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Bronchial Inflammation in Alpha-1-Antitrypsin Deficiency

A thesis submitted by

Adam T. Hill, MB ChB with commendation, MRCP

for the degree of Doctor of Medicine

to

**The Faculty of Medicine
University of Glasgow**

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**To my wife Lucy
and my boys Graham and Alasdair**



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SUMMARY

Chronic bronchitis was first recognised as a disabling disorder in 1808. It is often a feature of chronic obstructive pulmonary disease, and recent studies have shown that the neutrophil and in particular neutrophil elastase, released by activated neutrophils, is implicated in the pathogenesis of chronic bronchitis including the facilitation of bacterial colonisation. The degree of neutrophil influx has been shown to be associated, not only with worse lung function, but also the rate of progression of airflow obstruction. Many patients with chronic bronchitis are colonised with bacteria in the stable clinical state but it is not known how this affects upper airways inflammation, disease progression, frequency of exacerbations, or quality of life.

Secretory leukoprotease inhibitor is thought to be the most critical anti-elastase in the upper airways whereas alpha-1-antitrypsin is thought to be less important, although more important at the alveolar level protecting against the development of emphysema. Patients with homozygous PiZ alpha-1-antitrypsin deficiency have decreased circulating (15-20% normal) and alveolar concentrations of alpha-1-antitrypsin which facilitate the development of early onset and rapidly progressive emphysema. About 30-40 % of these patients also have chronic bronchitis although the nature of the upper airways inflammation has not been studied.

There have been few studies assessing the complex interplay of inflammatory cells and appropriate mediators in patients with chronic bronchitis. The first study in this thesis investigated patients with chronic bronchitis and a wide spectrum of neutrophil influx to

assess the relationships between: neutrophil influx (as reflected by sputum myeloperoxidase concentration) and the chemoattractants interleukin 8 and leukotriene B4; active neutrophil elastase and the chemoattractants (interleukin 8 and leukotriene B4), its own inhibitor secretory leukoprotease inhibitor, and bronchial protein leak (leakage of albumin from serum into the airways); and finally FEV₁ (% predicted) and myeloperoxidase, interleukin 8 and leukotriene B4, secretory leukoprotease inhibitor, and bronchial protein leak.

The results showed that both interleukin 8 and leukotriene B4 correlate with the degree of neutrophil influx and elastase activity, although the relationship with interleukin 8 and neutrophil influx appeared to be curvilinear. Elastase activity that exceeded 50nM was associated with decreased levels of secretory leukoprotease inhibitor and increased levels of bronchial protein leak. Patients with chronic bronchitis without alpha-1-antitrypsin deficiency showed a negative correlation between FEV₁ (% predicted) and myeloperoxidase, interleukin 8 and leukotriene B4, and bronchial protein leak. Although the interrelationships were often significant, they were complex, and further understanding the interaction of various mediators will require the development of specific antagonists and appropriately designed intervention studies.

The second study investigated the effect of bacterial colonisation, bacterial load, and bacteria themselves on upper airways inflammation in patients with chronic bronchitis. This study highlighted that bacterial colonisation of sputum exceeding 10⁵ colony forming units/ml was associated with neutrophil influx in the upper airways, which increased with increasing bacterial load. Patients with a bacterial load of less than 10⁶

colony forming units/ml had a similar degree of upper airways inflammation to samples where no pathogen or only mixed normal flora was isolated. Finally the study revealed that patients colonised with *Pseudomonas aeruginosa* had a more intense upper airways inflammatory response than patients colonised with *Moraxella catarrhalis* or non-typeable *Haemophilus influenzae*.

The third study assessed upper airways inflammation in patients with chronic bronchitis with and without PiZ alpha-1-antitrypsin deficiency in the stable clinical state, to determine the importance of alpha-1-antitrypsin in the upper airways and the effects of continued smoking. Patients with alpha-1-antitrypsin deficiency had excessive upper airways inflammation despite being younger, including a greater proportion of ex-smokers and more being on inhaled steroids (all of which would tend to limit the degree of inflammation). The results highlight the importance of alpha-1-antitrypsin in the upper airways. Ex smokers had lower levels of interleukin 8 compared to current smokers and was associated with less neutrophil influx (as determined by myeloperoxidase). The results suggest that the reduction in interleukin 8 levels was responsible for the reduced neutrophil influx and this mechanism may explain the slower rate of progression of lung disease compared to continuing smokers.

The final study assessed upper airways inflammation in patients with and without alpha-1-antitrypsin deficiency during an acute exacerbation. This study revealed that there was excessive upper airways inflammation in patients with alpha-1-antitrypsin deficiency which was probably due to the low baseline alpha-1-antitrypsin levels and the reduced acute phase response.

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The work reported in this thesis was undertaken while I was a clinical research fellow in the Department of Medicine, University of Birmingham. I carried out the experiments described, analysed the data, and prepared the publications which have arisen from them. None of the work presented here has been submitted in any previous application for a higher degree.

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1. INTRODUCTION

1.1 THE NORMAL BRONCHIAL TREE

The airways have important functions beyond gas conduction. This includes the warming and humidification of inhaled air, and acts as the primary host defence system cleansing inhaled air of potentially harmful dust particles and micro-organisms. The primary host defence system is relatively non-specific and includes:

1. nervous reflexes that cause bronchoconstriction and cough.
2. integrity of the bronchial epithelium and the efficiency of the mucociliary escalator, that prevent bacterial adherence, penetration, and facilitate their removal.
3. lung secretions proteins including lysozyme and lactoferrin that prevent bacterial replication (Jacquot et al, 1987)(Ellison et al, 1991), as well as Immunoglobulin A (IgA) that prevent epithelial adherence of bacteria and viruses (Stockley, 1997).
4. cellular immune reactions to remove allergenic particles from the airways.
5. airway macrophages, the resident phagocytes that facilitate bacterial and particulate removal (Stockley, 1995).

The normal bronchial tree is kept sterile and animal experiments have shown that these defences are able to clear bacterial loads of up to 2×10^5 colony forming units per ml (cfu/ml) (Onofrio et al, 1983) with little or no activation of the secondary host defences.

1.1.1 Development of the airways

On the 26th day after ovulation, the lung first appears as an epithelial bud (Boyden, 1977), which is derived from endoderm, and will form the epithelium of the airways and the acini. As it elongates, it becomes invested in mesenchyme derived from mesoderm (Thurlbeck, 1981) which develops into the connective tissue, cartilage, smooth muscle and vessels of the lung. In the first few weeks of development, nerve fibres arising from ectoderm migrate into the mesenchyme to give the lung its motor and sensory connections (Longman, 1975). The developing lung bud divides into 2 halves and elongates, growing caudally on either side of the oesophagus. By about the 33rd day the trachea has become separated from the foregut, and pouches representing the 5 lobes are apparent. Subsequent dichotomous division leads to the development of the full adult complement of segments by the 41st day and to completion of the bronchial tree as far as the terminal bronchioles by the 16th week (Bucher et al, 1961).

1.1.2 Secretion of the airways

The bulk of the bronchial secretions is secreted by the serous and mucous cells of the bronchial glands, with important contributions from the goblet cells as well as from the serous and clara cells of the airway epithelium. Even in the normal airways, however, the bronchial secretions are contaminated by surfactant from alveoli and by fluid transudate from plasma. Sputum is a pathological secretion and consists of a mixture of bronchial secretions, cells and cellular debris, cleared organisms and saliva that is spontaneously expectorated.

1.1.2.1 Airway mucus

The functions of bronchial mucus include: waterproofing, diminishing water loss from the respiratory tract; protecting the bronchial epithelium by forming a barrier between it and particles in the inhaled air; and defence both by removing inhaled particles as a result of ciliary activity, and by acting as a vehicle for immunoglobulins and other protective proteins.

Mucus is present over the bronchial epithelium as a continuous sol layer (or periciliary liquid), in which cilia beat, and a gel layer (on the surface of the cilia) (Richardson et al, 1992) which is not continuous at the bronchiolar level, but forms almost a complete covering of the epithelium in the larger airways.

The depth of the sol layer is about 5-10 μm , which is just sufficient for the cilia during their effective stroke enabling them to propel the gel layer over the surface with their tips. The thickness of the gel phase is uncertain but values are between 2 and 20 μm . The gel is moved by mucociliary transport, being faster in the larger airways (human trachea is about 10mm min^{-1} with that in the bronchioles about 10 times slower). It is thought that the sol layer is probably derived largely from the clara cells at the bronchiolar level in the airway epithelium with some contribution from a fluid transudate (serum proteins including albumin, fibrinogen, alpha-1-antitrypsin, and immunoglobulins "leak" from the blood vessels in the mucosa due to transudation during airway inflammation (Persson, 1992)). The mucus gel phase of secretion is derived from several sources including goblet cells and serous cells in the airway

epithelium (Kim, 1994), clara cells at the bronchiolar level (Widdicombe et al, 1982), and the submucosal glands (Richardson et al, 1992)(Shimura et al, 1994).

There are many proteins identified in sputum sol phase (some of which include albumin, lysozyme, lactoferrin, immunoglobulins, alpha-1-antitrypsin, alpha-1-chymotrypsin, and secretory leukoprotease inhibitor (Stockley, 1997)). The gel phase consists of about 95% water, 1% salt, 1-3% proteins and mucoglycoproteins, and 1-3% proteoglycans and lipids (Boat et al, 1994).

The physical properties of mucus are provided mainly by mucins, which are high molecular mass (up to 15 MDa) mucoglycoproteins (King et al, 1994), although proteoglycans of lower molecular mass also contribute to viscosity. Phospholipids may be important in weakening adhesion of the mucus to the epithelium (Rubin et al, 1992)(Puchelle et al, 1992).

Immunoglobulin A is an important component of bronchial mucus and predominates in the upper respiratory tract (Morgan et al, 1980), with Immunoglobulin G (IgG) and M contributing little (Burnett, 1986). In the lung most of the IgA is dimeric (linked by a peptide called the J chain), 70% is of the IgA1 subclass and the remainder IgA2 subclass. The dimeric form can prevent the epithelial adherence of bacteria and viruses but in addition other studies have shown that secretory IgA can interact with other arms of the immune system (enhancing macrophage phagocytosis (Richards et al, 1985) and facilitating antibody dependent cell mediated cytotoxicity in synergism with IgG (Shen

et al, 1981)). In addition it also plays a role in increasing the strength and stability of the mucus itself.

The immunoglobulins are relatively large molecules and transudation from plasma is therefore limited. Thus most of the immunoglobulins detected in lung secretions represent those made locally (secreted originally by B lymphocytes and plasma cells around the bronchial glands (Soutar, 1976)), facilitated by a special transport system for the passage of dimeric IgA into the lumen of the lung. The secretory component (N terminal sequence of a transmembrane Fc_{α} receptor) (Mostov et al, 1980) is expressed on the basal surface of bronchial epithelial cells and strongly binds dimeric IgA (to form an irreversible complex) and some unbound receptor. The complex is internalised by endocytosis, sorted in an endosomal compartment and incorporated into transcytotic vesicles. These vesicles translocate to the apical surface (Breitfeld et al, 1989), fuse with the membrane and the amino terminus, with the IgA attached, is cleaved (Musil et al, 1987), and the secretory IgA molecule or free secretory component (both identified in lung secretions) is released (Stockley et al, 1981). The remainder of the polymeric IgA receptor (transmembrane segment and cytosol tail) is then transported to lysosomes for degradation (Stockley et al, 1981).

Other proteins such as lysozyme and lactoferrin participate in airway bacterial defence (Jacquot et al, 1987)(Ellison et al, 1991), whereas molecules such as catalase and glutathione that are detected in the lower respiratory tract may play a role in the oxidant and anti-inflammatory properties of airways secretions (Cantin et al, 1990). The anti-

protease secretory leukoprotease inhibitor is produced locally in the lung by epithelial cells (Maruyama et al, 1994), and is present in serous glands (De Water et al, 1986)(Mooren et al, 1983) and clara cells (Sallenave et al, 1993). It acts as the main bronchial inhibitor against neutrophil elastase (Kramps et al, 1988), but in addition has broad spectrum antibiotic activity that includes anti-retroviral, bactericidal, and antifungal activity (Tomee et al, 1998).

1.1.2.2 Mucus and bacteria

The mucus gel acts as a barrier for bacteria, which adhere to it and can then subsequently multiply within it (Puchelle et al, 1992). Mucins have chemical receptors that can bind to the adhesins on many bacteria but other mucus receptors that may be active in binding to bacterial adhesins include glycolipids, which are found in airway secretions. The adhesins on common respiratory pathogens include the pilin proteins in fimbriae, mucoid exopolysaccharide, haemagglutinins, internal lectins, exoenzyme S and non pilus protein components (Widdicombe et al, 1990)(Girod et al, 1992). The respiratory pathogens that bind strongly to mucus include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Colonised bacteria may also promote further mucus secretion (Adler et al, 1986)(Somerville et al, 1992). This increase has been shown in response to several bacterial species *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, and the active secretory agents have been established as proteases and rhamnolipids (Adler et al, 1986)(Somerville et al, 1992). This process could be advantageous to the host if it promotes greater clearance of

mucus from the airway by cough (although this could also lead to bacterial spread) but a disadvantage of the increased mucus secretion would be blockage of the smaller airways and a further nidus for bacterial multiplication.

1.1.3 Mucociliary clearance

1.1.3.1 Cilia

The ciliated cell predominates in the tracheo-bronchial epithelium, although reduced numbers are present at the bronchiolar level (Shimura et al, 1994)(Widdicombe et al, 1995). There are approximately 200-300 cilia per cell, each is 4-6 μm long and about 0.1-0.2 μm in diameter, and beats at 1000 beats per minute with co-ordination between adjacent cells (Sleigh et al, 1988). Microvilli project between the cilia and are thought to play a role in fluid absorption and therefore control the depth of the periciliary fluid layer within which the cilia beat.

The tips of the cilia end in small claws that engage the overlying mucus gel, enabling it to be transported (Jeffery et al, 1975). In the cross section of the cilium there are 9 outer microtubular doublets and two central single microtubules linked by dynein arms (consist of an ATPase protein), nexin links, and spokes (Figure 1-1). The bending of the cilia depends on the active sliding of the peripheral microtubules relative to each other, based on the dynein bridges. ATP is the source of energy for this process and dynein acts as an ATPase protein.

In man the ciliary beat frequency appears uniform in the large and small airways (15-18Hz). The ciliary beat cycle has 2 components: movement towards the larynx which is the effective stroke followed by a recovery stroke in the opposite direction when the cilia disengage from the mucus and bend as they recover (Sleigh et al, 1988). The action of the cilia both in a single cell and between adjacent cells is co-ordinated in health (Sanderson et al, 1981).

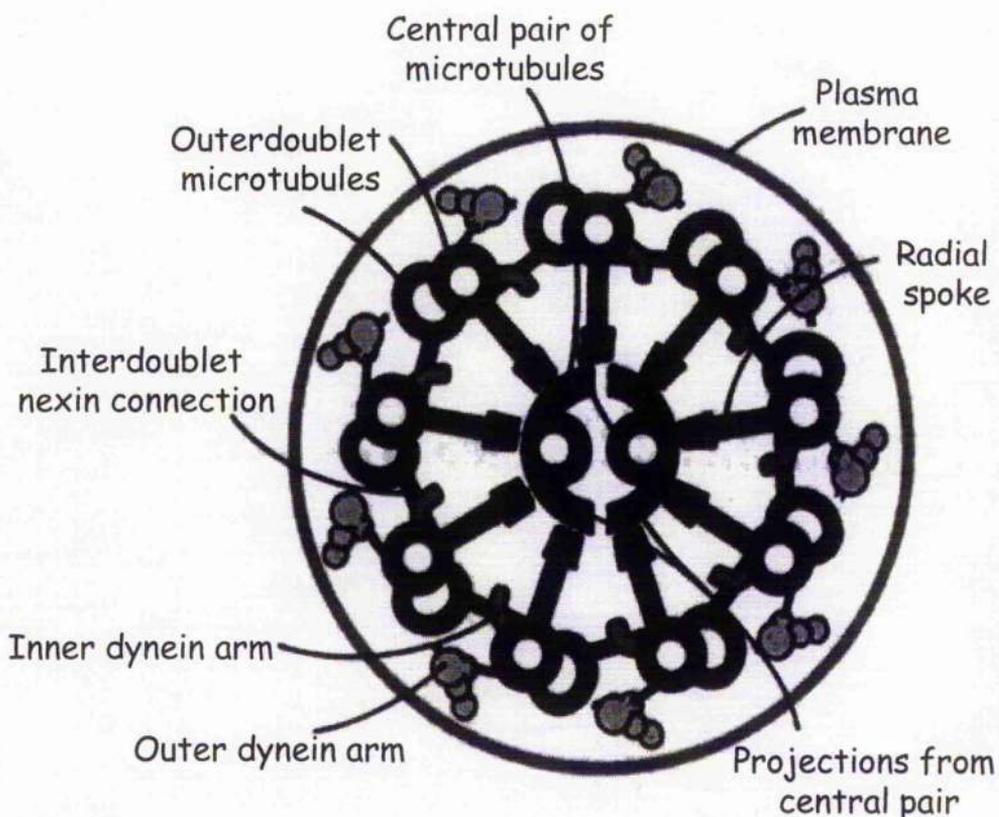


Figure 1-1: Diagrammatic cross section of a cilium.

The intracellular control of ciliary action depends on ATP, cAMP, cGMP, and protein kinase C, in addition to ionic calcium (Wanner, 1994). All increase ciliary beat frequency except for protein Kinase C which decreases it. Baseline ciliary activity does not seem to be under nervous control, but acetylcholine and beta₂ adrenoceptor agonists increase ciliary beat frequency. Vasoactive intestinal polypeptide stimulates ciliary movement and may coact with acetylcholine as both are released from parasympathetic nerves. The sensory neuropeptides, for example substance P, stimulates ciliary activity,

so this action may be a feature of neurogenic inflammation when the sensory nerves are excited.

Most inflammatory mediators affect ciliary activity. It is increased by prostaglandins E1 and E2, leukotrienes C4 and D4, and bradykinin whereas platelet activating factor is said to inhibit ciliary beating (Wanner, 1994).

Some of the proteases, including neutrophil elastase and the elastases from *Pseudomonas aeruginosa* inhibit cilia (Widdicombe et al, 1990)(Wilson et al, 1987). Other bacterial products such as rhamnolipid and pyocyanin (Wilson et al, 1987) also inhibit cilia as do oxygen radicals. Some of these effects are due to activation of protein kinase C.

1.1.3.2 Mucociliary transport

The clearance of mucus with any entrapped material depends, in part, only on ciliary activity (Hasani et al, 1989)(Puchelle et al, 1980)(Yeates et al, 1975)(Pavia, 1984). Other factors include cough, amount of mucus present, viscoelasticity of the mucus, and adhesiveness to the airway epithelium. Mucociliary transport (Hasani et al, 1989)(Pavia, 1984) has 2 phases, a fast phase taking a few hours in healthy people due to clearance by cilia and cough and a slower phase with a half life of weeks or months that represents alveolar clearance.

Inflammatory mediators may have actions that are different for ciliary beat frequency and mucociliary transport (Wanner, 1994). For example histamine increases mucociliary transport in man, but has little action on the ciliary beat.

In airway diseases, structural damage to the epithelium and secretion of mucus with increased adhesiveness may be more important factors in determining mucociliary transport than changes in ciliary beat frequency (Puchelle et al, 1992)(Girod et al, 1992) (mucociliary transport is impaired in chronic bronchitis when the ciliary beat of individual epithelial cells may be normal).

1.1.4 The airway wall

This comprises epithelial, lymphoid, muscular, vascular, and nervous elements interspersed in a connective tissue support arranged as a) a lining mucosa of surface epithelium, basement membrane, and supporting elastic lamina propria; b) the submucosa in which lie the glands, muscle and cartilage; c) adventitial coat.

If the layer of the mucus overlying the epithelium is inadequate to protect the mucosa, the airway epithelium presents the next important barrier (Laitinen et al, 1991)(Munakata et al, 1991). Smooth muscle becomes 3-5 times more responsive to contractile drugs such as luminal acetylcholine, histamine and 5 hydroxytryptamine (Munakata et al, 1991)(Barnes, 1991) following removal of the epithelium. Experiments with B₂ adrenoceptor agonists are far more effective when applied to the serosal side of the airway wall than when presented at the mucosal side (Munakata et al, 1991). This difference in sensitivity disappears when the epithelium is removed suggesting the epithelium is acting as a barrier. Measurement of the permeability of radiolabelled tracers through the epithelium or the airway mucosa supports the view that the epithelium can be a major barrier (Hanafi et al, 1994)(Wells et al, 1995) especially for hydrophilic molecules. In isolated ferret trachea, epithelial damage by platelet activating factor increases the permeability coefficient about 8 fold (Hanafi et al, 1994). In vivo where penetration has only to be through the epithelium to reach the copious vascular network, the coefficient of permeability to DTPA is increased 50 fold (Morikawa et al, 1991) by epithelial destruction. With lipophilic agents, on the other hand, destruction of the epithelium makes no difference to the permeability (Hanafi et al, 1994). The epithelium is also a strong barrier to macromolecules and, in healthy airways,

horseradish peroxidase penetrates the epithelium only up to the tight junctions between cells (Hogg, 1990). If tight junctions are opened by the effect of histamine or cigarette smoke for example, the peroxidase penetrates through the epithelium to the basement membrane (Hogg, 1990)(Hulbert et al, 1981).

1.1.4.1 Bacterial-epithelial interaction

Many bacteria adhere more to mucus than to undisrupted epithelium provided the latter is healthy (Puchelle et al, 1992)(Widdicombe et al, 1990)(Girod et al, 1992). Most respiratory pathogens, apart from *Mycoplasma pneumoniae* and *Bordatella pertussis* will not adhere to epithelium until it has been damaged by toxins or proteases (Tuomanen et al, 1983)(Bredt et al, 1982). Airway pathogens such as Non typeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* adhere, however, powerfully to damaged cells, both ciliated and non ciliated (Widdicombe et al, 1990)(Plotkowski et al, 1989)(Feldman et al, 1992). Bacteria use a wide range of adherence mechanisms, examples include lectin like substances which are found embedded in the outer membrane, the secretion of exopolysaccharide, or the expression of hair like outgrowths called fimbriae (Wilson et al, 1997).

