocol**

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Prepare all reagents and working standards as directed in the previous sections.

Remove excess microtiter plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

Add 50µl of Assay Diluent HD1D to each well.

Add 200µl of standard or sample per well. Cover with the adhesive strip provided. For shaker protocol: Incubate for 2 hours at room temperature on a horizontal orbital microtiter plate shaker (0.12" orbit) set at 500 ± 50 rpm. For Benchtop protocol: Incubate for 14-20 hours at room temperature on the benchtop.

Wash 4 times

Add 200µl of IL-6 Conjugate to each well. Cover with a new adhesive strip. For shaker protocol: Incubate for 2 hours at room temperature the shaker. For Benchtop protocol: Incubate for 6 hours at room temperature on the benchtop.

Wash 4 times

Add 50µl of Substrate solution to each well. Cover with a new adhesive strip. For shaker protocol: Incubate for 60 minutes at room temperature on the shaker. Do not wash the plate. For benchtop protocol: Incubate for 60 minutes at room temperature. Do not wash plate.

Add 50µl of Amplifier Solution to each well. Cover with a new adhesive strip. For shaker protocol: Incubate for 45 minutes at room temperature on the shaker. Do not wash the plate. For benchtop protocol: Incubate for 30 minutes at room temperature. Do not wash plate.

Add 50µl of Stop solution to each well. Addition of Stop Solution does not affect colour in the wells.

Determine the optical density of each well within 30 minutes, using a microtiter plate reader set to 490nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

The booklet also includes how to make up the standards (what volumes) and how to calculate the results. The wash step is described in great detail.

Appendix 3b **TNFα ELISA kit Protocol**

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Prepare all reagents and working standards as directed in the previous sections.

Open the resealable foil pouch containing the microtiter plate. Remove any excess microtiter plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

Add 50µl of Assay Diluent to each well.

Add 200µl of standard or sample per well. Cover with the provided adhesive strip. For shaker protocol: Incubate for 3 hours at room temperature on a horizontal orbital microtiter plate shaker (0.12" orbit) set at 500 ± 50 rpm. For Benchtop protocol: Incubate for 14-20 hours at 2-8°C.

Wash 4 times

Add 200µl of TNFα Conjugate to each well. Cover with a new adhesive strip. For shaker protocol: Incubate for 2 hours at room temperature the shaker. For Benchtop protocol: Incubate for 3 hours at room temperature on the benchtop.

Wash 4 times

Add 50µl of Substrate solution to each well. Cover with a new adhesive strip. For shaker protocol: Incubate for 45 minutes at room temperature on the shaker. Do not wash the plate. For benchtop protocol: Incubate for 60 minutes at room temperature. Do not wash plate.

Add 50µl of Amplifier Solution to each well. Cover with a new adhesive strip. For shaker protocol: Incubate for 45 minutes at room temperature on the shaker. Do not wash the plate. For benchtop protocol: Incubate for 30 minutes at room temperature. Do not wash plate.

Add 50µl of Stop solution to each well. Addition of Stop Solution does not affect colour in the wells.

Determine the optical density of each well within 30 minutes, using a microtiter plate reader set to 490nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction

will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

The booklet also includes how to make up the standards (what volumes) and how to calculate the results. The wash step is described in great detail.

Appendix 3c sL-selectin ELISA kit Protocol

Bring all reagents and samples to room temperature before use. It is recommended that all samples, Standards and Parameter Control serum be assayed in duplicate.

Dilute all samples at least 1 in 100 with Sample Diluent. For example, 10µl sample + 990 µl Sample Diluent.

For most samples (serum or plasma) a dilution of 1 in 100 should be adequate (see Technical Hints). For cell culture supernate samples a dilution of 1 in 25 or 1 in 50 may be used.

Parameter Control Serum must be diluted after reconstitution 1:100 with Sample Diluent, prior to assay (see step 1).

Remove excess microtiter plate strips from the frame and store in the resealed pouch with the silica gel sachet.

Add 100µl Standard, diluted sample or diluted Parameter control to each well in duplicate. A plate template is included on the back of the Data Card to record plate layout of Standards, samples and Control Serum.

Cover the plate with a plate sealer provided and incubate at room temperature for 1 hour.

Add 100ml Anti-L-selectin-HRP Conjugate to each well with sufficient force to ensure mixing. Conjugate is red coloured to facilitate correct addition.

Cover the plate with a new plate sealer provided and incubate at room temperature for 30 minutes.

Aspirate or decant contents from each well and wash by adding 400µl of Wash Buffer per well. Repeat the process 5 times for a total of 6 washes. After the last wash, aspirate or decant the contents and remove any remaining Wash Buffer by tapping the inverted plate firmly on clean paper towelling.

Add 100µl Substrate to each well. Cover the plate with a new plate sealer provided and incubate at room temperature for 30 minutes.

Add 100µl of Stop Solution to each well. The Stop Solution should be added to the wells in the same order as the Substrate.

Determine the optical density (OD) of each well within 30 minutes using a microtiter plate reader or photometer set at 450nm with a correction wavelength of 620nm. If the wavelength correction facility is not available read plates at 450nm and then separately at 620 nm. Subtract the OD_{620} from the OD_{450}.

A Section detailing calculating of results follows.

Appendix 3d **G-CSF ELISA kit Protocol**

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Remove excess microtiter plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

Add the appropriate Assay Diluent to each well. For serum/plasma samples: add 100µl of Assay Diluent HD1-3

Add the appropriate amount of standard or sample to each well. For serum/plasma samples: add 100µl of standard or sample per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature.

Wash

Add 200µl of G-CSF Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

Wash

Add 50µl of Substrate solution to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.