1.1.4.2 Surface epithelium

This includes the surface lining of the airway epithelium and submucosal glands. The epithelium consists of pseudostratified, ciliated, columnar cells. There are many different epithelial cell types including the ciliated cell (described above), goblet cell, serous cell, clara cell, endocrine, basal and brush cells, and finally transitional forms (Jeffery et al, 1975)(Breeze et al, 1977)(Jeffery, 1983). There are also cells involved in

the immune response that may migrate through the epithelial basement membrane, some of which remain within the surface epithelium whereas some pass into the airway lumen (McDermott et al, 1982). The terminal processes of nerve fibres whose cell bodies lie deep to the epithelium, also pierce the epithelial basement membrane and are thought to initiate airway reflexes such as cough and bronchoconstriction (Richardson et al, 1981).

1.1.4.3 Migratory cells

In health, neutrophils are rarely found in the airways but are rapidly recruited in inflammatory states. In the normal healthy epithelium the following mononuclear migrating cells are found: a) intraepithelial lymphocytes and b) intraepithelial mast cells. The intraepithelial lymphocyte may occur alone or in groups (McDermott et al, 1982) and functions to remove allergenic particles from the airways. They may be either T or B cells, although few B lymphocytes are found in this compartment (Pabst, 1997). The T cell surface markers normally identified in the bronchial epithelium include the universal lymphocyte marker CD3+ cells, as well as the more specific CD4+ and CD8+ cells (preponderance of CD8+ cells compared with CD4+ cells in the epithelium (Bradley et al, 1976) but conversely the CD4+ cells predominate over CD8+ cells in the lamina propria (Azzawi et al, 1990)). The immunostimulation of resting T lymphocytes is thought to require the initial presentation of antigen in association with class II major histocompatibility complex (Ia) molecules expressed by normal dendritic cells (Holt, 1993). It appears that T cell immunity is regulated by a balance of signals from resident Ia positive dendritic cells and macrophages (Holt et al, 1989)(Holt et al, 1990).

The intraepithelial mast cell and subepithelial mast cell is morphologically and functionally distinct from the mast cell present in the connective tissue (Enerback, 1986). Both are capable of releasing mediators of inflammation which affect epithelial and vascular permeability. A greater number of these cells are found in the lung tissues of smokers (Lamb et al, 1982).

1.1.4.4 Submucosal glands

These are numerous (4000 glands are present in the human trachea (Tos, 1970)) and are probably the major source of tracheobronchial mucus. Each gland unit is of the tubuloalveolar type and in humans it is composed of 4 regions, the lumina of which are continuous (Meyrick et al, 1969)(Meyrick et al, 1970): a) the ciliated duct is continuous with the surface epithelium; b) a wider collecting duct of cells of indeterminate morphology or of eosinophilic cells packed with mitochondria c) mucous tubules and acini; 4) serous acini. Although mucins of both acidic and neutral type are produced, other secretions such as lysozyme (Bowes et al, 1977), lactoferrin (Bowes et al, 1981), secretory component of IgA (Brandtzaeg, 1974) and low molecular weight antiproteinase secretory leukoprotease inhibitor (Kramps et al, 1981) are also found in submucosal glands, particularly in serous acini. In addition lymphocytes and plasma cells which are responsible for the production of IgA (Soutar, 1976) are found in the gland. The submucosal gland mass increases in chronic bronchitis, which is the result of cell proliferation within each secretory acinus rather than of increase in the number of gland units (Douglas, 1980).

1.2 CHRONIC BRONCHITIS

1.2.1 General background

1.2.1.1 Historical background

The recognition of chronic bronchitis as a common and often gravely disabling disorder dates from the early nineteenth century. Bronchitis was first described by Badham in 1808 (Badham, 1808) and then subsequently by Mackintosh in 1831 (Mackintosh, 1831) and Laennec in 1834 (Laennec, 1834). In 1923 a detailed review of morbidity and mortality statistics was undertaken by Collis (Collis, 1923). He commented that despite records in England and Wales showing that bronchitis was the most important of all diseases when morbidity and mortality were taken together, "those called upon to treat it do not find it sufficiently interesting to study closely". Renewed interest in bronchitis however came after a severe smog in 1952 (5th to 9th December). Within 1 week, there were 4000 extra deaths which occurred mainly in people already suffering from chronic respiratory or cardiovascular disease. Since this period there has been intensive research into the epidemiology, aetiology, and natural history of chronic bronchitis.

Until 1959, there was diagnostic confusion between the use of the terms chronic bronchitis and emphysema. In 1959 a group of British workers (Ciba Guest Symposium (Symposium, 1959)) proposed that the diagnostic confusion could be alleviated by defining emphysema on an anatomical basis and chronic bronchitis on a clinical basis. They defined emphysema as enlargement of air spaces with destructive changes in their walls and chronic bronchitis as chronic or recurrent excessive mucus production in the bronchial tree that was not attributable to other lung diseases. Although this gained wide acceptance, the term chronic bronchitis continued to be used without qualification to

indicate on one extreme of regular production of small quantities of sputum without any abnormality of lung function and at the other extreme severe airflow obstruction. In 1965, the Medical Research Council (Medical Research Council, 1965) addressed this issue, and proposed that chronic bronchitis be classified into "simple" chronic bronchitis with mucoid hypersecretion, chronic or recurrent mucopurulent bronchitis (expectoration that has occurred on most days during at least 3 consecutive months for more than two successive years), and chronic obstructive bronchitis.

1.2.1.2 Prevalence

The college of general practitioners conducted a survey of sample practices through Britain aged 40-64 years (Practitioners, 1961), and found that the prevalence of chronic bronchitis was 17% for males and 8% for females. These figures were similar to figures from New Hampshire, USA, where the prevalence was 22% for males and 9% for females (Ferris et al, 1962). A much lower prevalences has been found in parts of Africa where there are low rates of smoking (Cookson et al, 1978).

1.2.1.3 Burden

Chronic bronchitis and emphysema is the fourth leading cause of mortality in the USA with age adjusted death rate of 21 per 100,000 (Ventura et al, 1997) (UK (1994) around 12/100,000 mortality rate (Organisation, 1996)). Mortality statistics are, however, based on the underlying cause of death listed on death certificates, and because chronic bronchitis and emphysema are often not listed as the underlying cause of death, mortality statistics may underestimate the true burden of disease (Mannino et al, 1997). Mortality is related to the number of cigarettes smoked per day with more than a twofold

difference between light (<15 cigarettes per day) and heavy (>25 cigarettes per day) smokers in the British physicians study (Doll et al, 1976). In the Seven Countries study chronic bronchitis and emphysema was found to be a clear predictor of excess all cause mortality in the populations studied (Menotti et al, 1997).

1.2.1.4 Demographic characteristics

Men have a higher prevalence of chronic bronchitis in most population based studies, and this difference persists after adjustment for smoking but not after adjustment for occupational exposures (Sherrill et al, 1996). It is not clear whether men or women are more susceptible to cigarette smoke. A recent finding, however, from two population studies of nearly 14,000 Danish adults, followed for up to 16 years, revealed that female smokers had greater losses in lung function and were at significantly increased risk of being hospitalised for chronic bronchitis than male smokers (Prescott et al, 1997).

Prevalence and mortality data for chronic bronchitis based on race and ethnicity, suggests that blacks, Hispanics, and Asian/Pacific Islanders are at lower risk than whites for developing chronic bronchitis (Petty et al, 1997). Many potential factors could account for these racial disparities including differences between the age structure, smoking habits, occupational exposures, education, income, and genetic make up of the population.

1.2.1.5 Aetiology of chronic bronchitis

1.2.1.5.1 Smoking

Chronic bronchitis has been mainly associated with the inhalation of cigarette smoke (pipe and cigar smokers have a much lower prevalence of the disease and less impairment of function (Olsen et al, 1960)(Higgins, 1959)). A sample survey in urban and rural populations in Britain found, that the prevalence of chronic bronchitis in males aged 55-64 was 17.7% for heavy smokers, 13.9% for light smokers, 4.4% for ex-smokers, and nil for non smokers (Higgins, 1959). Prevalence studies in at least 10 countries have confirmed the association between smoking and chronic bronchitis (Fletcher, 1968).

The most important evidence relating smoking and mortality was published by Doll and Peto (Doll et al, 1976). They found that the death rate for chronic bronchitis was significantly higher for cigarette smokers than non smokers and increased with the amount smoked (bronchitis mortality per 100,000 British male doctors was 3 for non smokers and 114 for subjects smoking ≥ 25 cigarettes per day). Recent population based studies in China have provided further evidence of a causal link between smoking and death from chronic bronchitis. Lam et al (Lam et al, 1997) found, in a cohort study of factory workers followed up over 20 years, an adjusted relative risk of chronic bronchitis mortality of 4.1 in male and 26.6 in female ever smokers compared with never smokers. Another prospective study of factory workers in Shanghai followed up for 16 years indicated a relative risk for chronic bronchitis mortality of 2.5 for male smokers compared with never smokers, and a strong dose response relationship (Chen et al, 1997).

Pathogenesis

Smoke induces mucosal gland hypertrophy (Megahed et al, 1967)(Greenberg et al, 1967)(Thurlbeck et al, 1967) and increased mucus secretion. This, together with the inhibitory effect of cigarette smoke on the bronchial ciliary blanket (Goodman et al, 1978), predisposes to the accumulation of mucus in the bronchial tree.

Cigarette smoke recruits inflammatory cells in the lungs and studies have shown increased numbers of neutrophils in broncho-alveolar lavage fluid from smokers (Reynolds et al, 1974)(Hunninghake et al, 1983). In addition the lungs of smokers with airflow obstruction contain more neutrophils than smokers without airflow obstruction (Stanescu et al, 1996)(Di Stefano et al, 1998) and the number of neutrophils recovered are related to the amount smoked (Bosken et al, 1992). These observations are consistent with a major role for the neutrophil in the pathogenesis of chronic bronchitis. In vitro and in vivo work have demonstrated that neutrophil elastase, released from the activated neutrophil, can cause epithelial damage (Amitani et al, 1991), reduce ciliary beat frequency (Smallman et al, 1984), produce mucus gland hyperplasia (Snider et al, 1985), stimulate mucus secretion (Sommerhoff et al, 1990), and inactivate many critical lung host defences (Solomon, 1978)(Tosi et al, 1990). Thus neutrophil elastase alone can reproduce most of the typical features of chronic bronchitis.

There may be several ways in which cigarette smoke could increase neutrophil traffic into the lungs and hence release of neutrophil elastase (Janoff et al, 1983):

a) neutrophil chemotaxis

Cigarette smoke has been shown to stimulate bronchial epithelial cells to produce interleukin 8, an important neutrophil chemoattractant (Mio et al, 1997), and indeed current smokers have been found to have raised interleukin 8 levels (Keatings et al, 1997). Nicotine itself has been shown to be a neutrophil chemoattractant in vitro (Totti et al, 1984), and finally cigarette smoke can stimulate alveolar macrophages to release neutrophil chemoattractants (Hunninghake et al, 1983).

b) upregulation of adhesion molecules

Smokers with airflow obstruction, have increased expression of adhesion molecules including E selectin on submucosal vessels (Di Stefano et al, 1994), intercellular adhesion molecule-1 (ICAM-1) in bronchial epithelium (Di Stefano et al, 1994), and sputum leukocyte beta 2 integrins (CD11b and CD18) (Maestrelli et al, 1996). All of these adhesion molecules are critical in the process of neutrophil migration from the vascular space into the lung.

c) reduction in neutrophil transit time

In vitro studies have demonstrated that oxidants (Drost et al, 1992), but not nicotine (Aoshika et al, 1994), from cigarette smoke reduce neutrophil deformability. The reduced deformability would reduce the neutrophils ability to pass through the

pulmonary circulation. This possibility is supported by in vivo studies confirming a delay in transit time in lungs immediately after smoking (Bosken et al, 1991).

d) inactivation of alpha-1-antitrypsin

Cigarette smoke can inactivate alpha-1-antitrypsin in vitro probably by oxidation of the active site methionine (Carp et al, 1978), and indeed a 40% reduction of functional alpha-1-antitrypsin has been demonstrated in broncho-alveolar lavage fluids of human smokers (Gadek et al, 1979). Local inactivation of alpha-1-antitrypsin could also be the result of oxidants released from activated neutrophils and macrophages (Clark et al, 1981)(Harris et al, 1970). It has been proposed that this process may increase the likelihood of lung damage due to proteinase release. Other studies, however, have not confirmed a reduction in functional alpha-1-antitrypsin (Boudier et al, 1983)(Stone et al, 1983)(Stockley et al, 1984), except perhaps transiently (Abboud et al, 1985) and this mechanism remains unproven.

1.2.1.5.2 Atmospheric pollution

Atmospheric pollution has been implicated as an aetiological factor. Studies in Britain demonstrated an increased prevalence of and mortality from chronic bronchitis with increased urbanisation (Practitioners, 1961)(Reid, 1964)(Holland et al, 1965)(Holland et al, 1965)(Lambert et al, 1970). An increase in bronchitic symptoms has been shown to relate to an increase in pollution (Waller, 1978), and studies in Canada, USA, and Japan have confirmed the relationship between pollution and bronchitis (Service, 1967)(Oshima et al, 1964).

The precise factor or factors in atmospheric pollution responsible for the effects remain unknown. Animal models have demonstrated that many of the features of chronic bronchitis can be mimicked after exposure to tobacco smoke (Lamb et al, 1969)(Jeffery et al, 1981) and inhalation of sulphur dioxide (Lamb et al, 1968). Smoke may be the most important as the two great London smogs of 1952 and 1962 were both associated with a rise in bronchitis mortality (Reid, 1964). However although the sulphur dioxide concentrations were similar in both years, the smoke concentration was much lower in 1962 as was the associated mortality (Reid, 1964).

Since the introduction of the Clean Air Act, there has been substantial reduction in smoke levels and lesser reductions in sulphur dioxide in Britain (Waller, 1978)(Economics, 1977). In Sheffield where there has been a substantial decrease in pollution, a study showed that patients during the later, less polluted period, had less productive cough, fewer winter illnesses, less severe breathlessness, and only a third of the rate of decline in FEV₁ compared with those who were studied in the earlier highly

polluted period (Howard, 1974). Recent studies in the USA have also shown a persisting increased mortality from non malignant cardiopulmonary disease (although not specifically chronic bronchitis mortality) associated with urban air pollution (Dockery et al, 1993) although lower than 30 years earlier when pollution was higher.

1.2.1.5.3 Infection

Childhood infections

Evidence relating childhood respiratory infection and chronic respiratory symptoms in adult life was published by Colley (Colley et al, 1973) who showed that chronic winter cough was more common at the ages of 20 and 25, both for smokers and non smokers, who had a lower respiratory tract illness before the age of 2. Similarly in Arizona, Lebowitz and Burrows (Lebowitz et al, 1977) found a significant relationship between poor respiratory function and a history of childhood respiratory illness or recurrent respiratory infections in adult life. Nevertheless caution should be employed in assuming that the association between childhood respiratory infections and adult chronic bronchitis is causal as both may relate to poor social circumstances or a genetic predisposition.

Exacerbations

Both virus (Grist, 1967)(Buscho et al, 1978) and bacterial infection have been implicated in exacerbations of chronic bronchitis (Turk et al, 1967)(May et al, 1973)(Stuart-Harris, 1968)(Tager et al, 1975). Long term studies in England by Fletcher (Fletcher et al, 1977) and in Canada by Bates (Bates, 1973) found that deterioration in lung function occurred independently of respiratory infections. Fletcher and Peto

(Fletcher et al, 1977) in their 8 year prospective study of working men in London found that broncho-pulmonary infection caused an acute decline in lung function but that recovery was complete. Although these authors found an association between mucus hypersecretion, increased frequency of infection and lower absolute levels of FEV₁ they concluded that neither mucus hypersecretion nor bronchial infection caused FEV₁ to decline more rapidly, as after adjustment for age, smoking and FEV₁ left no independent correlation between indices of mucus hypersecretion or bronchial infection and annual decline in FEV₁. The epidemiological data, however, that indicated a lack of effect of broncho-pulmonary infections and an accelerated decline in FEV₁ was derived from men with mild airflow obstruction and few individuals with severe obstruction. Once obstruction has developed hypersecretion and/or infections may therefore accelerate the subsequent decline in FEV₁. This was demonstrated by a large community study in Copenhagen (Lange et al, 1990) in which hypersecretion was associated with a relative risk of death from chronic bronchitis of 1.2 if the FEV₁ was 80% predicted but a risk of 4.2 if the FEV₁ was 40% predicted. With the known potential for an infection to cause lung tissue damage, it seems surprising that bronchial infection does not cause the FEV₁ to decline more rapidly and further studies are clearly indicated.

1.2.1.5.4 Genetic factors

Preliminary studies from Eriksson in 1964 confirmed that pulmonary emphysema was related to homozygous PiZ alpha-1-antitrypsin deficiency (Eriksson, 1964) and that chronic bronchitis was common with reported prevalences from 20-59% (Eriksson, 1965)(Brantly et al, 1988)(Tobin et al, 1983). The role of alpha-1-antitrypsin deficiency in the pathogenesis of lung disease is discussed in greater detail later.

Alpha-1-antitrypsin deficiency however accounts for less than 1% of cases of chronic bronchitis in the USA (Bresnitz, 1997). Other genes, however, may influence the development of chronic bronchitis and the susceptibility to develop lung disease with smoking or other environmental factors is likely to depend on the coincidence of several gene polymorphisms that act together.

Few polymorphisms have been studied in patients with chronic bronchitis, although both polymorphism in the 5' promoter region of the tumour necrosis factor-alpha (TNF- α) gene (Huang et al, 1997) and the microsomal epoxide hydrolase gene (Smith et al, 1997) have been found to be more prevalent in patients with chronic bronchitis and emphysema. The polymorphism of the TNF- α gene would result in an increased transcription of TNF- α on activation (Wilson et al, 1997)(Kroeger et al, 1997) and increase in TNF- α concentrations (Louis et al, 1998), which may be a mechanism to promote neutrophil influx in such patients. The polymorphism of the microsomal epoxide hydrolase gene results in a slow metabolising form of the enzyme (Hassett et al, 1994) that metabolises highly reactive epoxide intermediates that may be formed in cigarette smoke, and such patients may thus be more susceptible to the effects of cigarette smoke.

Recent data by Silverman and colleagues demonstrated that after adjusting for age and pack-years of smoking, odds ratios for reduced FEV₁ and chronic bronchitis were increased in current or ex-smoking first-degree relatives of early-onset probands with

chronic bronchitis, suggesting that other non identified genetic factors are likely to be important (Silverman et al, 1996)(Silverman et al, 1998).

1.2.1.5.5 Occupation

A recent population based study in New Zealand found that chronic bronchitis and airway obstruction were significantly associated with working with vapours, gases, dust, or fumes (Fishwick et al, 1997). Elevated odds ratios for the development of chronic bronchitis were found in specific occupational groups including bakers, chemical processors, and spray painters. Hendrick (Hendrick, 1996), in a recent review of the occupational lung disease literature, concluded that employment in certain occupations was likely to influence the development of chronic bronchitis, possibly due to complex adverse interactions with smoking and other environmental agents.

1.2.1.5.6 Airway hyper-responsiveness

This is known to be a risk factor for accelerated rate of decline in FEV₁ (Silverman et al, 1996) but its role in the development of chronic bronchitis is unclear. Prospective data from a 24 years follow up of a cohort of more than 2600 adults in the Netherlands showed that increased airways hyper-responsiveness was a positive predictor of the development of chronic respiratory symptoms including cough, phlegm, dyspnoea, asthma attacks, and persistent wheeze, and was a negative predictor of the remission of these symptoms (Xu et al, 1997).

1.2.2 Pathogenesis of chronic bronchitis

1.2.2.1 Pathology

There is airway wall inflammation (Mullen et al, 1985) associated with hypersecretion of mucus, goblet cell hyperplasia, enlargement of tracheo-bronchial submucosal glands, and a disproportionate increase in acidic mucus (Reid, 1954)(Wright et al, 1983)(Thurlbeck, 1990)(Jeffery, 1997). The mucus gland size can be assessed by measuring the thickness of the gland layer in histological sections compared to that of the bronchial wall (Reid index) (Reid, 1960) or alternatively by measurement of the absolute gland area or by the volume proportion of the glands, both of which correlate better with ante-mortem sputum production than the Reid index (Jamal et al, 1984). The mucus secreting goblet cells are increased in the bronchi and bronchioles (readily blocked by their secretions) but not in the terminal or respiratory bronchioles (Thurlbeck, 1976)(Lumsden et al, 1984). Other epithelial changes may include atrophy, focal squamous metaplasia, ciliary abnormalities, and decreases in both ciliated cell number and mean ciliary length (Jeffery, 1998).

1.2.2.1.1 Cellular changes

Early studies have shown that the inflammatory process present in the bronchial mucosa of subjects with chronic bronchitis is characterised predominantly by macrophages and activated T lymphocytes (Mullen et al, 1985)(Fournier et al, 1989)(Ollerenshaw et al, 1992)(Saetta et al, 1993). Such patients had an increased number of cells expressing interleukin-2 receptor and very late activation antigen-1, representing T lymphocytes at different stages of activation (Saetta et al, 1993). Bronchial biopsies have shown that this

inflammatory process may persist even after smoking cessation, in subjects who continue to have symptoms of chronic bronchitis (Turato et al, 1995).

The T lymphocytes that were increased in patients with chronic bronchitis were CD3+ (Saeita et al, 1993) with no significant differences in CD4+ and CD8+ T lymphocytes in the lamina propria. A further study by O'Shaughnessy and colleagues (O'Shaughnessy et al, 1997), however, have shown that compared to non smoking controls, patients with chronic obstructive bronchitis had increased numbers of macrophages, and CD3+ and CD8+ T lymphocytes in the lamina propria, and that the CD8+ T lymphocytes negatively correlated with FEV₁ (% predicted) (O'Shaughnessy et al, 1997).

The influence of airflow obstruction, was addressed by comparing patients with chronic bronchitis with and without airflow obstruction, showing that patients with chronic bronchitis with airflow obstruction have greater number of macrophages and T lymphocytes (CD3+ cells) and that the T lymphocytes negatively correlated with FEV₁ (Di Stefano et al, 1996). Di Stefano and colleagues studied smokers with chronic bronchitis with a wider range of airflow limitation and found that patients with chronic bronchitis with severe airflow limitation (FEV₁ <50% predicted) had increased numbers of neutrophils, macrophages, and NK lymphocytes in the lamina propria and that FEV₁ inversely correlated with the numbers of neutrophils, macrophages, and NK lymphocytes (Di Stefano et al, 1998). The authors postulated that neutrophils may be of importance in disease progression (Di Stefano et al, 1998).

1.2.2.1.2 Adhesion molecules

Adhesion molecules are critical in the process of neutrophil migration from the vascular space into the lung. It has been shown that in patients with chronic bronchitis with airway obstruction, there is an increased vascular expression of adhesion molecules E-selectin on submucosal vessels, ICAM-1 on bronchial epithelium (Di Stefano et al, 1994), and increased beta-2-integrin (Mac-1) up-regulation on sputum neutrophils (Maestrelli et al, 1996). In addition Mac-1 negatively correlated with FEV₁/VC ratio, and the authors suggested that Mac-1 expression on sputum neutrophils represented a marker for smokers who develop chronic airflow obstruction (Maestrelli et al, 1996). In addition further studies by Riise and colleagues found that patients with chronic bronchitis had increased levels of circulating ICAM-1 both in serum and in bronchial lavage, and higher circulating E-selectin in serum (Riise et al, 1994), the latter correlated significantly with FEV₁ (% predicted) (Riise et al, 1994). These studies suggest the involvement of these adhesion molecules may be of importance in the pathogenesis of chronic bronchitis.

1.2.2.1.3 Exacerbation

This cellular profile may change during exacerbations, and bronchial biopsies taken during such episodes show a marked eosinophilia and a milder increase in the number of neutrophils, activated CD3+ T lymphocytes, and TNF- α positive cells (Sactta et al, 1994).

1.2.2.2 Bronchial and bronchoalveolar lavage data

Bronchial lavage and bronchoalveolar lavage fluid from patients with chronic bronchitis recovers increased numbers of neutrophil granulocytes (Spurzem et al, 1991)(Thompson et al, 1989). Further studies have confirmed not only the increased activity of neutrophils but also demonstrated an increase of eosinophils in patients with chronic bronchitis, although the importance of the eosinophils in the pathogenesis of bronchial disease has yet to be established (Riise et al, 1995)(Pesci et al, 1998). Balbi and colleagues demonstrated increased number of neutrophils and eosinophils in bronchoalveolar lavage specimens during exacerbations of chronic bronchitis (Balbi et al, 1997).

1.2.2.2.1 Association with airflow obstruction and progression

Early studies postulated that airway inflammation may play a pathogenetic role in airway obstruction in patients with chronic bronchitis, since all spirometric measurements of airway obstruction were significantly lower in chronic bronchitic patients with neutrophilia (Thompson et al, 1989). Similarly sputum induction studies have demonstrated that the % neutrophils negatively correlated with the FEV₁ (% predicted) (Keatings et al, 1997). Further studies have shown that increased neutrophils in the airways are related to the progression of airflow obstruction (Stanescu et al, 1996).

1.2.3 Neutrophil migration into the lung

These studies highlight the importance of the neutrophil in the pathogenesis of chronic bronchitis, but to have this effect the neutrophils have to migrate into the lung. Because mature neutrophils cannot divide, the neutrophils in lung tissues are derived directly from the blood. A variety of integrated processes results in movement of neutrophils from the circulation to sites of inflammation. These involve adherence to the vascular endothelium, diapedesis between the endothelial cells, and migration through the connective tissue in the interstitial space to the site of the chemoattractant release within the airways. At present it is unknown whether these processes are directly applicable to both the bronchial and pulmonary circulation and whether differences exist between the two.

1.2.3.1 Cell differentiation and release

The neutrophil is the most abundant leukocyte in the body (numbers in the circulation normally exceed 5×10^{11}). The normal neutrophil differentiates in the bone marrow, from pluripotent stem cells and is released into the circulation as a mature cell (Bainton et al, 1971).

It is thought to differentiate through several distinct stages: myeloblast, promyelocyte, myelocytes, metamyelocyte, band cells and finally to mature neutrophils (the azurophil granules are synthesised and the enzymes packaged at the promyelocyte stage (Bainton et al, 1971)). This differentiation process takes between 10-14 days and these mature cells remain in the marrow for approximately 2 days before being released in to the circulation, where they do not survive long, half life about 8 hours (Bainton et al, 1971).

About half of the neutrophils are freely circulating but the rest are marginated on the endothelium (Abramson, 1993). The marginated cells can be mobilised rapidly and thus provide an immediately available source of extra cells which may be required during episodes of acute inflammation (during episodes of infection, the increased granulopoiesis along with mobilisation of neutrophils, results in neutrophilia to combat infections (Athens et al, 1965)).

1.2.3.2 Neutrophil rolling, triggering and strong adhesion

1.2.3.2.1 Neutrophil adhesion to endothelium

A critical step in cell migration is the adherence of freely circulating neutrophils to specific areas of endothelial cells near the tissues that have become inflamed. This involves the specialised adhesion molecules that are expressed on the surface of both the neutrophil and the endothelial cells.

Adhesion of circulating neutrophils to the endothelial cells is necessary for subsequent trans-endothelial migration and involves 3 stages: neutrophil rolling, triggering and strong adhesion (Adams et al, 1994)(Springer, 1994).

Neutrophil rolling is the first crucial step in halting the progress of neutrophils through the circulation and is brought about by at least 3 different cell surface receptors called selectins (Bevilacqua et al, 1993)(Tedder et al, 1995)(Symon et al, 1996). Neutrophils express L-selectin constitutively (McEver et al, 1995)(Von Andrian et al, 1992)(Tedder et al, 1995) and the counter receptors are expressed on endothelial cells.

Several studies have shown that neutrophil rolling on endothelial cells is L-selectin mediated (von Andrian et al, 1993, von Andrian et al, 1991, Von Andrian et al, 1992) and the rapid binding of this adhesion molecule to endothelial cells is believed to allow the slow rolling of neutrophils along venular walls at the shear force rates exerted by flowing blood (Von Andrian et al, 1992) (Symon et al, 1996).

Endothelial cells express 2 selectins, E-selectin and P-selectin (Albelda et al, 1994). P-selectin is stored within the Weibel-Palade bodies in endothelial cells and translocates rapidly to the plasma membrane under the influence of inflammatory mediators, including thrombin, histamine, complement fragments, oxygen derived free radicals and cytokines (Springer, 1994)(Tedder et al, 1995). In contrast E-selectin is not pre-stored, but is synthesised rapidly by endothelial cells and transported to the plasma membrane in response to inflammatory cytokines including Interleukin-1 (IL-1), Interleukin 8 (IL8) and tumour necrosis factor- α (TNF- α) (Albelda et al, 1994). Thus weak selectin mediated attachment of neutrophils to endothelium occurs selectively at sites where these inflammatory mediators are being expressed.

Neutrophil rolling on endothelial cells at the very early stages of an inflammatory response, are dependant initially on L-selectin followed by P or E selectin (Wardlaw, 1990)(Hogg et al, 1995)(Tedder et al, 1995).

The importance of the selectin mediated neutrophil rolling is seen in leukocyte adhesion deficiency II which is characterised by a failure to express sialyl Lewis X, the counter

receptor for E and P selectin. This leads to a failure of neutrophil migration to inflammatory foci resulting in recurrent bacterial infection (Etzioni et al, 1992)(von Andrian et al, 1993).

The interactions of the selectins with their counter receptors are not strong, and a second step of triggering and strong adhesion is required.

1.2.3.2.2 Triggering and strong adhesion

Neutrophils rolling has the effect of bringing the cells into close contact with endothelial cells at regions of inflammation, and consequently into contact also with factors that trigger or activate the neutrophil receptors-integrins, responsible for strong adhesion (Von Andrian et al, 1992).

The integrins are a family of trans-membrane glycoproteins. They are heterodimeric proteins, each consisting of one α -subunit and one β subunit, which are bound non-covalently. Subfamilies of integrins share common β subunits and different subfamily members have distinct α -subunits (Montefort et al, 1991)(Wardlaw, 1990)(Osborn, 1990)(Albelda et al, 1994).

The most important neutrophil integrin (β_2 integrin) is Mac-1 or CR3 (CD11b/CD 18) (Anderson et al, 1985). Another β_2 integrin LFA-1 (CD 11A/CD 18) is expressed by neutrophils but is probably less important than Mac-1 for strong binding (Anderson et al, 1985).

Strong adhesion appears to be primarily mediated by the β_2 integrins LFA-1 and Mac-1. This is emphasised in the leukocyte adhesion deficiency syndrome I where patients have an autosomal recessive genetic defect in the β_2 integrin subunit CD18 (Osborn, 1990) causing reduced or absent expression of all the β_2 integrins (Anderson et al, 1985). The neutrophils in these patients have an inability to adhere and migrate to the site of inflammation, and thus such patients suffer from recurrent infections (although the neutrophils demonstrate normal effector functions).

Once the β_2 integrins have been activated, firm adhesion of neutrophils is dependant on their subsequent binding to one of their counter receptors the intercellular adhesion molecules (ICAMs) on the surface of the endothelium. Mac-1 binds to ICAM-1 (CD54) and LFA-1 binds to ICAM-1, ICAM-2, and ICAM-3 (Springer, 1994). One amino acid sequence known to be recognised by Mac-1 and LFA-1 is Arg-Gly-Asp (Ruoslahti et al, 1987), which is found on several proteins including C3bi (Mac-1 also has a binding site for bacterial lipopolysaccharide (Wright et al, 1989)).

Another family of integrins, the β_1 integrins (Wardlaw, 1990) is also present on neutrophils, known as the "very late antigens" VLA-5 & VLA-6. VLA-5 binds to fibronectin and VLA-6 to laminin (Wardlaw, 1990). These adhesion proteins have been thought to be important in the migration of leukocytes (principally eosinophils, monocytes, and lymphocytes) through the endothelium and the extracellular matrix (Montefort et al, 1991).

The expression of integrins and ICAMs is modulated by a variety of pro-inflammatory factors. Integrin adhesiveness is increased on neutrophils by factors including host cell derived cytokines such as IL8, and compounds derived from micro-organisms such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) and other formulated peptides. Similarly endothelial cells respond to pro-inflammatory agents. Whereas ICAM-2 appears to be constitutively expressed (Abramson, 1993) by endothelial cells and synthesis is not believed to be regulated, ICAM-1 expression can be increased. Endothelial expression of ICAM-1 is upregulated by cytokines such as IL-1, TNF- α and by bacterial lipopolysaccharide (Burnett, 1997)(Khair et al, 1994), again factors likely to be released at sites of inflammation. Thus strong adhesion of neutrophils to endothelial cells, through integrin-ICAM interactions and associated changes in neutrophil shape (flattening and spreading), is regulated by common pro-inflammatory factors, ensuring that this process is confined to areas of tissue where the cells are required.

Interestingly adhesion molecule expression has been shown to be down regulated on neutrophils that have migrated in response to a chemotactic signal in vitro (Harvath et al, 1995) which might represent the breakdown of cell adhesion in order for migration to occur.

1.2.3.2.3 Transmigration

The final step from tethering and rolling, firm adhesion involves transmigration. Transmigration into tissues is a process that requires both a chemotactic stimuli and engagement of adhesion receptors. Although inflammatory mediators can augment

leukocyte adhesion to the endothelium, transmigration requires a chemotactic gradient. Studies have implicated PECAM-1, a member of the Immunoglobulin (Ig) superfamily to be one of the major molecules involved in transmigration (Albelda et al, 1994). If devoid of PECAM, there is a reduced migration rate (Kim et al, 1998) of up to 70-90% (Muller, 1993).

1.2.3.3 Chemoattractants

Neutrophils are recruited from the circulation to the lung in response to a chemotactic signal. Blood neutrophils are not homogeneous with respect to their chemotactic responses. There appears to be two subpopulation, one responsive, and the other not (Harvath et al, 1982) although the implications are currently unknown.

The chemotactic signal will be greatest at the point of release and decreases as it diffuses away. The exquisite sensitivity of the cell to a changing concentration gradient results in movement up the gradient towards the source of the signal, but the rate of movement will decrease after the optimum concentration is encountered. Thus neutrophils will accumulate at or near the origin of the signal. The cell will only then continue directional movement if the concentration changes, or in response to a different chemotactic signal (Foxman et al, 1997).

1.2.3.3.1 Interleukin 8

Interleukin 8 (IL8) is present in patients with chronic lung disease, where it is thought to play a key role in neutrophil recruitment (Richman-Eisenstat et al, 1993)(McElvany et al, 1992). Recent data confirms that IL8 levels are raised in patients with chronic bronchitis (Riise et al, 1995)(Pesci et al, 1998)(Richman-Eisenstat et al, 1993)(Yamamoto et al, 1997), and correlates with myeloperoxidase (Riise et al, 1995)(Pesci et al, 1998)(Yamamoto et al, 1997) (a marker of neutrophil influx). This relationship supports the concept that IL8 is an important lung chemoattractant for neutrophils.

IL8 is a 16kDa protein that is a member of the C-X-C family of cytokines. It is made and released by bronchial epithelial cells (Nakamura et al, 1992), monocytes/macrophages (Standiford et al, 1992), and even the neutrophils themselves (McCain et al, 1994).

The regulation of IL8 production in the lung is complex but recent studies have started to determine the processes involved. It is known that tumour necrosis factor- α (TNF- α) can increase the expression of the IL8 gene (Khair et al, 1994), and certainly lung secretions contain TNF- α (Keatings et al, 1997). In addition bacteria can also induce IL8 expression by epithelial cells (Khair et al, 1994)(Inoue et al, 1994). At present it is uncertain however whether the effect of bacterial products on IL8 expression is direct or via TNF- α .

1.2.3.3.2 Leukotriene B4

Leukotriene B4 (LTB₄) is a biologically active fatty acid synthesised in myeloid cells from arachidonic acid. LTB₄ was isolated and purified in 1978 from neutrophils (Borgeat et al, 1979), but subsequently it was chemically synthesised and its ability to activate neutrophils was identified in 1980 (Ford-Hutchinson et al, 1980). LTB₄ is now known to be a major chemotactic product of activated neutrophils (Ford-Hutchinson et al, 1980) and macrophages (Hubbard et al, 1991).

Leukotriene B4 induces recruitment and activation of neutrophils as well as monocytes, eosinophils, and lymphocytes. LTB₄ promotes both neutrophil adherence to venule walls (via the integrin Mac-1 and ICAM-1) as well as the chemotactic process (Crooks et al, 1998).

Synthesis of LTB₄ from arachidonic acid (Figure 1-2) is catalysed by 5-lipoxygenase and leukotriene A₄ hydroxylase and is increased by inflammatory mediators including endotoxin, complement fragments, TNF- α , and interleukins such as IL8 (Crooks et al, 1998). 5 lipoxygenase activating protein, is an 18 kDa nuclear membrane protein, which is an essential co-factor for 5-lipoxygenase pathway degradation (Crooks et al, 1998).

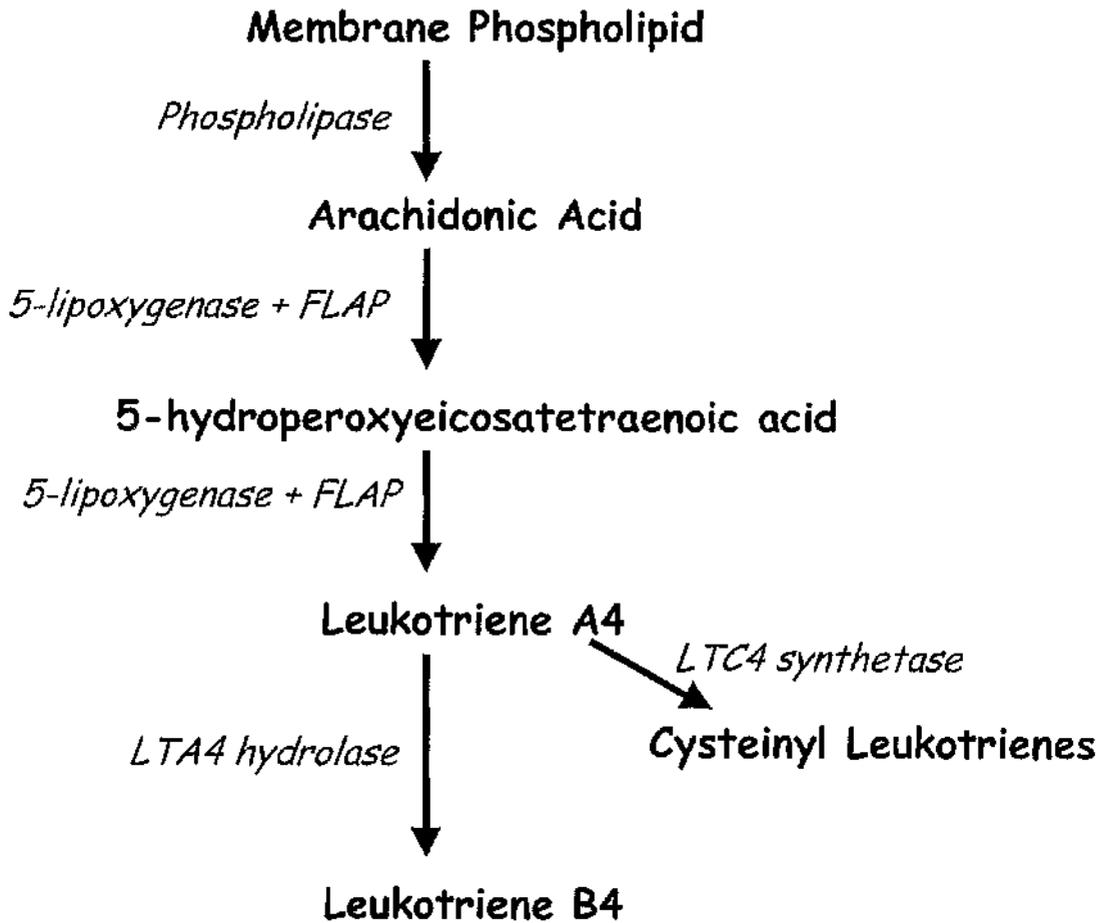


Figure 1-2: The pathway of LTB₄ production from membrane phospholipid. 5 lipoxygenase activating protein (FLAP) is an essential cofactor for 5 lipoxygenase.

1.2.3.3.3 Role of IL8 and LTB₄

The exact role of these chemoattractants in lung disease is currently uncertain. As described above IL8 was thought to be the major chemoattractant in bronchial disease (Richman-Eisenstat et al, 1993)(McElvany et al, 1992). On the other hand Hubbard et al described studies that indicated that LTB₄ was the major lung chemoattractant in alpha-1-antitrypsin deficiency (Hubbard et al, 1991). The LTB₄ was thought to be released from macrophages as a direct effect of elastase activity, which was not inactivated in the

lung because of the alpha-1-antitrypsin deficiency. There was, however, no information in these studies of the initial chemoattractant involved in recruitment of the neutrophils that would release the elastase to generate the LTB₄.

Studies by Mikami et al (Mikami et al, 1998) have provided some clarification of the role of IL8 and LTB₄ in bronchiectasis indicating that they both play a significant role in neutrophil chemotaxis. The authors demonstrated that up to 43% of the chemotactic activity was dependant on IL8 and 27% was dependant on LTB₄. In addition it was shown that the chemoattractants worked in an additive way for neutrophil recruitment (Mikami et al, 1998). However these workers also emphasised that some of the chemotactic activity of the lung secretion was due to an, as yet, unidentified protein.

1.2.3.3.4 Other potential chemoattractants

There are a variety of other chemoattractants that could play a role in the airways including:

- i. C5a generated from complement activation (Fick et al, 1986).
- ii. modified alpha-1-antitrypsin (Stockley et al, 1990).
- iii. leukocyte elastase-inhibitor complexes (Banda et al, 1988).
- iv. protein and peptide components of damaged extracellular matrix, such as collagen (Senior et al, 1989) and laminin (Bryant et al, 1987).
- v. antigen-antibody complexes (Boyden, 1962).

- vi. factors including the formyl peptides such as fMLP from micro-organism (Casale et al, 1992) as well as lipopolysaccharide or endotoxin which can prime neutrophil functions including the chemotactic responses (Young et al, 1990).

1.2.3.3.5 Chemoattractants- summary

In summary a significant proportion of the chemotactic activity of lung secretions can be attributed to IL8 and LTB4. This is particularly true in patients with bronchiectasis, who usually have marked bronchial inflammation associated with significant neutrophil influx. Other potential chemoattractants listed above may also play a role and cells migrating in tissues may thus encounter multiple chemoattractant signals. Foxman et al (Foxman et al, 1997) have demonstrated that neutrophils can migrate "down" a local chemoattractant gradient in response to a distal gradient of a different chemoattractant. In addition cells can migrate effectively to a secondary distant agonist after migrating up a primary gradient into a saturating non-orienting concentration of an initial attractant. These mechanisms to navigate from one gradient to another in complex chemoattractant fields are likely to be necessary for successful localisation within sites of inflammation (Foxman et al, 1997).

1.2.3.3.6 Hierarchy of chemoattractants

The hierarchy of chemoattractants has been shown to be influenced by the quantity and type of chemoattractant present but also the barrier through which the neutrophil must migrate (Casale et al, 1992). These authors demonstrated that LTB4 was more potent than fMLP which was more potent than platelet activating factor (PAF) for migration

through endothelial cells but fMLP>LTB4>PAF for migration through epithelial cell barriers. Further studies have demonstrated the dominance of end target derived chemoattractants (C5a, fMLP) over regulatory cell derived chemoattractants (IL8, LTB4) (Campbell et al, 1997)(Kitayama et al, 1997). This supports a concept of hierarchy of chemoattractants and further studies have demonstrated that neutrophils can migrate away from a local IL8 or LTB4 source towards fMLP or C5a although not vice-versa (Foxman et al, 1997).

1.2.4 Role of proteinases and anti-proteinases

The neutrophil plays a key role in the pathogenesis of chronic bronchitis which is likely mediated through the neutrophil products released. In particular in vitro and in vivo studies have shown that neutrophil elastase can reproduce many of the features of chronic bronchitis, when an imbalance occurs in the airways between the enzyme and its inhibitors (Stockley, 1994).