Add 50µl of Amplifier Solution to each well. Cover with a new adhesive strip. Incubate for 30 minutes at room temperature.

Add 50µl of Stop solution to each well. Addition of Stop Solution does not affect colour in the wells.

Determine the optical density of each well within 30 minutes, using a microtiter plate reader set to 490nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

The booklet also includes how to make up the standards (what volumes) and how to calculate the results.

Appendix 3e **GM-CSF ELISA kit Protocol**

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Prepare all reagents and working standards as directed in the previous sections.

Remove excess microtiter plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

Add Assay Diluent to each well. For serum/plasma samples: add 100µl of Assay Diluent HD1-3

Add the appropriate amount of standard or sample per well. For serum/plasma samples: add 150µl of standard or sample per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature.

Wash 4 times

Add 200µl of GM-CSF Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

Wash 4 times

Add 50µl of Substrate solution to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker. Do not wash the plate.

Add 50µl of Amplifier Solution to each well. Cover with a new adhesive strip. Incubate for 30 minutes at room temperature.

Add 50µl of Stop solution to each well. Addition of Stop Solution does not affect colour in the wells.

Determine the optical density of each well within 30 minutes, using a microtiter plate reader set to 490nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is

not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

The booklet also includes how to make up the standards (what volumes) and how to calculate the results. The wash step is described in great detail.

Appendix 4 **Superoxide generation calculation**

Calculation of superoxide generation by neutrophils from the reduction of cytochrome C

This was used to convert the change in optical density at λ550nm to the nmol cytochrome C reduced. This concentration was expressed per million neutrophils for the 20 min, incubation period.

Reduced cytochrome C has a millimolar extinction coefficient of 29.5 (horse heart).

O.D. = k x c x l

O.D.	change in optical density at 550 nm	
k	extinction coefficient	29.5 $mM^{-1}cm^{-1}$
c	concentration of cytochrome C	2mM
l	light path	0.8cm

Results to be expressed as nmol Cytochrome C reduced per million neutrophils.

50µl HBSS without cytochrome C was mixed with 450µl of HBSS with cytochrome C at 2mM, therefore final concentration was 1.8mM.

e.g.; *Patient 1 'start'*

Blank = *0.284*

10-7 fMLP = *0.651* *Difference 0.369*

$0.369 = 29.5 \times c \times 0.8$

$c = \frac{0.369}{29.5 \times 0.8}$

$c = 0.0156nmol$

Cell count was 0.0177, therefore $\frac{0.0156}{0.2}$

17.7 nmol cytochrome C reduced / million neutrophils in 20 min.

Appendix 5.1 Variability in phagocytosis experiments

Inter assay variation:

The mean CV for each triplicate of the first 6 control experiments was;

	Patient	Control
Neutrophils present	14	17
neutrophils absent	16	7.4

Intra assay variation:

The corresponding % phagocytosis;	61	62
CV	27	17

Table

Variability in intracellular killing Experiments

Inter assay variation:

The mean CV for each triplicate of the first 6 control experiments was;

12 8 31 20 1 31 = 17 %

and for triplicates in the absence of neutrophils;

43 9 5 5 11 10 = 14 %

Intra assay variation:

The corresponding % killing;

70 78 91 74 82 89 = mean 81% CV 3%

Appendix 5.2

The number of colonies (colony forming units) on each plate was counted, and the mean for the three determinations taken.

e.g. NVP02C (No sputum)

Colony counts of 122, 137, 139 (neutrophils present) Mean = 133

477, 452 (absence of neutrophils) Mean = 465

The percentage *P.aeruginosa* was determined from these values

e.g. 133 / 465 = 0.289

Therefore 29 % of the *P.aeruginosa* remain.

Hence 71% were phagocytosed by the neutrophils

When the percentage phagocytosis had been calculated this varied from subject to subject, and sputum-sol only produced small variations. To make the results clearer, the percentage phagocytosis in the presence of sputum-sol was subtracted from the percentage phagocytosis in the absence of sputum-sol. These are the values quoted in Table 4.2.

e.g. % phagocytosis in the absence of sputum sol = 70%

% Phagocytosis in the presence of 1:10 sputum sol = 67%

Appendix 6 **Clinical data collection sheets for study group A**

Patient	Age at 1st study	FEV1 when stable	A Exacerbation	A Clinically Stable	B PMA	C FACS	D Adherence	E Metablolism
1	24	15	✓					
2	16	46	✓					
3	28	90	✓		✓	✓		
4	24	19	✓					
5	18	49	✓				✓	✓
6	31	94	✓			✓		
7	19	37	✓				✓	
8	24	36	✓	✓			✓	
9	27	107	✓	✓				
10	19	108	✓		✓			
11	22	67	✓					
12	25	59	✓		✓	✓		
13	28	37		✓				
14	29	47		✓			✓	
15	16	59		✓				
16	34	87		✓				
17	19	57		✓				
18	22	25		✓				
19	26	27		✓				
20	22	52		✓				
21	30	15		✓				
22	18	35		✓			✓	
23					✓		✓	
24	24	10			✓	✓		
25	27	17			✓			
26	19	26			✓			
27	23	62			✓			
28	17	76			✓			✓
29	19	43			✓			✓
30	24	8			✓			
31	24	39			✓		✓	

32	27	76			✓	✓		✓
33	18	45			✓			✓
34					✓		✓	
35					✓		✓	✓
36	25					✓		
37	24					✓	✓	
38	25					✓		
39								✓
40								✓
41								✓
42								✓
43							✓	✓
44							✓	✓
45								✓
46								✓
47								✓
48							✓	
49							✓	
50							✓	
51							✓	
52							✓	
53							✓	
54							✓	
55							✓	
56							✓	
57							✓	
58							✓	
59							✓	