1.2.4.1 Proteinases

1.2.4.1.1 Neutrophil Elastase

Proteolytic enzymes have been implicated in the pathogenesis of both chronic bronchitis and emphysema. Early studies by Gross and colleagues (Gross et al, 1964) revealed that a proteolytic enzyme (papain) was capable of producing lesions in experimental animals similar to the pathological changes in human emphysema. Subsequent studies have concentrated on the effects of relevant serine proteinases, especially human neutrophil elastase. This enzyme is produced during the early stages of neutrophil differentiation and stored within the azurophil granules (Sandborg et al, 1988). The enzyme is released when the neutrophil is activated, and can be rapidly neutralised by proteinase inhibitors such as alpha-1-antitrypsin (AAT) (Baumstark, 1967) and secretory leukoprotease inhibitor (Morrison et al, 1987).

When neutrophil elastase is instilled into the lungs of experimental animals, it produces lesions similar to human emphysema with enlargement of peripheral airspaces (Janoff et al, 1977), as a result of degradation of lung elastin (Kuhn et al, 1980).

In addition, *in vitro* and *in vivo* work have demonstrated that neutrophil elastase can cause epithelial damage (Amitani et al, 1991), reduce ciliary beat frequency (Smallman et al, 1984), produce mucus gland hyperplasia (Snider et al, 1985), stimulate mucus secretion (neutrophil elastase being the most potent secretagogue studied to date) (Sommerhoff et al, 1990), and inactivate many critical lung host defences (Solomon, 1978)(Tosi et al, 1990). Thus neutrophil elastase can produce pathological processes similar to both chronic bronchitis (Figure 1-3) and emphysema found in humans.

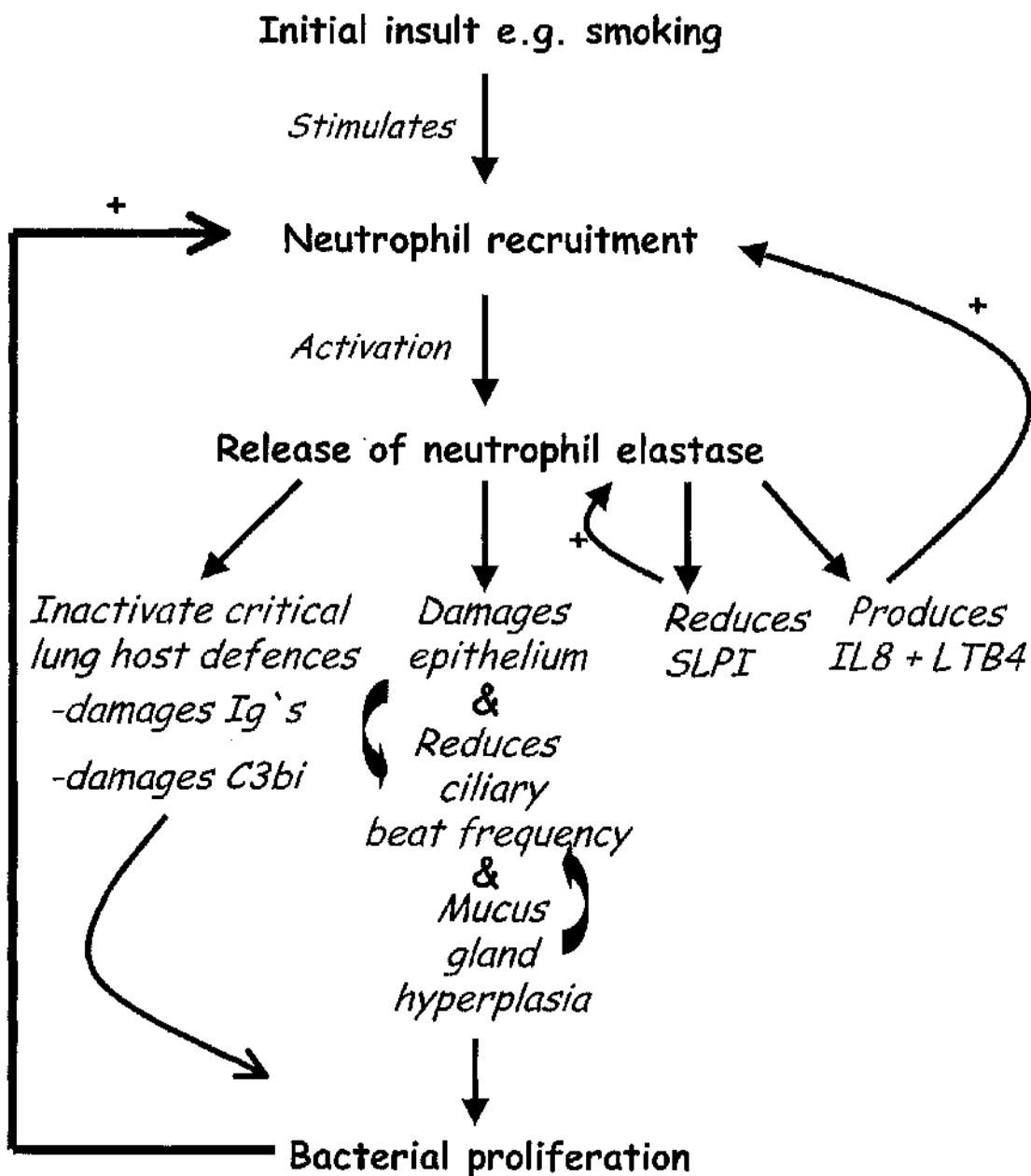


Figure 1-3: The potential damage from neutrophil elastase causing many of the features of chronic bronchitis. Amplification can be caused by neutrophil elastase inhibiting production of the proteinase inhibitor secretory leukoprotease inhibitor (SLPI) (Sallenave et al, 1994), stimulating the release of the chemoattractants [IL8 by epithelial cells (Nakamura et al, 1992) and LTB4 by macrophages (Hubbard et al, 1991)], and finally bacterial proliferation itself stimulating further IL8 release by epithelial cells (Khair et al, 1994). The symbol "+" indicates an amplification loop. Ig's = Immunoglobulins.

1.2.4.1.2 Other proteolytic enzymes

There are other proteolytic enzymes that could play a role in the pathogenesis of chronic bronchitis and emphysema. These include: 1) serine proteinases such as cathepsin G and proteinase 3 which are components of the azurophil granule of the polymorphonuclear phagocyte (Sandborg et al, 1988) and likely to be released at the same time as neutrophil elastase; 2) Cysteine proteinases such as Cathepsin B which has been identified within the human macrophage (Burnett et al, 1983) and epithelial cells (Howie et al, 1985); 3) Metalloproteinases such as the human collagenases (D'Armiento et al, 1992) and bacterial enzymes (Kilian et al, 1980). Despite this wide range of proteinases and their different classes, few have been studied in depth.

In experimental animals proteinase 3 (Kao et al, 1988), cathepsin B (Lesser et al, 1992), and collagenase (D'Armiento et al, 1992) have been shown to cause emphysema whereas cathepsin G (Sommerhoff et al, 1990)(Lucey et al, 1985) and bovine cathepsin B (Cardozo et al, 1992) cause mucus gland hyperplasia and hypersecretion. Cathepsin B is present in lung secretions as the pro-enzyme which can be cleaved by neutrophil elastase releasing active cathepsin B (Buttle et al, 1991) suggesting that if it plays a role in human disease it may be secondary to elastase release. Although neutrophil elastase remains the most potent secretagogue in the airways, more recent studies have shown that Proteinase 3 is also a potent secretagogue in the airways (Witko-Sarsat et al, 1999), and may play a role in the pathogenesis of bronchial disease.

1.2.4.1.3 Summary

Several proteinases may play a role in the pathogenesis and features of bronchial disease. However the evidence to date suggests that neutrophil and its enzyme neutrophil elastase is likely to be a major mediator of bronchial disease and the mechanisms that summarise this processes of importance are shown in Figure 1-3.

1.2.4.2 The anti-proteinase screen

For neutrophil elastase to have these effects, it has to overcome the anti-elastases that protect the tissues. Several potent inhibitors of neutrophil elastase are present in lung secretions and are believed to play a major role in the protection of lung tissue from the destructive effects of these enzymes.

1.2.4.2.1 Alpha-1-antitrypsin

Alpha-1-antitrypsin (AAT) is secreted by hepatocytes and cells of the monocytic lineage (Perlmutter et al, 1989). Although alveolar macrophages are able to produce AAT (Barbey-Morel et al, 1987) most of the lung AAT is believed to be derived from plasma by simple transudation.

Lung AAT has been considered to be the most important of the lung anti-elastases particularly in the lower airways, since patients with AAT deficiency are particularly prone to develop severe emphysema at a younger age than non deficient subjects whom are susceptible to cigarette smoke (Tobin et al, 1983).

Structure

Alpha-1-antitrypsin is a 52 kDa glycoprotein consisting of a folded polypeptide chain of 394 amino acids, with three carbohydrate side chains accounting for 12% of the total molecular mass (Carrell et al, 1982). Alpha-1-antitrypsin belongs to the serpin family because it is an inhibitor of serine proteases (Carrell, 1986).

The serpins share the unusual property of being able to change from one conformational form to another (Carrell et al, 1994) which has enabled them to evolve and become the

most effective protease inhibitors in human plasma. The advantage that this molecular mobility of the serpins provides in higher organisms is that it allows the inhibitor not only to snare its target protease but also to tightly entrap it thereby effectively inactivating both proteins. The formation of a tight complex between a protease and its inhibitor is a physiological requirement as the complex may circulate for minutes or even hours before it is catabolically removed from the bloodstream and it would be detrimental to release the active enzyme again (Laurell et al, 1963). A disadvantage of having a mobile molecular mechanism however is that it makes the serpins more vulnerable to dysfunctional mutations (Stein et al, 1995).

The serpin molecule is made up of nine helices and three β pleated sheets; the dominant feature of the molecule being the five stranded A sheet and the reactive centre loop of the molecule arising from it.

AAT Variants

Alpha-1-antitrypsin is a polymorphic protein with over 100 phenotypes described to date. Most of these occur because of single amino acid substitutions although these do not usually affect protein concentration or function. The variants have different electrophoretic properties because of these amino acid differences that are detectable by isoelectric focussing (Hoffmann et al, 1977). The position of the isoelectric points of the variants identifies its phenotype which is described alphabetically. Alpha-1-antitrypsin is codominantly expressed with both alleles usually producing protein identifiable in the serum (Fagerhol et al, 1981)(Brantly et al, 1988). The most common phenotype is

referred to as M (approximately 92% in England) (Fagerhol et al, 1981), and has at least four subtypes, although none are thought to be directly responsible for ill health. The other common variants are the S and Z phenotypes (approximately 5% and 2% in England) (Fagerhol et al, 1981), which are readily distinguished by iso-electric focussing and often by reduced serum concentration.

The AAT gene locus is located on chromosomal segment 14q 32.1 (Darlington et al, 1982) and the variants arise from genetic coding errors which lead to a variety of changes including single amino acid substitutions in the primary chain, such as (a) glutamine to lysine at position 342 in the Z variant (Yoshida et al, 1976) and (b) glutamine to valine at position 264 in the S variant (Owen et al, 1976). Some abnormalities of the AAT gene totally prevent any production and secretion of AAT (Null genes) although these variants are rare (<1% of the population) (Fagerhol et al, 1981)(Talamo et al, 1973)(Garver et al, 1986).

Effects of Z variant

The commonest deficiency associated with a tendency to develop lung disease is the Z mutation identified by its low concentration (<20% normal) and retarded mobility in iso-electric focussing (Fagerhol et al, 1981). The homozygous Z variant results in normal synthesis of AAT (Bathurst et al, 1983) but only 15% is secreted into the circulation. The remaining 85% is blocked in the hepatocyte secretory pathway (Forman et al, 1984) and most of this blocked AAT is degraded intracellularly. However some accumulates and is recognised as the classical large intracellular inclusions seen as PAS positive bodies on liver biopsy.

The Z variant of antitrypsin has a mutation at the hinge of the reactive loop that facilitates entry of the loop into the A sheets. This results in the loss of the constraints that hold the reactive loop in its external position and facilitates transition of the molecule to the partially incorporated locking form (Stein et al, 1995). These intermediate forms are susceptible to intracellular catabolism (Le et al, 1990) and also to extended linkage, forming long polymers that prevents protein secretion (Lomas et al, 1992). This explains why the majority of the Z protein accumulates in the liver and it is the formation of tangles of these polymers, visible on electron microscopy, that form the characteristic liver inclusions.

Inheritance

Initial clinical studies suggested that the inheritance of AAT deficiency behaved as an autosomal recessive condition (Eriksson, 1964). Whereas this may be true for the clinical phenotype, the genetic components (alleles) are co-dominantly expressed so that each allele contributes to the total AAT serum concentration (Lieberman et al, 1972). The mean alpha-1-antitrypsin concentration expressed as percentage predicted of the normal level (MM=100%) of the common phenotypes are MM=100%, MS=75%, MZ=57%, SS=52%, SZ=37%, and ZZ=16% (Lieberman et al, 1972). However the phenotype is dependant on iso-electric focusing of the plasma protein. Since null genes do not contribute to the phenotype it has become convention to describe the phenotypes PiM, PiS or PiZ for cases which are apparently homozygous but where the null gene has not been excluded. In reality more than 95% of patients with severe AAT deficiency are

PiZ homozygotes, and have serum AAT levels of 2.5 to 7 μM (mean 16% of normal) (Society, 1995).

Role of AAT

Alpha-1-antitrypsin (52 kDa) in serum is capable of inactivating a variety of serine proteinases (neutrophil elastase, cathepsin G, and proteinase 3) providing about 90% of the inhibitory capacity of serum against such enzymes (Baumstark, 1967).

Most lung AAT is derived from plasma by simple diffusion (Stockley et al, 1979), and AAT is present in both the bronchial (Stockley et al, 1979) and broncho-alveolar regions (Olsen et al, 1975) of the lung. The concentration of AAT in sputum is 1-2% of that found in plasma (Stockley et al, 1990) approximately 0.3 μM and values in epithelial lining fluid are calculated to be 1-3 μM (Morrison et al, 1987). However unlike plasma AAT, airways protein is not fully active as a proteinase inhibitor being about 30% active in non-infected mucoid sputum (Morrison et al, 1986) and 50% active in lavage fluids (Morrison et al, 1987) although some workers have suggested that it is fully active in epithelial lining fluid (Gadek et al, 1979).

Inactivation of AAT in sputum secretions and lavage fluids is the result of several mechanisms:

- 1) about 20% is enzyme/inhibitor complexes in sputum (Morrison et al, 1986) and lavage (Stockley et al, 1984) and at least some of this is likely to be with neutrophil elastase (Stockley et al, 1979).

- 2) proteolytic cleavage near the active site accounts for a further 20% in sputum (Morrison et al, 1986) and is also seen in lavage (Stockley et al, 1984). AAT can be inactivated by a variety of enzymes that cause limited cleavage at or near the active site including enzymes from bacteria (Moriyama et al, 1979), the macrophage (Banda et al, 1985), and also by neutrophil elastase itself (Stockley et al, 1984).

- 3) the remaining 60% in sputum was present as its native molecular size although a portion remained inactive as an inhibitor, probably due to oxidation at the active site methionine (Morrison et al, 1986). Again similar results have been identified in lavage fluids from patients (Stockley et al, 1984).

- 4) The form and function of lung AAT harvested by bronchoalveolar lavage has however been the subject of much debate. Early studies suggested that cigarette smoke decreased AAT function by 40% (Gadek et al, 1979) although this has not been confirmed by others (Stone et al, 1983)(Boudier et al, 1987)(Afford et al, 1988). Furthermore these other studies have found a degree of inactivation of AAT in healthy non-smokers and as little as 40% of the protein may remain active as an inhibitor (Afford et al, 1988). The nature of the inactivation has yet to be defined as most of the AAT was the same size as the native protein and there was little evidence of oxidation of the active site (Afford et al, 1988).

Acute phase response (PiM)

Some serum proteins including AAT, alpha-1-antichymotrypsin, and C reactive protein (CRP) demonstrate acute phase responses in the presence of inflammatory processes. For instance following surgery in PiM individuals, AAT concentration rapidly rise to a maximum value after 1-4 days, rising on average by 120% although the range was wide (17-300%) (Crockson et al, 1966). C reactive protein rose within 24 hours also reaching its highest concentrations between 1-4 days later (mean 2 days) with an average increase of approximately 3000% (range= 33-7900%) (Crockson et al, 1966). Further studies assessing the acute phase response in surgical trauma confirmed that the response was rapid and the CRP and alpha-1-antichymotrypsin begin to rise within 8 hours, although AAT rose only after 24 hours (Aronsen et al, 1972).

Other studies have confirmed that AAT concentration can rise in PiM individuals, on average up to two fold, following typhoid vaccination (Kueppers, 1968) or after 6 months of oral contraceptive therapy (Laurell et al, 1967). In addition serum AAT levels increase throughout pregnancy, in a linear fashion until it reaches about twice the normal value at the time of parturition (Ganrot et al, 1967). Six weeks after parturition, the serum AAT concentration returns to normal (Ganrot et al, 1967).

Acute phase response (PiMZ. & PiZ)

Patients with heterozygous (PiMZ) AAT deficiency show an acute phase response in AAT following stilboestrol therapy (Lieberman et al, 1973), pregnancy (Ganrot et al, 1967), and typhoid vaccination (Kueppers, 1968). The serum concentrations rise 2 fold and probably reflects the activity of the normal M gene. The mean percentage increase

and time course was identical to that seen in normal subjects who were homozygote for the M allele. However the absolute concentration reached at the peak of the response was lower in heterozygote patients than in the PiM patients.

Nevertheless although patients with partial deficiency have a demonstrable acute phase response, initial studies with stilboestrol and typhoid vaccination indicated that patients with PiZ AAT deficiency had a much reduced response. Some further insight has been gained from the treatment of PiZ patients with danazol therapy, an impeded androgen, which induces the hepatocytes to increase the production of AAT (Gadek et al, 1980). The authors found that 53% of patients had a greater than 20% rise in serum AAT levels with a mean percentage rise of 52% for the group classified as responders (Wewers et al, 1986). However, not all the patients responded and again the peak concentrations achieved were much lower than patients with partial deficiency or normal AAT.

Lung AAT

Most lung AAT is derived from plasma by simple diffusion (Stockley et al, 1979). The concentration of AAT in the lung is therefore partially dependent upon the plasma concentration of AAT as well as the degree of airways inflammation (Stockley, 1984). As AAT is an acute phase protein, the lung concentrations of AAT will be higher during episodes where there is an acute phase response, as may occur in the presence of infection irrespective of inflammation (Stockley et al, 1979). However when the lung is inflamed, further protein leakage will occur due to airway vascular injury and these 2 processes will have an additive effect on the concentration of lung AAT. This is likely to be critical in the protection of the lung since such episodes are likely to be associated

with increased neutrophil influx and hence elastase release. Failure of an appropriate acute phase response might therefore enhance the degree of elastase induced lung damage.

Prevalence of PiZ AAT deficiency

The prevalence of PiZ AAT deficiency varies throughout the world and is thought to be rare in Black or Asian populations (Pierce et al, 1975)(Lieberman et al, 1976). The highest prevalence has been found by screening Swedish new-born infants (one in 1,670) (Sveger, 1976). In the UK, estimates vary from one in 2,047 to one in 3,450 (Cook, 1975)(Blundell et al, 1975)(Arnaud et al, 1979) whereas in the USA the prevalence is even more variable, with figures as low as one in 5,097 found from screening 100,000 new-born infants in Oregon (O'Brien et al, 1978) and 1 in 2,857 from screening blood donors in St Louis (Silverman et al, 1989).

Clinical manifestations and natural history of PiZ AAT deficiency

The current knowledge regarding the clinical manifestations and natural history of AAT deficiency is unclear as less than 5% of expected number of patients with PiZ AAT deficiency have been identified (Silverman et al, 1989)(Organisation, 1996). Once identified patients with AAT deficiency have a reduced life expectancy. Larsson (Larsson, 1978) reported a greatly reduced survival following an 11 year follow up of PiZ patients and in addition smoking PiZ individuals had an even lower life expectancy than non-smoking PiZ individuals. This study was supported by Brantly and colleagues (Brantly et al, 1988) who studied 120 PiZ patients with AAT deficiency and reported that the actuarial survival to age 60 was 16% compared with an expected age-matched

US survival of 85%. In both these studies, whilst referral bias may have effected mortality rates, the presence of PiZ AAT deficiency appears to be a significant factor determining survival.

More recent studies by Seersholm and colleagues (Seersholm et al, 1994), however, have indicated that differences exist between index cases (identified because of the presence of lung disease) and non-index cases (identified through family studies). The overall median life expectancy was 54.5 years. However, the median survival of the index cases (49.4 years) was significantly less than the non index cases (69.3 years) regardless of smoking history. In addition the authors found that life expectancy of the non-index subjects who had never smoked was similar to that of the normal Danish population (Seersholm et al, 1994) indicating that deficiency alone has a relatively good prognosis.

Effect of smoking in patients with PiZ AAT deficiency

Cigarette smoking is known to have a major adverse effect on subjects with AAT deficiency (Eriksson, 1965) and this has been confirmed by several other studies (Brantly et al, 1988)(Kueppers et al, 1974)(Tobin et al, 1983)(Black et al, 1978)(Janus et al, 1985). In addition, Larsson (1978) showed not only that the median age of onset of dyspnoea was younger in PiZ smokers (40 years) compared to non smokers (53 years), but that smoking PiZ individuals had a significantly lower life expectancy than PiZ non-smokers (Larsson, 1978).

In a recent study of 225 PiZ never smokers, Piitulainen and colleagues (1997) concluded that some individuals showed a significant decline in lung function, after 50 years of age. The authors found that additional risk factors that determined the development of airflow obstruction were male gender, previous asthmatic symptoms, and a history of occupational exposure to airway irritants. However this study also confirmed that a significant proportion of patients had well preserved lung function even into old age (Piitulainen et al, 1997).

Chronic bronchitis and emphysema in PiZ AAT deficiency

Studies of the patients indicate that chronic bronchitis and emphysema is the most prevalent clinical disorder and liver disease is the second most prevalent (Organisation, 1996). The relationship between AAT deficiency and lung disease is becoming clarified. Although early studies suggested it was a common association (Tobin et al, 1983), other studies however have now indicated that this may reflect a selection bias (Seersholm et al, 1994) in which studies have focused on index cases. Nevertheless there remains a general belief that AAT deficiency is a risk factor for the development of emphysema.

Emphysema in PiZ AAT deficiency

Preliminary studies from Eriksson in 1964 described pulmonary emphysema as a common complication of PiZ AAT deficiency (Eriksson, 1964). In the archetypal case of emphysema, patients had an insidious onset of progressive shortness of breath between the ages of 25 and 40, associated with increasing evidence of airflow obstruction (Hutchison, 1988)(Brantly et al, 1988). Radiologically the striking feature is the predominant destruction of the lower zones of the lungs (Eriksson, 1964)(Kueppers

et al, 1974)(Tobin et al, 1983) which occurs in over 90% of those with an abnormal chest radiograph (Gishen et al, 1982). This is in contrast to the upper zone or more uniformly distributed emphysema found in patients with normal AAT phenotype.

Pathologically, the emphysema has been described as panacinar or bullous as opposed to the centrilobular emphysema found more commonly in patients with emphysema without AAT deficiency (Eriksson, 1964)(Thurlbeck et al, 1970)(Orell et al, 1972). Histological examination of post mortem specimens indicated that chronic bronchitis was common being present in 11/17 cases and 4/6 pathological specimens had cylindrical bronchiectasis (Orell et al, 1972).

The pathogenesis of emphysema is becoming clarified. Initial lavage studies revealed that PiZ patients had increased numbers of neutrophils in the lower airways, suggesting a greater potential elastase burden, compared with smoking subjects with emphysema who have normal levels of AAT (Morrison et al, 1987). In addition patients with PiZ AAT deficiency have reduced levels of immunoreactive AAT in both sputum (Morrison et al, 1987) and lavage fluids (Morrison et al, 1987)(Gadek et al, 1981). Therefore the mechanism for the development of lung disease was initially thought to be predominantly due to the low AAT in the presence of an increased neutrophil burden and that the development of lung disease was a direct consequence of poorly controlled neutrophil elastase activity.

As neutrophils migrate from the vascular space into the airways in response to chemoattractants such as LTB₄ (Hubbard et al, 1991), they must pass through a compact

interstitium of connective tissue. In order to achieve this migration it is believed that the cells need to digest a path creating a natural hole through the matrix as has been elegantly demonstrated for eosinophils (Okada et al, 1997). Studies by Campbell and colleagues have shown that when neutrophils are in close contact with connective tissue they are able to digest matrix proteins even in the presence of super-normal concentrations of proteinase inhibitors (Campbell et al, 1982) which suggests the importance of the ability of these cells to digest connective tissue in order to migrate. It is believed that AAT acts as an inhibitor that prevents destruction of connective tissue beyond the limits of the cell and its close contact with the substrate.

Studies with AAT isolated from deficient subjects (PiZ) have shown that Z AAT has a reduced ability to inhibit connective tissue degradation by neutrophils compared to the M protein even at equivalent concentrations. This difference has been attributed to the slightly lower association rate constant of PiZ AAT ($1.2 \pm 0.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ versus $5.3 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for normal AAT (PiMM)) (Llewellyn-Jones et al, 1994). This provides one explanation for excessive connective tissue destruction by migrating neutrophils in subjects with AAT deficiency thereby leading to the development of extensive emphysema.

However more recently studies by Liou and Campbell, both on theoretical (Liou et al, 1995) and experimental grounds (Liou et al, 1996), have emphasised that the azurophil granule contains exceedingly high concentrations of neutrophil elastase (5.33 mM). As these granules are exocytosed (when the cell is activated), the elastase would start to

diffuse away from the granule leading to a gradual drop in concentration until it equals the physiological concentration of the inhibitors surrounding them. At this point connective tissue degradation would cease. Studies predict and show that the area of enzyme activity is well restricted until the concentration of surrounding AAT falls below $10\mu\text{M}$. At this point there would be, and is, an exponential rise in the area of persistent activity of neutrophil elastase. The implications of this observation are that for subjects with PiZ AAT deficiency where the concentration is less than $10\mu\text{M}$ (average around $5\mu\text{M}$), each neutrophil that enters the interstitium of the lung would produce an excessive area of connective tissue degradation, due to the inability of the critically low concentration of AAT to prevent elastase activity for a wide area around the activated neutrophil.

The above studies have provided an understandable mechanism which explain how emphysema can develop excessively in subjects with AAT deficiency. The addition of cigarette smoking is likely to enhance this process since the lungs of smokers contain more neutrophils than non smokers (Reynolds et al, 1974) that will have migrated through the interstitium causing some connective tissue degradation.

Other factors, however, are also likely to be important. Silverman et al (Silverman et al, 1989) and Larsson (Larsson, 1978) identified lower respiratory tract infection as a risk factor for chronic airflow obstruction in adults with PiZ AAT deficiency, again a situation where neutrophil migration is likely to increase. In addition it is likely that there is some other familial component or risk factor involved in the development of

lung disease, and a history of COPD or asthma in a parent may be important suggesting a genetic tendency (Silverman et al, 1989).

Bronchial disease in PiZ AAT deficiency

The prevalence of chronic bronchitis as defined by the Medical Research Council (1965) is variable affecting from 20 - 59 % of patients (Eriksson, 1965)(Brantly et al, 1988)(Tobin et al, 1983). Early pathological studies demonstrated the presence of bronchiectasis in patients with AAT deficiency (Orell et al, 1972) and more recently a study by King and colleagues revealed 6 out of 14 PiZ patients had a diagnosis of bronchiectasis identified by computed tomography (King et al, 1996). Further studies are needed with larger cohorts of deficient patients to determine whether bronchiectasis is really associated more frequently with PiZ AAT deficiency.

The pathogenesis of emphysema related to PiZ AAT deficiency is now better understood, but there remains a paucity of information about bronchial disease in such patients apart from prevalence figures quoted above.

The bronchial disease is responsible for a significant proportion of the morbidity of patients with COPD, especially during acute exacerbations (Seemungal et al, 1998). Again there is little data in subjects with AAT deficiency, even though it is recognised that lower respiratory tract infection is a risk factor for the development of chronic airflow obstruction in such patients (Larsson, 1978). It is possible therefore to hypothesise that during infections, such patients may be particularly susceptible to lung damage by proteinases due to the low baseline concentration of AAT, and the absence of

a physiologically important acute phase response (Kueppers, 1968) resulting in excessive neutrophil mediated lung injury.

1.2.4.2.2 Other proteinase inhibitors

Although AAT is the main serum inhibitor of neutrophil elastase, secretory leukoprotease inhibitor (SLPI) is thought to be the major bronchial inhibitor of this enzyme (Morrison et al, 1987).

Secretory leukoprotease inhibitor

Studies in the 1970's identified a low molecular mass (12 kDa) inhibitor of neutrophil elastase that was in bronchial secretions, subsequently called secretory leukoprotease inhibitor. In addition to its anti-elastase function, SLPI may also have a broad spectrum antibiotic functions that include anti-retroviral, bactericidal, and antifungal activity (Tomee et al, 1998). SLPI is not glycosylated, and is produced locally in the lung by epithelial cells (Maruyama et al, 1994), and is present in serous glands (De Water et al, 1986)(Mooren et al, 1983) and clara cells (Sallenave et al, 1993).

Regulation of the production of SLPI is only partly understood. Early studies suggested it was constitutively expressed and that its production was constant (Dijkman et al, 1986). However, more recent studies have shown that corticosteroids increase its concentration in the bronchial secretions in vivo (Stockley et al, 1986) and its production by epithelial cells in vitro (Abbinante-Nissen et al, 1995). Furthermore, its secretion may actually decrease during infection (Piccioni et al, 1992) suggesting negative control, and

recent studies have confirmed that neutrophil elastase can reduce SLPI secretion by epithelial cells (Sallenave et al, 1994).

Because it is the major inhibitor of neutrophil elastase in bronchial secretions (accounting for up to 90% of all inhibition (Morrison et al, 1987)) it is probably the most important inhibitor protecting the upper airways from neutrophil elastase. The role of this protein in the lower airways however has been more controversial. Early studies suggested that the only inhibitor of neutrophil elastase in the peripheral airways was AAT (Gadek et al, 1981). This observation is at variance with other studies that have not only demonstrated the presence of SLPI in the lower airways (Boudier et al, 1983), but shown that it is functionally active (Boudier et al, 1983)(Afford et al, 1988) and can be produced by type II pneumocytes (Sallenave et al, 1994). These data suggest that SLPI is likely to have a role at the alveolar level. Furthermore SLPI may be secreted from the basolateral surface of epithelial cells (Dupuit et al, 1993) and can be identified in the lung interstitium (Willems et al, 1986), suggesting it may also have a protective role at that site as well as in the airways.

Other elastase inhibitors

Other protease inhibitors have been identified in the lung with the ability to inhibit neutrophil elastase although their role is less clear. These inhibitors include elafin (Sallenave et al, 1992) a 7kDa inhibitor produced locally in the lung by type II pneumocytes (Sallenave et al, 1994) and α_2 macroglobulin which is a serum derived inhibitor of neutrophil elastase although its large mass (725 kDa) largely prevents its

diffusion into lung secretions in effective amounts unless the lung is also inflamed (Stockley et al, 1979). Furthermore, even when present, α_2 macroglobulin contributes little to the overall lung antineutrophil elastase screen (Stockley, 1986).

1.2.5 Influence of bacterial colonisation

The normal bronchial tree is kept sterile through a sophisticated system of defence which consists of primary and secondary mechanisms. Patients with chronic bronchitis, however, are frequently colonised by bacteria even in the stable clinical state (Monso et al, 1995), but little is known how this influences the bronchial inflammation or natural history.

The complex interactions between bacteria and the lung and host defences can result in several distinct clinical situations.

1) If the bacterial load is low, the primary defences can sterilise the bronchial tree without activation of the secondary host defences. This has been shown in animal models revealing the primary host defences are able to clear bacterial loads of up to 2×10^5 colony forming units per ml (cfu/ml) (Onofrio et al, 1983) with little or no activation of the secondary host defences.

2) Bacteria may cause acute infections probably due to a higher bacterial load. In this instance the primary host defences become overwhelmed, and the secondary defences are activated. The net effect of these processes results in sterilisation of the bronchial tree and the process resolves. The processes involved in the secondary host response are currently poorly understood but may consist of 3 distinct phases.

Initiation:

It is plausible that airway bacterial load can initiate the secondary host response, as bacterial products may stimulate bronchial epithelial cells to secrete interleukin 8 (Bedard et al, 1993)(Khair et al, 1994) and activated alveolar macrophages by the phagocytic process may be a source of interleukin 8 (Standiford et al, 1992) and leukotriene B4 (Bigby et al, 1987)(Hubbard et al, 1991). Both these chemoattractants are thought to be of major importance in neutrophil recruitment (Mikami et al, 1998), but in addition further neutrophil migration will be further enhanced by stimulation of endothelial adhesion molecules by the pro inflammatory cytokines (Khair et al, 1994)(Wardlaw, 1990). In addition to the neutrophil, monocytes recruited to sites of inflammation may also play an important role in the inflammatory process. A subpopulation (20-30%) of circulating monocytes have neutrophil-like pro inflammatory properties (P phenotype), including avid adherence to the extracellular matrix, the ability to produce reactive oxygen species, high neutrophil elastase content, and proteolytic activity against elastin and fibronectin (Owen et al, 1994). These pro inflammatory monocytes have considerably higher phagocytic activity than other monocytes and also lack the HLA-DR antigen, which means they cannot participate in specific immune responses. It is this neutrophil like pro inflammatory subpopulation that is recruited rapidly by chemoattractants to sites of inflammation (Owen et al, 1994), where they can either promote resolution of inflammation or contribute to tissue injury in a similar way to the neutrophil. Finally the inflammatory response that forms a key part of this secondary defence system results in leakage of additional proteins, such as immunoglobulins and complement factors from the circulation which will also facilitate bacterial phagocytosis and death (Stockley, 1995).

Amplification

Activation of neutrophils following migration results in the release of further chemoattractants including both LTB₄ (Ford-Hutchinson et al, 1980) and IL8 (Takahashi et al, 1993) which will add to the chemoattractant concentration in the airway. In addition the release of neutrophil elastase from activated neutrophils has been reported to increase IL8 production by epithelial cells (Nakamura et al, 1992) and induces production of LTB₄ by alveolar macrophages (Hubbard et al, 1991) again adding to the chemoattractant gradient, further increasing neutrophil influx.

Infection related indirect mechanisms may also amplify and perpetuate neutrophil influx, including: 1) chemoattractants and active leukocyte elastase released from neutrophils (Takahashi et al, 1993)(Doerfler et al, 1989)(Nakamura et al, 1992); 2) leukocyte elastase-inhibitor complexes (Banda et al, 1988); and 3) products of local extracellular matrix degradation (Senior et al, 1980).

Resolution of the inflammatory response

The mechanisms involved in switching off the secondary host defences have been least studied but may involve some of the mechanisms described below.

- a) As the bacterial load is reduced, this would be expected to decrease the bacterial stimulus to the inflammatory processes (reducing release of the chemoattractants IL8, LTB₄, and other factors such as TNF- α and neutrophil elastase). These changes would reduce neutrophil recruitment and this down-regulation may also

be facilitated by the release of anti-inflammatory cytokines such as interleukin 10 (Poulter, 1997).

- b) The acute phase response may also be important with serum AAT levels increasing up to threefold as part of the systemic effect of lung inflammation (Crockson et al, 1966). This along with increased lung endothelial and epithelial permeability, would result in major leakage of this proteinase inhibitor into the lung which will tend to reduce the amount of free neutrophil elastase (Stockley, 1995). Such a change would potentially reduce IL8 and LTB4 production.
- c) Other acute phase proteins such as alpha-1-chymotrypsin rises several fold within 8 hours (Aronsen et al, 1972). This protein is thought to be a major inhibitor of neutrophil chemotaxis (Lomas et al, 1995), which could "down-regulate" the inflammatory process by a direct effect on reducing neutrophil influx.
- d) Apoptosis represents a granulocyte fate which by a number of mechanisms would tend to limit inflammatory tissue injury and promote resolution rather than progression of inflammation: apoptosis is responsible for macrophage recognition of senescent neutrophils with retention of the integrity of the plasma membrane; the apoptotic neutrophil loses its cytotoxic and secretory capacity; the macrophage possesses a huge phagocytic capacity for apoptotic neutrophils which it rapidly ingests and degrades without disgorging neutrophil contents; and the macrophage utilizes a novel phagocytic recognition mechanism which fails to trigger the

release of pro-inflammatory macrophage mediators during the phagocytosis of apoptotic neutrophils (Haslett et al, 1994). The release of pro-inflammatory cytokines Interleukin 6 (IL6) (Afford et al, 1992) and TNF- α (Hachiya et al, 1995) may paradoxically promote apoptosis, leading to phagocytosis of the neutrophils by resident macrophages (Haslett et al, 1994).

3) All these and, almost certain, other processes result in a return of the lung to its normal sterile state. However in some patients sterilisation does not occur and persistent colonisation is the result. In such patients there is a balance between the efficacy of the host defence mechanisms and bacterial replication which achieves a position of stability. This usually occurs in patients with an abnormal host defence e.g. patients where mucociliary clearance may be impaired as in patients with chronic bronchitis (Monso et al, 1995) where bacteria may be permanently present in the secretions even in the stable clinical state. Little is known, however, how this influences the bronchial inflammation or natural history in such patients.

The bacteria isolated are thought to be of low pathogenicity (non-typable *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus parainfluenzae*) but may themselves facilitate the process of colonisation by interfering with host defence. For instance bacterial products can interfere with neutrophil chemotactic responses inhibiting neutrophil migration and therefore down-regulating the inflammatory response (Cundell et al, 1993). In addition bacteria may generate features

of chronic bronchitis and impair other features of the primary host defences, thus facilitating bacterial persistence in the airways. The processes involved may include:

- i. Interference with ciliary function via cilia toxins such as pyocyanin and hydroxyphenazine (Wilson et al, 1987).
 - ii. The generation of excess mucus production (Li et al, 1997).
 - iii. The production of epithelial damage (Amitani et al, 1991).
 - iv. Damage to immunoglobulin A (IgA) by specific proteases (Kilian et al, 1980).
 - v. Change their antigenic response to avoid the immune response (Grocneveid et al, 1988), thus facilitating their survival.
- 4) Finally in some instances colonisation is associated with a major degree of inflammation which may be detrimental and produce a vicious circle (Figure 1-4) of ongoing bronchial inflammation, further facilitating bacterial persistence. In this situation there is usually an overactive response of both the immune system and inflammatory response.

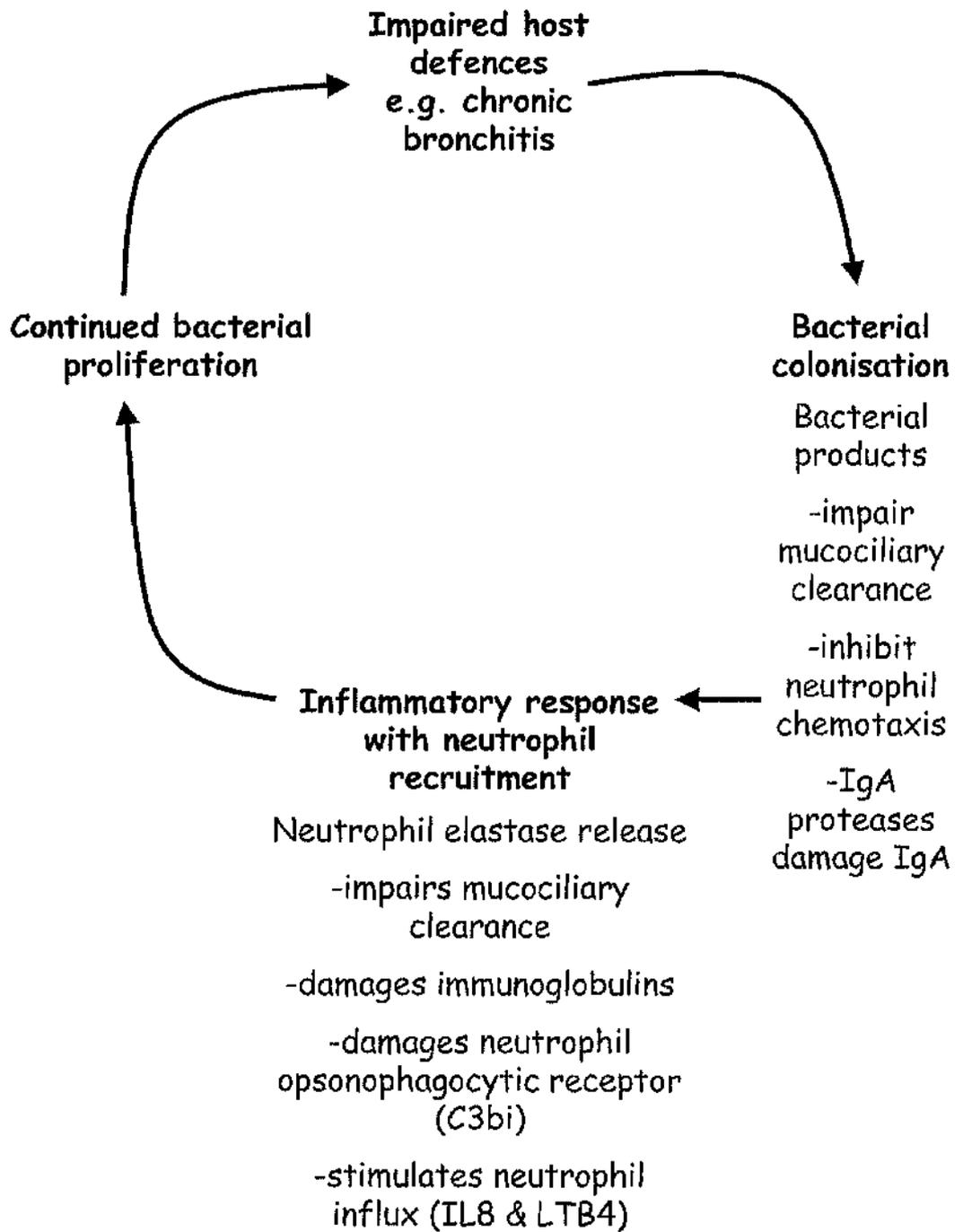


Figure 1-4: The vicious cycle of bronchial inflammation and bacterial colonisation.

Immune system

Patients who are chronically colonised often have increased serum immunoglobulin concentrations with increased immunoglobulins in their secretions (Bilton et al, 1992) (including both the IgA₁ and IgA₂ subclasses (Burnett et al, 1987)). The lung tissues contain increased numbers of T lymphocytes, predominantly with CD8+ cell population (Lapa e Silva et al, 1989). In some patients the host immune response may be made less efficient by the production of the wrong subclass of antibodies that prevent effective phagocytosis thus protecting the important clearance mechanisms (Fick et al, 1986).

Inflammatory response

Activation of secondary host defences and the development of inflammation should result in clearance of the bacterial load. However, if this secondary response fails to sterilise the bronchial tree continued bacterial proliferation can occur despite a marked inflammatory response (Stockley, 1995) thereby resulting in a vicious circle of tissue damage (Figure 1-4). In this vicious circle (Figure 1-4), both the bacteria (described earlier) and the inflammatory response itself (thought to be mediated by neutrophil elastase) may play a role in the persistence of bacterial colonisation by reducing the efficacy of the host defences in the respiratory tract whilst perpetuating the inflammation.

In particular neutrophil elastase can (Figure 1-3):

- i. lead to excess mucus production (Sommerhoff et al, 1990).
- ii. cause mucus gland hyperplasia (Snider et al, 1985).
- iii. cause bronchial epithelial damage (Amitani et al, 1991).
- iv. reduce ciliary beating (Smallman et al, 1984).
- v. decrease phagocytic clearance by cleavage of immunoglobulins preventing opsonisation (Doring et al, 1986).
- vi. destroy phagocytic receptors on the neutrophil itself (C3bi) thereby reducing opsonophagocytosis (Tosi et al, 1990).
- vii. stimulate epithelial cells to produce IL8 (Nakamura et al, 1992) and macrophages to produce LTB4 (Hubbard et al, 1991) thus amplifying the neutrophil response.
- viii. Perpetuate its own function by decreasing the concentration of SLPI (Sallenave et al, 1994).

1.3 AIMS OF THESIS

The aims of the thesis were several fold:

1. To examine the interrelationship of sputum inflammatory markers in patients with chronic bronchitis in a stable clinical state including:
 - a) the relationship of myeloperoxidase (a measure of neutrophil influx) to elastase activity (a measure of the potential damage).
 - b) the relationship of the neutrophil chemoattractants interleukin 8 and leukotriene B4 to both myeloperoxidase and elastase activity.
 - c) the relationship of elastase activity to its major bronchial inhibitor secretory leukoprotease inhibitor, and airway epithelial leakage (as assessed by the sputum/serum albumin ratio %).
 - d) the relationship between FEV₁ (% Predicted) and inflammation as reflected by neutrophil numbers (myeloperoxidase) or protein leak.

2. To investigate the relationship between bacterial colonisation and upper airways inflammation from patients with chronic bronchitis in a stable clinical state to determine:
 - a) whether patients who are colonised have more upper airways inflammation than those not colonised.
 - b) whether there is a threshold effect between bacterial load and recruitment of the secondary host defences.
 - c) whether different bacteria have a similar effect on upper airways inflammation.

3. To study upper airways inflammation from patients with chronic bronchitis in a stable clinical state and investigate:
 - a) whether alpha-1-antitrypsin deficiency influences upper airways inflammation.
 - b) whether any effect is influenced by smoking.
 - c) whether bacterial colonisation has an influence in patients with and without homozygous PiZ alpha-1-antitrypsin deficiency.

4. Finally I wished to study upper airways inflammation during exacerbations in patients with chronic bronchitis with and without alpha-1-antitrypsin deficiency:
 - a) to determine whether upper airways inflammation in PiZ alpha-1-antitrypsin deficiency is greater than patients without deficiency.
 - b) to study airways inflammation during an exacerbation and compare this to the stable state in patients with PiZ alpha-1-antitrypsin deficiency.
 - c) to study the acute phase response of alpha-1-antitrypsin during the acute exacerbation in patients with PiZ alpha-1-antitrypsin deficiency.
 - d) to study the time course to recovery in patients with PiZ alpha-1-antitrypsin deficiency.

2. GENERAL METHODS

2.1 ETHICAL APPROVAL AND CONSENT

All studies reported in this thesis had ethical approval from the local ethics committee at the Queen Elizabeth Hospital, Birmingham. All patients gave signed consent.

2.2 SAMPLE COLLECTION & ANALYSIS

2.2.1 Sputum and serum collection

Patients provided a sputum sample that had been collected into a sterile container during the four hours following rising from bed. Patients delivered the sputum sample or the sputum was collected and processed within 2 hours. Serum was obtained at the time of sputum collection.

2.2.2 Sputum characteristics

Each sputum was assigned a value based upon its colour, by visual comparison with a 9 point chart (Bronkotest, Heredilab, Inc., Salt Lake City, USA). Available integers were 0 (water clear) to 8 (corresponding to the deepest green colour observed in cystic fibrosis) (0-2 = Mucoid, 3-5 = Mucopurulent, 6-8 = Purulent) (a copy of the colour chart is in the diary card at the end of the thesis).

2.2.3 Quantitative sputum bacteriology

A portion of the sputum collected was immediately removed for determination of the number of viable organisms present as described previously (Pye et al, 1995). Results are expressed as colony forming units per millilitre of sputum (cfu/ml). For each sample, the predominant organism present was determined (Pye et al, 1995).

2.2.4 Sputum and serum processing

The remaining sputum sample was ultracentrifuged at $50,000 \times g$ for 90 minutes at 4°C . The sol phase was removed and stored at -70°C until it was analysed. Venous blood was collected into a plain vacutainer tube, allowed to clot and then centrifuged at $1500 \times g$ for ten minutes at 25°C . The serum was then stored at -70°C until analysis.

2.3 BIOCHEMICAL ASSAYS

2.3.1 Myeloperoxidase from sputum sol phase

Measurement of sputum myeloperoxidase activity (MPO) was used as an assessment of neutrophil influx. MPO activity was measured from sputum sol phase by chromogenic substrate assay relative to a preparation of lysed neutrophils. Ten microlitres of standard or sample were added to the wells of a microtitre plate, followed by 150 microlitres of 1mg/ml (w/v) O-dianisidine dihydrochloride, 0.01% (v/v) 30% H₂O₂ in 50mM K₃HPO₄ and 0.5% w/v hexadecyl trimethyl ammonium bromide (pH 6) (Sigma Aldrich Co. Ltd. Poole, Dorset, UK). The plate was then incubated for 15 minutes at 25°C and the absorbance was then measured at 450nm using a Dynatech MR 5000 (Dynatech Corporation, Burlington, VT) and the MPO concentration interpolated from the standard curve and expressed as Units/ml.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-1. The standard curve is shown in Figure 2-1. The inter-assay coefficient of variation (standard deviation/mean (%)) was less than 10%.

Standards	Typical Sample Dilution	Lower Limit of Detection
0.0976 - 50 Units/ml	1 in 5 - 1 in 500	0.1 Units/ml

Table 2-1: The standards, sample dilution used, and the lower limit of detection for the myeloperoxidase assay is illustrated.

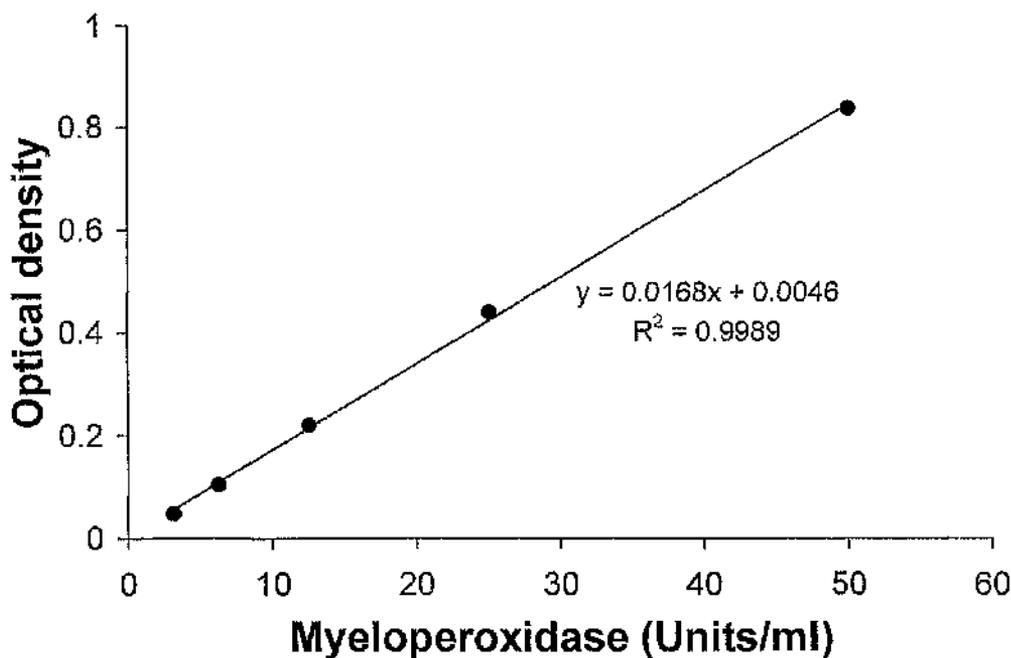


Figure 2-1: Standard curve for myeloperoxidase.

2.3.2 Neutrophil elastase activity from sputum sol phase

Neutrophil elastase standard was purified from empyema pus according to the method of Baugh and Travis (Baugh et al, 1976). The activity of the elastase standard was determined by active site titration using published kinetic constants (Nakajima et al, 1979). Neutrophil elastase activity present in the samples was measured spectrophotometrically using the synthetic substrate Succinyl-ala-ala-ala-paranitroanilide. 30 μ l of standard or sample were added to wells of a microtitre plate (Life Technologies, Ltd., Paisley, UK), followed by 150 μ l of 1mg/ml (w/v) Succinyl-ala-ala-ala-paranitroanilide (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) in 0.01M Tris-HCl, 0.5M NaCl, and 0.1% Triton X-100 (pH 8.6). The plate was then incubated

for 30 minutes at 37°C, following which the absorbance 410nm was measured and the elastolytic activity obtained by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-2. The lower limit of detection was 10 nM, and samples with less than this activity were considered to be zero for statistical purposes. The standard curve is shown in Figure 2-2. The inter-assay coefficient of variation was less than 1%.

Standards	Typical Sample Dilution	Lower Limit of Detection
0.8 nM - 0.1 μM	1 in 10 - 1 in 50	0.01 μM

Table 2-2: The standards, sample dilution used, and the lower limit of detection for the elastase assay is illustrated (Substrate Succinyl-ala-ala-ala-paranitroanilide).

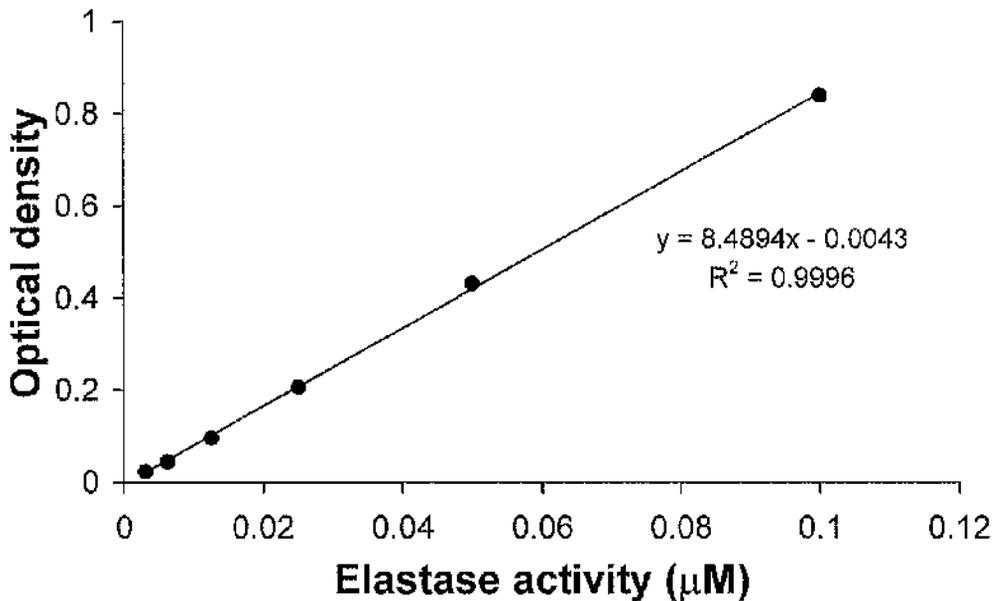


Figure 2-2: Standard curve for elastase.

Later studies used the substrate methoxysuccinyl-ala-ala-pro-val-paranitroanilide (MeOSAAPVpNa) (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) which was more sensitive at the bottom range of the assay. For this assay 20 μ l of standard or sample were added to wells of a microtitre plate (Life Technologies, Ltd., Paisley, UK), followed by MeOSAAPVpNa (0.3mM) in phosphate buffer (0.2M, pH 8.0). The reaction was allowed to continue for one hour at 37°C and then stopped by the addition of 200 μ l of 1 N acetic acid. The absorbance was read at 410nm and the elastolytic activity obtained from the standard curve by interpolation.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-3. The inter-assay coefficient of variation was less than 1% and the lower limit of detection was 0.8 nM (samples with less than this activity were considered to be zero for statistical purposes).

Standards	Typical Sample Dilution	Lower Limit of Detection
0.8 nM - 0.1 μ M	Pure sample - 1 in 5	0.8 nM

Table 2-3: The standards, sample dilution used, and the lower limit of detection for the elastase assay is illustrated (Substrate MeOSAAPVpNa).

2.3.3 Chemoattractants from sputum sol phase

2.3.3.1 Interleukin 8

IL8 was measured by ELISA using a commercially available Quantikine kit (R&D Systems Europe Ltd, Abingdon, UK). One-hundred microlitres of assay diluent RD1-8 were added to each well, followed immediately by 50 μ l of standard or sample (dissolved in calibrator diluent RD5P). One-hundred microlitres of IL8 conjugate were then added to each well. The wells were covered with an adhesive strip and the plate was incubated for 2.5 hours at 25°C. Twenty millilitres of wash buffer concentrate were dissolved in 480 ml of distilled water to produce 500ml of wash buffer. Each well was washed six times with wash buffer. Two hundred microlitres of substrate solution (equal volumes of colour reagents A and B) were then added to each well and the plate was incubated for 30 minutes at 25°C. The reaction was stopped by using 50 μ l of 1M sulphuric acid. The absorbance at 450nm with a 570nm correction was measured using a Dynatech MR 5000 microplate reader, and the IL8 concentration calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-4. The standard curve is shown in Figure 2-3. The inter-assay coefficient of variation was 7%, and samples spiked with pure IL8 resulted in greater than 85% recovery Figure 2-4.

Standards	Typical Sample Dilution	Lower Limit of Detection
0.004 - 0.25 nM	1 in 100 - 1 in 500	0.008 nM

Table 2-4: The standards, sample dilution used, and the lower limit of detection for the interleukin 8 assay is illustrated.

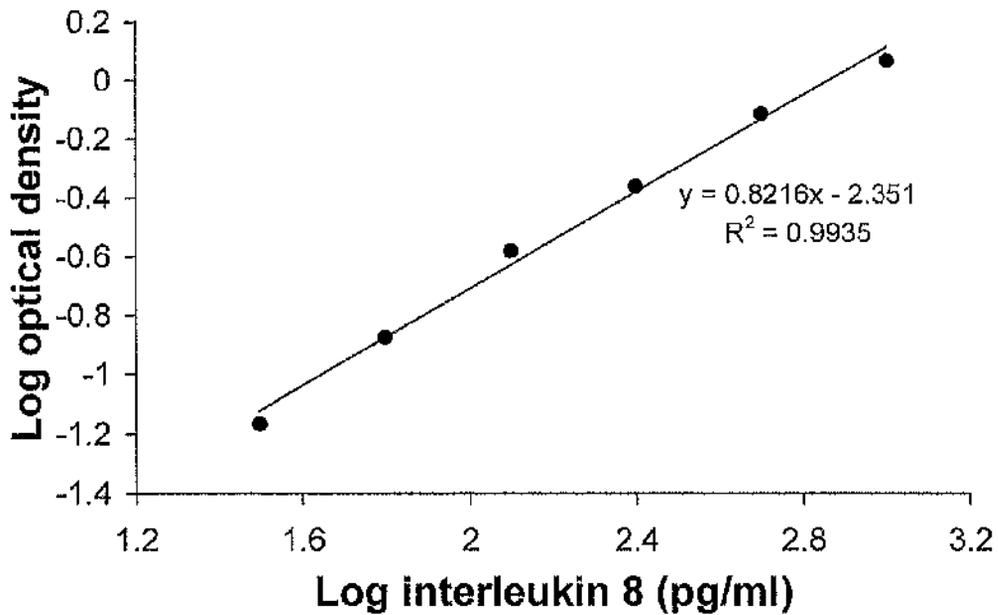


Figure 2-3: Standard curve for interleukin 8.

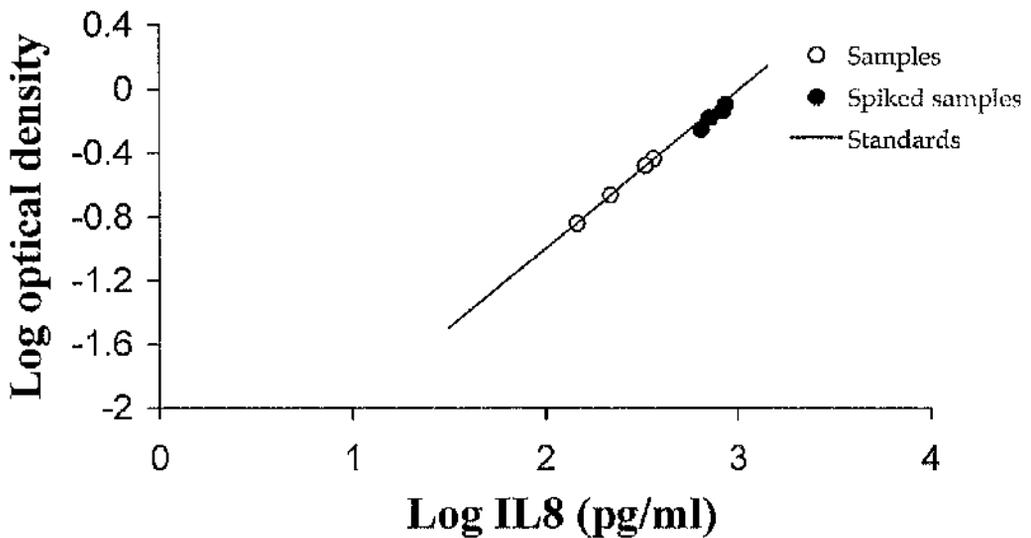


Figure 2-4: The optical density of the ELISA reaction (vertical axis) is plotted against the IL8 concentration (horizontal axis). The open circles are 4 sample results and the closed circles are spiked with known amounts of IL8. Results lie close to the line of the standard curve indicating good recovery of the sample spike.

2.3.3.2 Leukotriene B4

LTB4 was measured by ELISA using a commercially available kit (Amersham International plc, Buckinghamshire, UK). One-hundred microlitres of assay buffer were added to the non-specific-binding wells, followed immediately by 50 µl of standard or sample. Fifty microlitres of LTB4 antiserum was then added to all wells except the non-specific-binding wells and the plate covered with an adhesive strip. The plate was then incubated for 2 hours at 25°C whilst shaking at 150 rpm on a rotary shaker (Fisher Scientific, Loughborough, UK). Fifty microlitres of LTB4 peroxidase conjugate was then added to every well and the plate covered with an adhesive strip and the plate was then further incubated for 1 hour at 25°C whilst shaking at 150 rpm on a rotary shaker (Fisher Scientific, Loughborough, UK). Each well was washed four times with 370µl of wash buffer. One-hundred microlitres of substrate solution was then added to each well and the plate covered with an adhesive strip. The plate was incubated for 20 minutes at 25°C whilst shaking at 150 rpm on a rotary shaker (Fisher Scientific, Loughborough, UK). The reaction was then stopped by using 100 µl of 1M sulphuric acid. The absorbance at 450nm with a 570nm correction was measured using a Dynatech MR5000 microplate reader and the LTB4 concentration calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-5. The standard curve is shown in Figure 2-5. The inter-assay coefficient of variation was <6%, and samples spiked with pure LTB4 resulted in greater than 85% recovery (Figure 2-6).

Standards	Typical Sample Dilution	Lower Limit of Detection
0.028 - 0.9 nM	1 in 30 - 1 in 50	0.17 nM

Table 2-5: The standards, sample dilution used, and the lower limit of detection for the leukotriene B4 assay is illustrated.

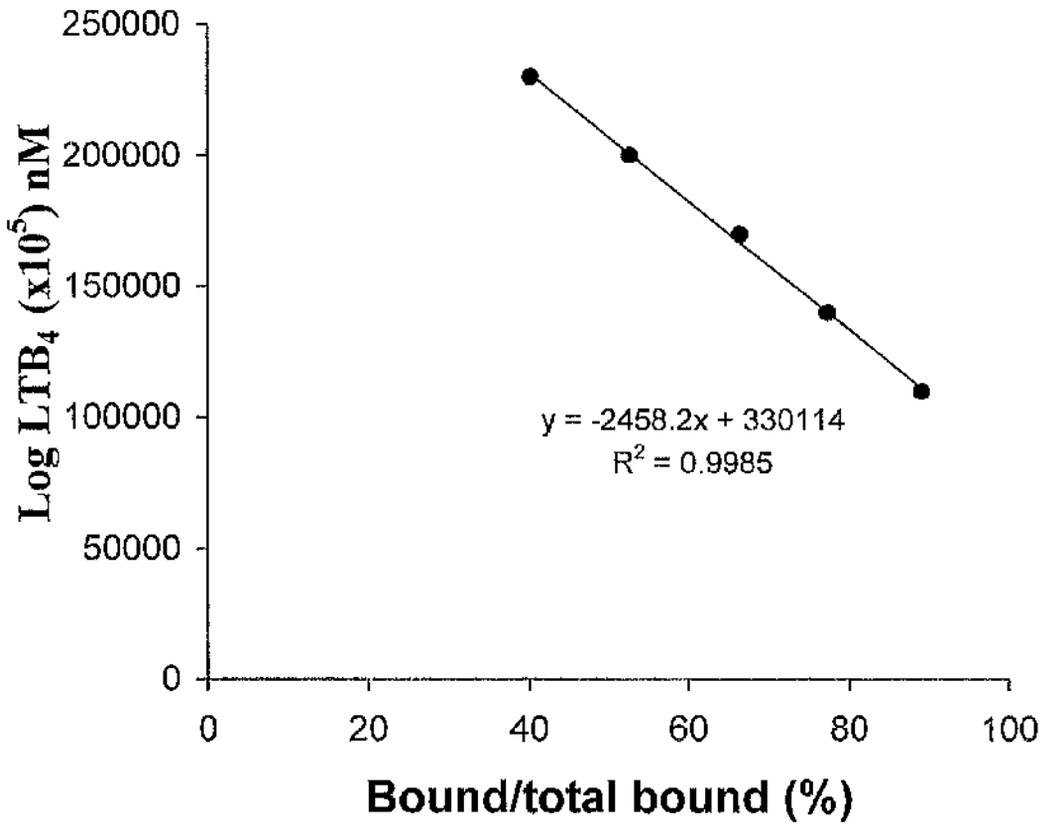


Figure 2-5: Standard curve for LTB4. The bound/total bound LTB4 (horizontal axis) is plotted against the LTB4 concentration (vertical axis).

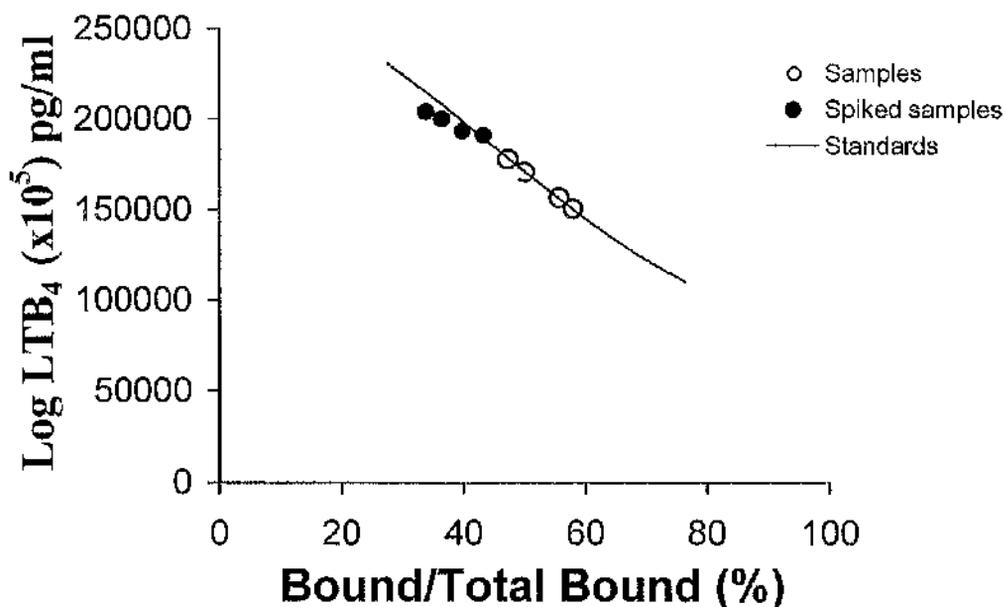


Figure 2-6: The bound/total bound LTB₄ (horizontal axis) is plotted against the LTB₄ concentration (vertical axis). The open circles are 4 sample results and the closed circles are spiked with known amounts of LTB₄. Results lie close to the line of the standard curve indicating good recovery of the sample spike.

2.3.4 Elastase inhibitors from sputum sol phase

2.3.4.1 Secretory leukoprotease inhibitor

SLPI was measured by ELISA using a commercially available Quantikine kit (R&D Systems Europe Ltd, Abingdon, UK). One-hundred microlitres of assay diluent RD1Q were added to each well, followed immediately by 100µl of standard or sample. The plate was covered with an adhesive strip and incubated for 2 hours at 25°C. Twenty millilitres of wash buffer concentrate were dissolved in 480 ml of distilled water to produce 500ml of wash buffer. Each well was then washed four times with 370 µl of wash buffer. Two-hundred microlitres of SLPI conjugate were then added to each well, the wells covered with an adhesive strip, and the plate was incubated for 2 hours at

25°C. Each well was washed four times with 370 µl of wash buffer. Two hundred microlitres of substrate solution (equal volumes of colour reagents A and B) were then added to each well and the plate was incubated for 20 minutes at 25°C. The reaction was stopped by using 50 µl of 1M sulphuric acid. The absorbance at 450nm with a 570nm correction was measured using a Dynatech MR 5000 microplate reader, and the SLPI concentration calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-6. The standard curve is shown in Figure 2-7. The inter-assay coefficient of variation was <10%, and samples spiked with pure SLPI resulted in greater than 85% recovery (Figure 2-8).

Standards	Typical Sample Dilution	Lower Limit of Detection
8 - 333 pM	1 in 10,000 - 1 in 100,000	1,125 pM

Table 2-6: The standards, sample dilution used, and the lower limit of detection for the SLPI assay is illustrated.

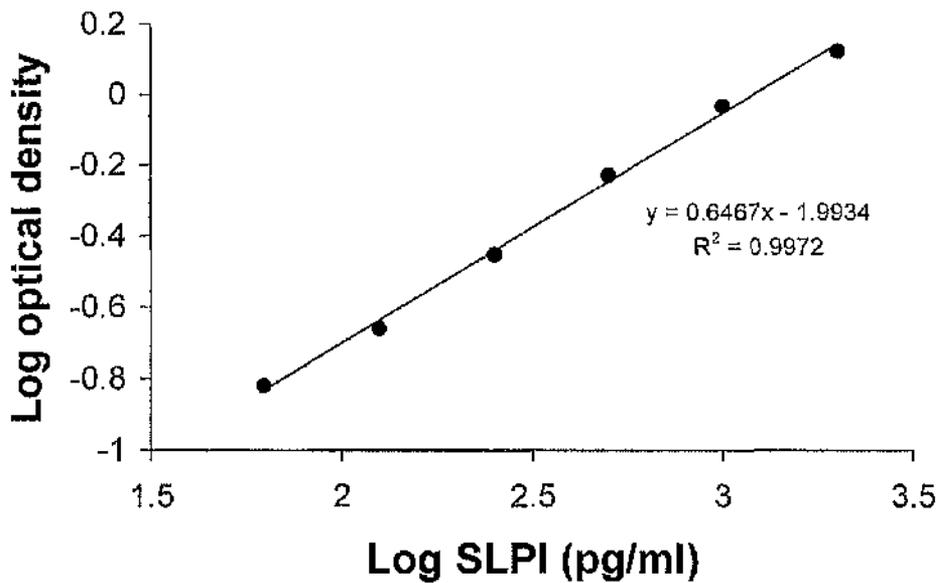


Figure 2-7: Standard curve for SLPI.

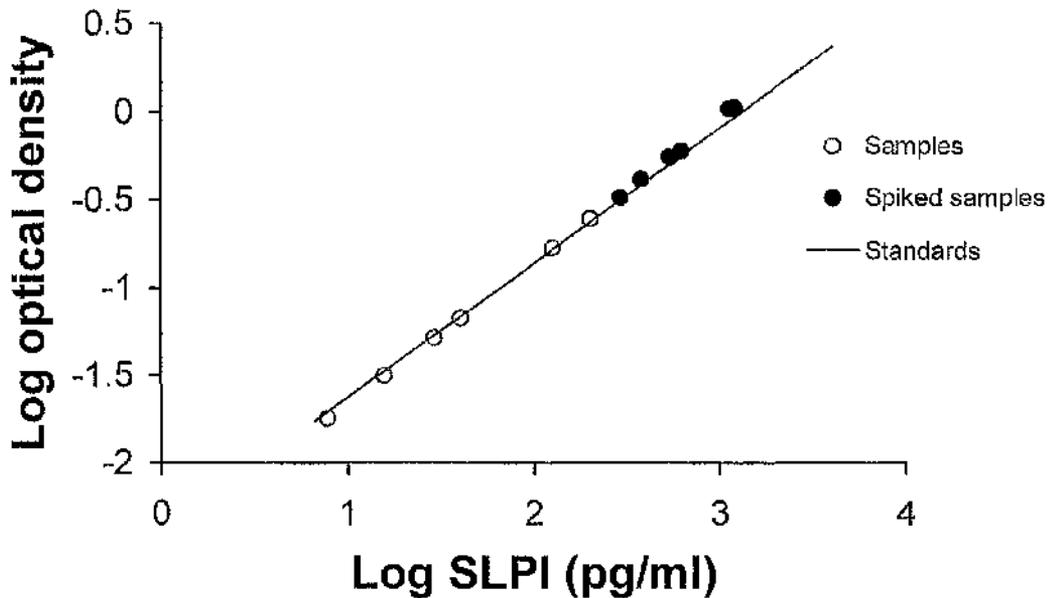


Figure 2-8: The optical density of the ELISA reaction (vertical axis) is plotted against the SLPI concentration (horizontal axis). The open circles are 6 sample results and the closed circles are spiked with known amounts of SLPI. Results lie close to the line of the standard curve indicating good recovery of the sample spike.

2.3.4.2 Alpha-1-antitrypsin

AAT was measured by ELISA relative to a commercially available serum standard (The Binding Site Limited, Birmingham, UK). Two hundred microlitres of goat antihuman AAT antibody (The Binding Site Limited, Birmingham, UK) in 0.05M sodium carbonate / bicarbonate pH 9.6 was added to a MAXISORP (Nunc) microtitre plate and incubated overnight (4 °C). The plate was then washed three times with PBS containing 1% (v/v) Tween 20. Two hundred microlitres of standard or sample were added to the plate followed by a 60 minute incubation at 37 °C. The plate was then washed three times with PBS containing 1% (v/v) Tween 20. Two hundred microlitres of goat antihuman AAT antibody peroxidase conjugate (The Binding Site Limited, Birmingham, UK) in PBS containing 1% (v/v) Tween 20 was then added to each well, the plate was then incubated for 60 minutes at 37 °C. Each well was then washed three times with PBS containing 1% (v/v) Tween 20 (Sigma). Two hundred microlitres of 1mg/ml O-phenylamine dihydrochloride (Sigma-Aldrich Company Limited, Poole, Dorset, UK), 0.01% H₂O₂ in 1 volume 0.1M citric acid (Sigma-Aldrich Company Limited, Poole, Dorset, UK) / 2 volumes 0.1M Na₂HPO₄ pH 5 was then added to each well followed by an incubation for 10 minutes at 25 °C. The reaction was then stopped with 50µl of 0.5M citric acid. The plate was then read at 450nm and the AAT concentration calculated from the standard curve by interpolation.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-7. The standard curve is shown in Figure 2-9. The inter-assay coefficient of variation was less than 5%.

Standards	Typical Sample Dilution	Lower Limit of Detection
0.001 - 1.08 nM	1 in 500 - 1 in 100,000	1.68 nM

Table 2-7: The standards, sample dilution used, and the lower limit of detection for the alpha-1-antitrypsin assay is illustrated.

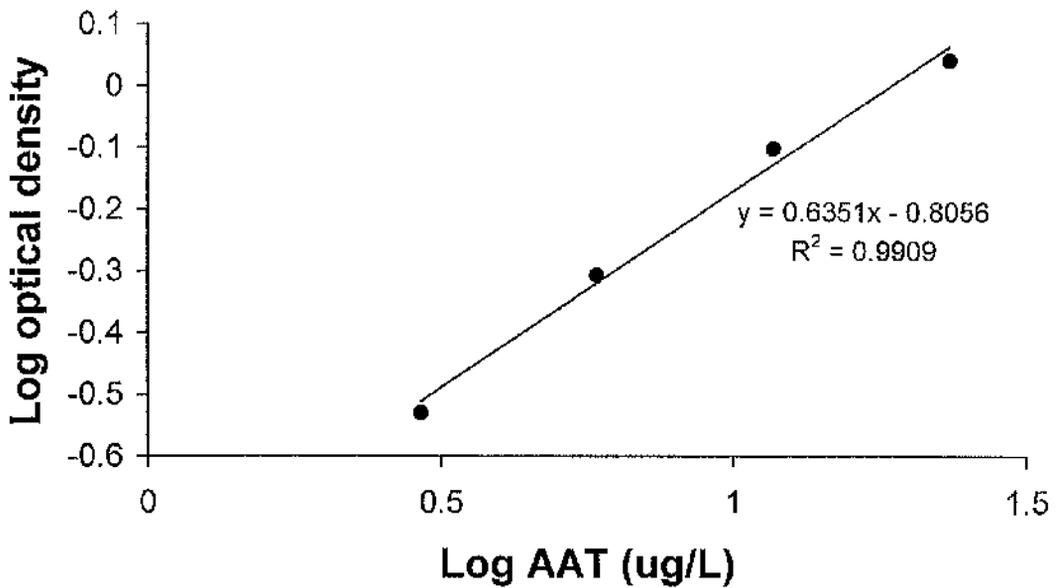


Figure 2-9: Standard curve for alpha-1-antitrypsin.

2.3.5 Protein leakage

The ratio of sol/serum albumin and sol/serum AAT was obtained for each patient sample and multiplied by 100 for convenience as described previously (Stockley, 1984). This value was used as a measure of protein transudation from plasma indicating the degree of lung airway leakage.

2.3.5.1 Measurement of albumin from sputum sol phase

Albumin from the sputum sol phase was measured by radial immunodiffusion using a commercially available kit (The Binding Site Limited, Birmingham, UK). Five microlitres of standard or sample was added to each well, and the plate was incubated for 72 hours at 25°C. The ring diameters were then measured using an eye piece graticule and the albumin concentration present in the samples calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-8. The inter-assay coefficient of variation was less than 5%.

Standards	Typical Sample Dilution	Lower Limit of Detection
16 - 160 mg/l	Pure to 1 in 20	10 mg/l

Table 2-8: The standards, sample dilution used, and the lower limit of detection for the sputum albumin assay is illustrated.

2.3.5.2 Serum albumin assay

Serum albumin was measured by radial immunodiffusion using a commercially available kit (The Binding Site Limited, Birmingham, UK). Five microlitres of standard or sample was added to each well, and the plate was incubated for 72 hours at 25°C. The ring diameters were then measured using an eye piece graticule and the albumin concentration present in the samples calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-9. The inter-assay coefficient of variation was less than 3%.

Standards	Typical Sample Dilution	Lower Limit of Detection
500 - 5,000 mg/l	1 in 20	300 mg/l

Table 2-9: The standards, sample dilution used, and the lower limit of detection for the serum albumin assay is illustrated.

2.3.6 Acute phase proteins

2.3.6.1 Serum Alpha-1-antitrypsin assay

AAT was measured by radial immunodiffusion using a commercially available kit (The Binding Site Limited, Birmingham, UK). Five microlitres of standard or sample was added to each well, and the plate was incubated for 72 hours at 25°C. The ring diameters were then measured using an eye piece graticule and the AAT concentration present in the samples calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-10. The inter-assay coefficient of variation was less than 2%.

Standards	Typical Sample Dilution	Lower Limit of Detection
5.4 - 53.8 µM	Pure	1.9 µM

Table 2-10: The standards, sample dilution used, and the lower limit of detection for the serum alpha-1-antitrypsin assay is illustrated.

2.3.6.2 Serum C reactive protein assay

Serum C reactive protein was measured by radial immunodiffusion using a commercially available kit (The Binding Site Limited, Birmingham, UK). Five microlitres of standard or sample was added to each well, and the plate was incubated for 72 hours at 25°C. The ring diameters were then measured using an eye piece graticule and the C reactive protein concentration present in the samples calculated by interpolation from the standard curve.

The standards, sample dilution used, and the lower detection of the assay is illustrated in Table 2-11. The inter-assay coefficient of variation was less than 2.5%.

Standards	Typical Sample Dilution	Lower Limit of Detection
5.2 - 52.0 mg/l	Pure	2 mg/l

Table 2-11: The standards, sample dilution used, and the lower limit of detection for the serum C reactive protein assay is illustrated.

2.4 Lung function testing

Full lung function testing including FEV₁, FVC, VC, TLC, RV, kCO and reversibility following nebulised β 2 agonist (salbutamol 5mg) were carried out in the stable clinical state (Society et al, 1994). They were expressed as % predicted for the patients age, sex, and height (Society et al, 1994).

**3. THE INTERRELATIONSHIP OF SPUTUM INFLAMMATORY
MARKERS IN PATIENTS WITH CHRONIC BRONCHITIS**

3.1 INTRODUCTION

The neutrophil and more specifically elastase released from its azurophil granules has been implicated as a major mediator of bronchial disease (Stockley, 1994). This serine proteinase is capable of generating many of the features of chronic bronchial disease including epithelial damage (Amitani et al, 1991), reduced ciliary beat frequency (Smallman et al, 1984), mucus gland hyperplasia (Snider et al, 1985), mucus secretion (Sommerhoff et al, 1990), and also to inactivate many of the critical lung host defences (Solomon, 1978)(Tosi et al, 1990). These latter effects may facilitate bacterial colonisation, which is often present in patients with chronic bronchitis (Monso et al, 1995).

However, for neutrophil elastase to have these effects, it has to overcome the anti-elastases that protect the tissues. SLPI is thought to be the most critical anti-elastase protecting the airways (Morrison et al, 1987) whereas alpha-1-antitrypsin is thought to be less important at this site, although critical at the alveolar level protecting against the development of emphysema (Gadek et al, 1981).

There have been few studies assessing the complex interplay of inflammatory cells and appropriate mediators in established lung disease. Recent limited studies have indicated that neutrophils are increased in the airway of patients with chronic bronchitis (Riise et al, 1995)(Pesci et al, 1998), and that the degree of neutrophil recruitment is related to the severity of airflow obstruction (Keatings et al, 1997), although separation of cause or effect has not been possible. The neutrophils or their product MPO is related to the

presence of a single chemoattractant, IL8 (Riise et al, 1995)(Pesci et al, 1998)(Yamamoto et al, 1997). Although these studies have suggested that IL8 may be the major factor influencing neutrophil recruitment, there are clearly other chemoattractants that may also play a role (Mikami et al, 1998). This concept is of importance since *in vitro* studies have indicated that chemoattractants may interact in an additive way (Mikami et al, 1998) and in addition there may be a hierarchical response influencing the cell and its migration pattern (Foxman et al, 1997).

Furthermore studies of the relationship between chemoattractants and neutrophils may be further complicated, although most of this concept is based on *in vitro* studies. Neutrophil activation can lead to the release of the chemoattractants IL8 (McCain et al, 1994) and LTB4 (Ford-Hutchinson et al, 1980) that could result in further neutrophil recruitment. Release of elastase from the neutrophil may stimulate production of IL8 from epithelial cells (Nakamura et al, 1992) and LTB4 from alveolar macrophages (Hubbard et al, 1991), and at the same time reduce production of its own natural inhibitor, SLPI (Sallenave et al, 1994) thereby perpetuating its function. Furthermore impairment of host defences by elastase could facilitate bacterial colonisation and endotoxin release from bacteria also provides a further potential mechanism for neutrophil recruitment by stimulating production of IL8 by epithelial cells (Khair et al, 1994).

The purpose of the present study was to investigate patients with chronic bronchitis and a wide spectrum of neutrophil influx to assess the interrelationship of the inflammatory markers: neutrophil influx (as reflected by the sputum MPO concentration) and the

chemoattractants (IL8 and LTB4); active neutrophil elastase to the chemoattractants, its own inhibitor SLPI, and bronchial protein leak (sputum/serum albumin ratio); FEV₁ (% Predicted) and MPO, elastase activity, IL8, LTB4, SLPI, and protein leak.

3.2 METHODS

3.2.1 Patient selection

Patients with a history of chronic bronchitis, as defined by the Medical Research Council (Medical Research Council, 1965), were studied in the stable clinical state. Patients that had an infection within the preceding 2 months of the study were excluded.

3.2.2 Processing of samples and lung function testing

The sputum and serum processing, the measurements of MPO, elastase activity, IL8, LTB₄, SLPI, and protein leakage (sputum/serum albumin ratio; %) is described in the general methods (Chapter 2). All subjects underwent standardised lung function testing (Society et al, 1994) at the time of the study.

3.2.3 Statistical analysis

Values are reported as mean (\pm standard error). Correlations between the inflammatory markers were assessed by the Spearman correlation coefficient (2 tailed). Samples were subdivided into 4 groups: those with no detectable elastase activity (Group A); those with small amounts of elastase activity (1-50nM) as Group B; those with a moderate level of elastase activity (50-100nM) as Group C; and those with high levels of elastase activity (>100nM) as Group D. The Kruskal-Wallis test was used to compare results between samples grouped by their elastase activity and where significant the Mann-Whitney U test for non paired data (2 tailed) was used to compare individual groups. A p value < 0.05 was considered to be significant.

3.3 RESULTS

The patients that entered the study included 101 samples from 55 subjects with chronic bronchitis (without alpha-1-antitrypsin deficiency), 61 samples from 40 patients with chronic bronchitis with homozygous (PiZ) alpha-1-antitrypsin deficiency, and 64 samples from 43 patients with idiopathic bronchiectasis diagnosed by high resolution computed tomography or bronchogram. Table 3-1 reveals the characteristics of the study population and the major results section reflects analysis of all samples as individual data points.

	Chronic bronchitis	Alpha-1-antitrypsin deficiency	Bronchiectasis
Number of patients	55	40	43
Mean age (years)	65.0	48.7	65.2
Range	(42-79)	(33-62)	(36-82)
Gender (M/F)	38/17	28/12	14/29
Current smoker	30	9	3
Ex- smoker	25	31	19
Never smoker	0	0	21
Mean FEV ₁ (% Predicted)	62.6 ± 3.0 (13.6-113.9%)	30.2 ± 2.5 (12-56%)	69.0 ± 3.8 (28.0-133.5%)

Table 3-1: Characteristics of the study population.

3.3.1 Sputum MPO and elastase activity

Myeloperoxidase activity was detectable in all but one sample and 147 samples (65.0%) had detectable elastase activity > 1 nM. The relationship between these two factors is summarised in Figure 3-1.

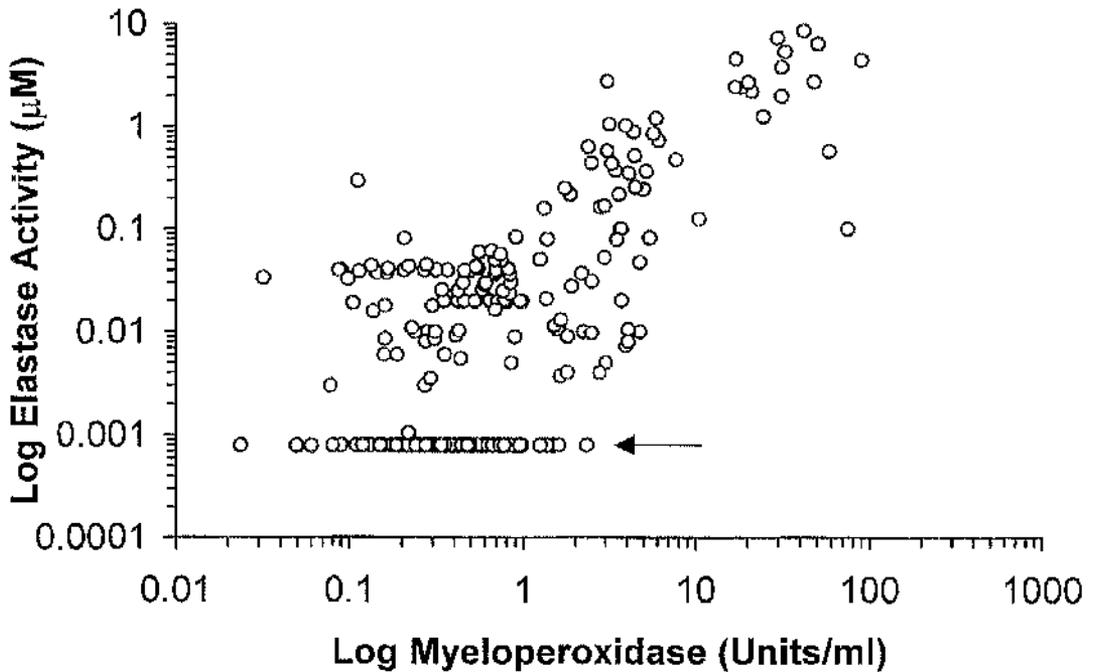


Figure 3-1: The relationship between sputum MPO (Units/ml) and elastase activity (μM) is shown on logarithmic scales. Each circle represents the data from an individual sample ($r = 0.68$, $n = 226$, $p < 0.001$). Samples where elastase was not detected are shown as $0.0008\mu\text{M}$ (arrowed).

3.3.2 Sputum chemoattractants and MPO

All samples (226) contained detectable amounts of both chemoattractants (range = 0.05-87.38 nM for IL8 and 0.34-263.34 nM for LTB4). Figure 3-2 and Figure 3-3 show the relationship between the levels of the chemoattractants IL8 and LTB4 and neutrophil influx (MPO). The overall data revealed a positive correlation between both chemoattractants and MPO activity (IL8; $r = 0.52$, $p < 0.001$ and LTB4; $r = 0.41$, $p < 0.001$). Similar correlations were present even when the outlying samples (MPO >10 Units/ml) were excluded (see Figure 3-2 and Figure 3-3).

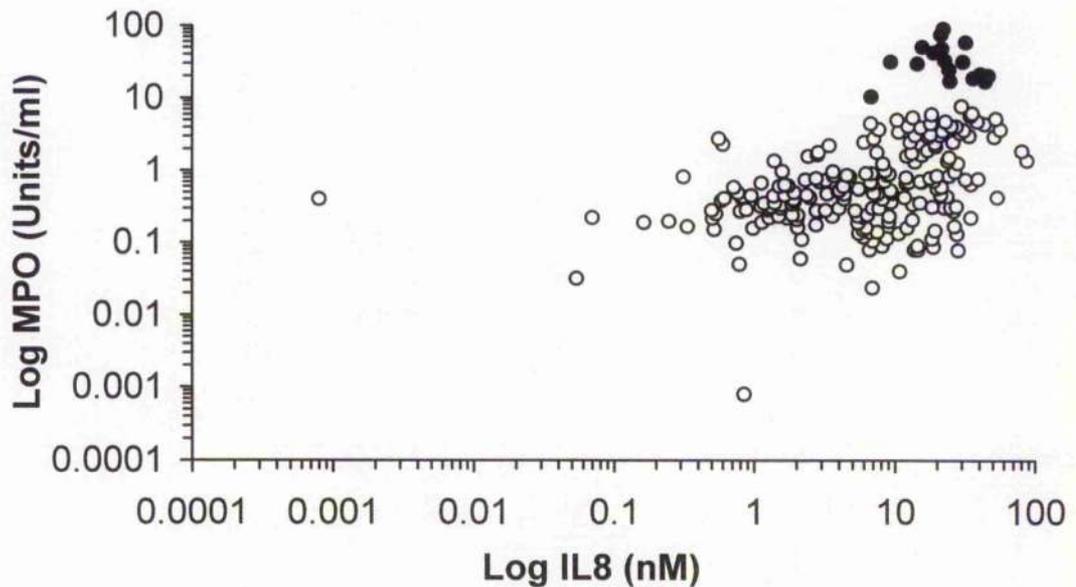


Figure 3-2: The relationship between sputum MPO (Units/ml) and the chemoattractant IL8 (nM) is shown ($r = 0.52$, $p < 0.001$). Each circle represents the result from a single sample (open circles represent samples with an MPO value <10 Units/ml and closed circles represent samples with an MPO value >10 Units/ml). After excluding these latter samples the relationship persisted ($r = 0.40$, $p < 0.001$).

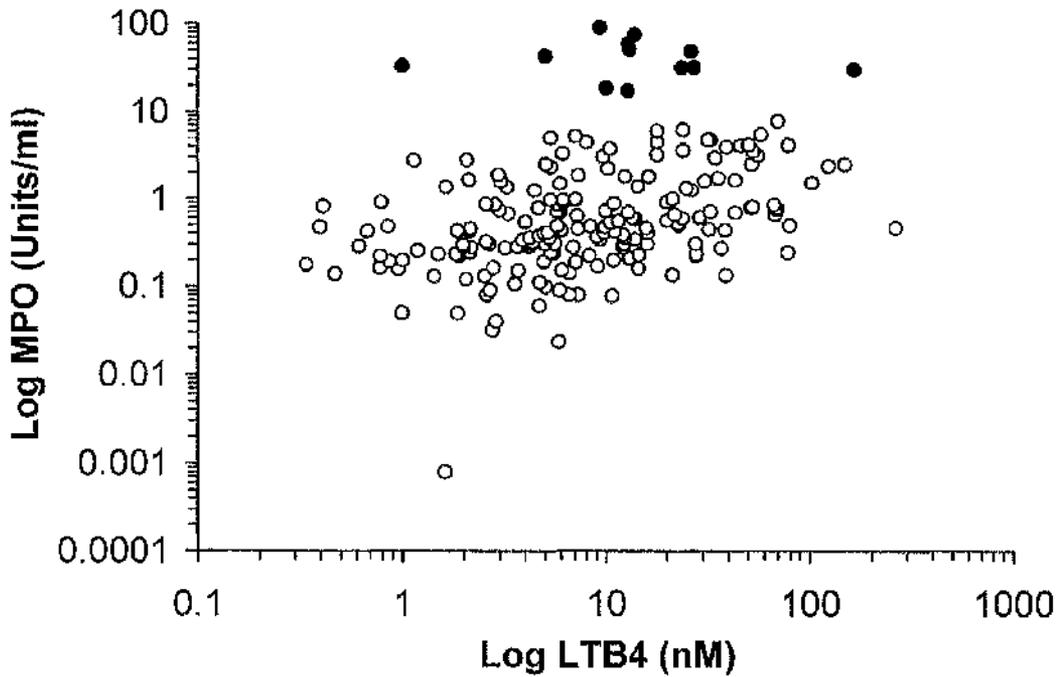


Figure 3-3: The relationship between sputum MPO (Units/ml) and the chemoattractant LTB4 (nM) is shown ($r= 0.41$, $p< 0.001$). Each circle represents the result from a single sample (open circles represent samples with an MPO value <10 Units/ml and closed circles represent samples with an MPO value >10 Units/ml). After excluding these latter samples the relationship persisted ($r= 0.43$, $p< 0.001$ respectively).

3.3.3 Sputum chemoattractants and elastase activity

The overall data revealed a positive correlation between both IL8 and LTB4 and elastase activity (IL8 $r= 0.55$, $p< 0.001$ and LTB4 $r= 0.41$, $p< 0.001$). However, in view of the wide scatter of elastase activity (range 0-8,564 nM), the samples were further subdivided into 4 groups based on the activity. Those with no detectable elastase activity were classified as Group A (n=79); those with small amounts of elastase activity (1-50nM) as Group B (n=92); those with a moderate level of elastase activity (50-100nM) as Group C (n=14); and those with high levels of elastase activity (>100nM) as Group D (n=41). The mean \pm SE levels of free elastase in each of the groups were: zero for Group A; 21.7 ± 1.4 nM for Group B; 71.1 ± 4.7 nM for Group C and 1739.7 ± 330.4 nM for Group D. Figure 3-4 demonstrates the average Interleukin 8 levels (\pm SE) and Leukotriene B4 for the four sample groups (A-D). Low concentrations of IL8 and LTB4 were found in patients with no detectable elastase (IL8 5.61 nM ± 0.67 ; LTB4 9.09 nM ± 1.47). However in the presence of elastase activity (Group B) greater amounts of these chemoattractants were found ($p< 0.001$ for IL8 and $p< 0.005$ for LTB4). The highest levels were found in patients in Group D (elastase activity > 100 nM) with an average IL8 concentration of 25.61 nM ± 2.48 and 36.51 nM ± 8.80 for LTB4 (both $p< 0.001$ compared with Group A).

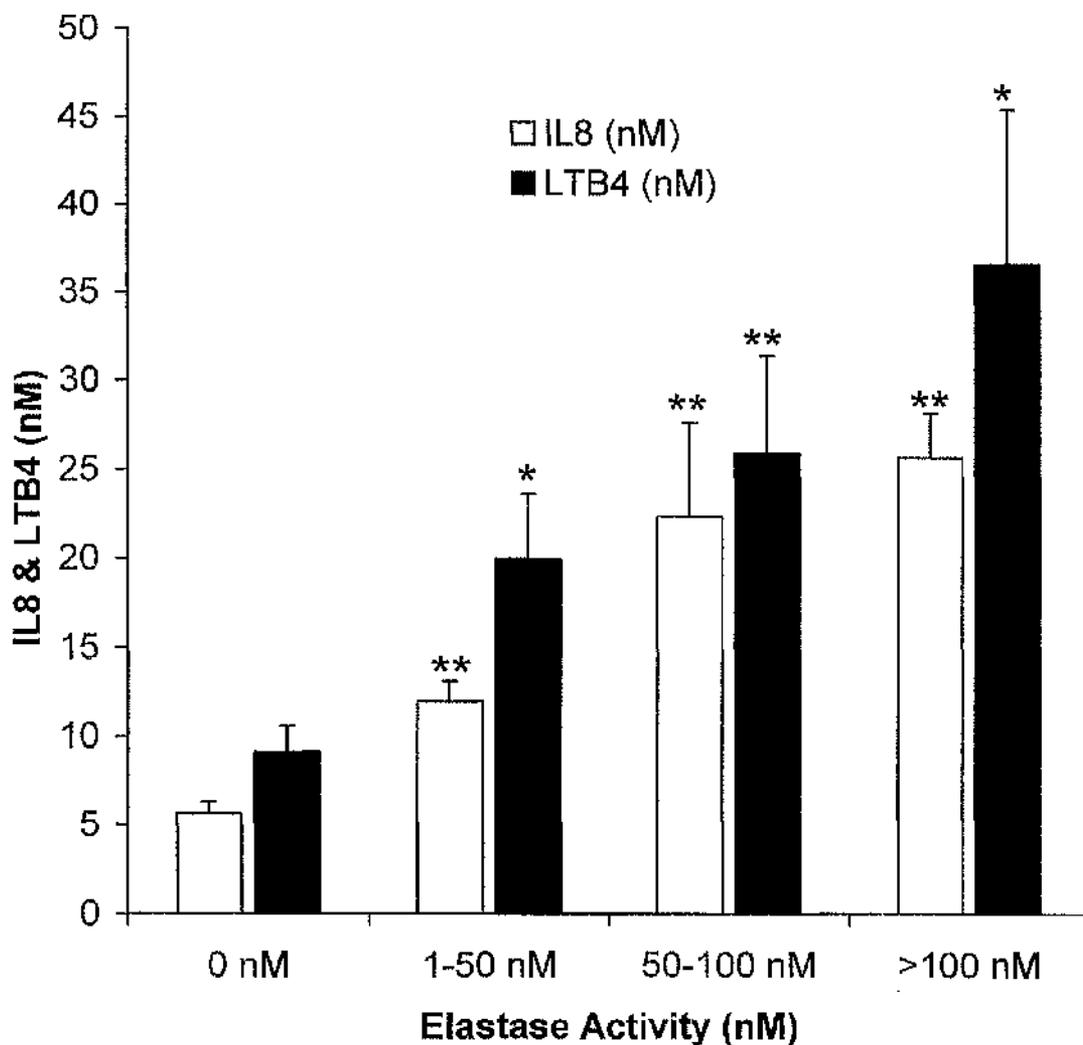


Figure 3-4: The relationship between the sputum chemoattractants (IL8 (nM) and LTB4 (nM)) and elastase activity (nM) is shown. Samples are grouped into those with no elastase activity, elastase activity between 1-50 nM, elastase activity between 50-100 nM, and those with elastase activity greater than 100 nM (see text). The histograms represent the mean \pm SE bar. The asterisks indicate values that are significantly greater than samples with no elastase activity (* $p < 0.005$; ** $p < 0.001$).

3.3.4 Sputum elastase activity and SLPI

SLPI was detectable in all samples (range 0.04-14.26 μM). There was a negative correlation between the SLPI concentration and elastase activity ($r = -0.49$, $p < 0.001$). Separation of samples into their elastase ranges indicated that SLPI concentrations were unaltered in samples with absent or low concentrations of elastase (Groups A&B). However the average concentration was reduced ($p < 0.005$) from $3.78 \mu\text{M} \pm 0.51$ (Group A) to $1.35 \mu\text{M} \pm 0.41$ in samples where elastase activity ranged from 50-100nM (Group C) and reduced further ($p < 0.001$) in samples containing $>100\text{nM}$ elastase activity ($0.66 \mu\text{M} \pm 0.16$). These results are summarised in Figure 3-5.

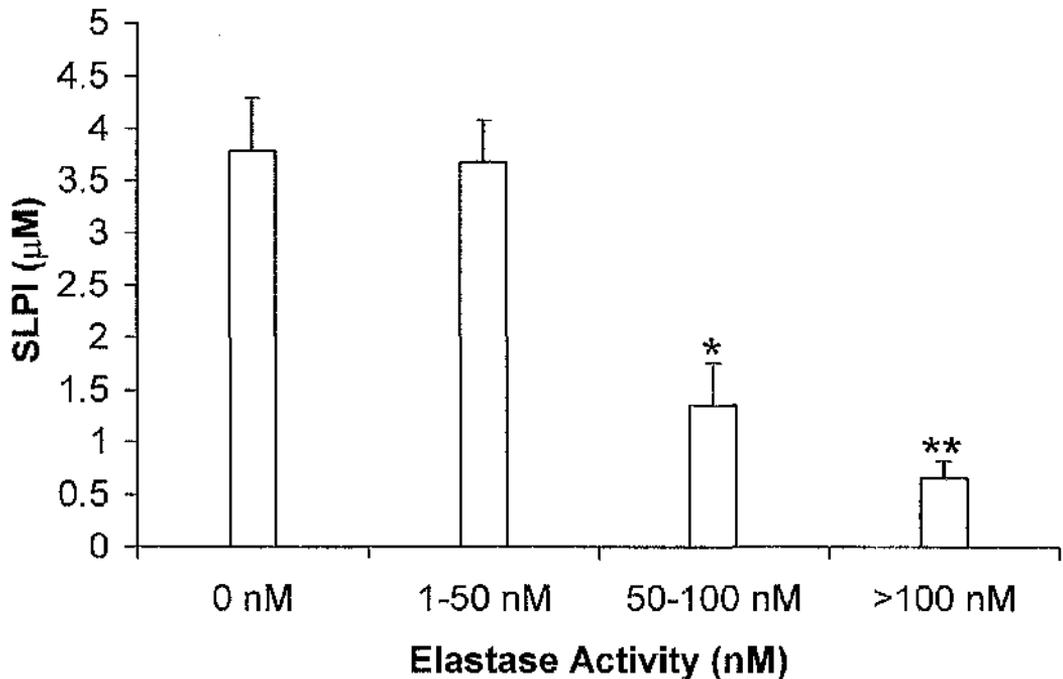


Figure 3-5: The relationship between sputum SLPI levels (μM) (mean \pm SE) and elastase activity (nM) is shown. The asterisks indicate group values that are significantly greater than those with no elastase activity (* $p < 0.005$; ** $p < 0.001$).

3.3.5 Sputum elastase activity and protein leakage

Protein leakage (as assessed by the sputum/serum albumin ratio) showed a positive correlation with elastase activity ($r= 0.36$, $p< 0.001$). In samples where elastase activity was undetectable (Group A) the degree of leakage was low ($1.02\% \pm 0.14$). Protein leakage was also low in Group B ($1.08\% \pm 0.10$), but increased in samples with elastase activity between 50 and 100 nM (Group C: $2.02\% \pm 0.51$, $p< 0.05$). Protein leakage was greatest ($2.54\% \pm 0.42$) in samples containing greater than 100nM elastase ($p< 0.005$ compared with Group A).

3.3.6 Effect of treatment

Patients with chronic lung disease are frequently treated with a variety of agents that may affect lung inflammation. No patients had taken oral steroids or antibiotic therapy within the preceding 2 months of sample collection. There were 90 of the 226 samples, however, were from patients on inhaled steroids with or without theophylline therapy. Exclusion of these samples did not alter the results seen for the group as a whole (Table 3-2).

MPO & Elastase activity	MPO & IL8	MPO & LTB4	Elastase activity & IL8	Elastase activity & LTB4	Elastase activity & Protein Leak	Elastase activity & SLPI
$r= 0.52$	$r= 0.44$	$r= 0.34$	$r= 0.57$	$r= 0.38$	$r= 0.46$	$r= - 0.52$
$p< 0.001$	$p< 0.001$	$p< 0.001$	$p< 0.001$	$p< 0.001$	$p< 0.001$	$p< 0.001$

Table 3-2: Correlations after excluding samples from patients on inhaled steroids with or without theophylline therapy.

3.3.7 Influence of replicate samples

Some patients provided more than 1 sample (usually 2). These were treated as individual data points (see methods). Nevertheless since it is possible that this approach could bias the interrelationships, the data was reanalysed for single samples from each patient and this had no significant effect on the correlation or statistics (Table 3-3).

MPO & Elastase activity	MPO & IL8	MPO & LTB4	Elastase activity & IL8	Elastase activity & LTB4	Elastase activity & Protein Leak	Elastase activity & SLPI
r= 0.43	r= 0.32	r= 0.43	r= 0.40	r= 0.30	r= 0.49	r= - 0.62
p< 0.001	p= 0.001	p< 0.001	p< 0.001	p< 0.001	p< 0.001	p< 0.001

Table 3-3: Correlations after excluding replicate samples.

3.3.8 Relationship with FEV₁ (% Predicted)

There was no correlation between the severity of lung function and neutrophil influx (MPO) or protein leakage for the group as a whole. Subset analysis of the individual patient groups was undertaken since the data would be skewed by the patients with idiopathic bronchiectasis who usually have well preserved lung function despite marked bronchial inflammation.

When the patient groups were studied independently there was still no correlation between FEV₁ and either neutrophil influx or protein leakage in the patients with bronchiectasis or AAT deficiency. In patients with chronic bronchitis without AAT deficiency, however, there was a significant negative correlation between FEV₁ and

MPO, elastase activity, chemoattractants, and protein leakage (Table 3-4). This relationship was retained when the analysis was confined to a single result (mean of replicates) from each patient (Table 3-4), with the exception of LTB₄. Figure 3-6 shows the relationship between FEV₁ (% Predicted) and protein leak (sol/serum albumin ratio (%)) is shown for patients with chronic bronchitis without AAT deficiency when the analysis was confined to a single result.

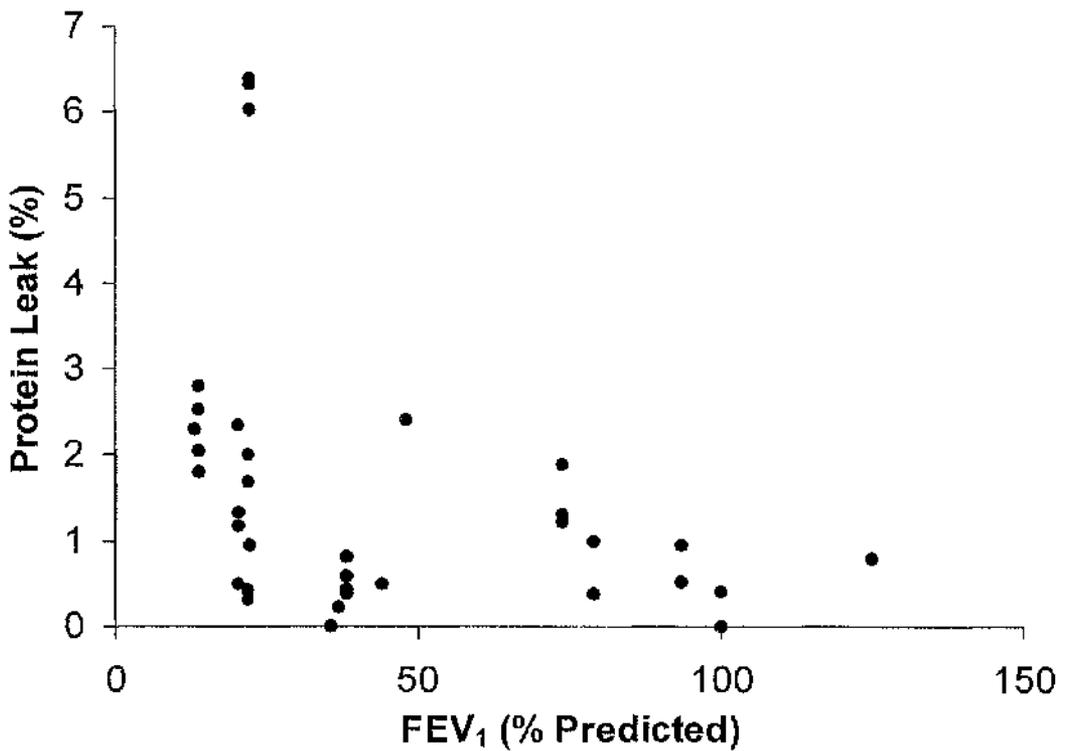


Figure 3-6: The relationship between FEV₁ (% Predicted) and protein leak (sol/serum albumin ratio (%)) is shown for patients with chronic bronchitis without AAT deficiency. Each point represents a value for each patient ($r = -0.54$, $p = 0.01$).

	Replicate samples		Single samples	
	r value	p value	r value	p value
MPO	- 0.37	< 0.001	- 0.31	< 0.005
Elastase activity	- 0.23	0.01	- 0.34	0.001
IL8	- 0.27	< 0.005	- 0.34	0.001
LTB4	- 0.22	0.02	- 0.16	0.1
SLPI	- 0.05	0.7	0.17	0.5
Albumin leakage	- 0.42	< 0.01	- 0.54	0.01

Table 3-4: Correlation with FEV₁ (% Predicted) and markers of bronchial inflammation for replicate and single samples (mean of replicates) from each patient.

3.4 DISCUSSION

The current data spans a wide range of myeloperoxidase activity used as a marker of the presence of bronchial neutrophils (Riise et al, 1995)(Pesci et al, 1998)(Stockley et al, 1991). Activation of these cells (as would occur during the migration process) leads to both MPO and elastase release (Ohlsson et al, 1977) as both are derived from the same granule. Indeed the data shows a good correlation between the MPO activity and elastase over the wide range, although this is less apparent at the lower end where many samples with detectable MPO had no detectable elastase activity. This is to be expected since the presence of bronchial inhibitors such as SLPI and alpha-1-antitrypsin would be expected to inhibit the low concentrations of elastase released when neutrophil numbers are low. Elastase activity was used in this study as *in vitro* studies have suggested the effects of elastase on inflammation are dependent on its activity.

Neutrophil recruitment is dependent upon the chemoattractants being released. Studies have shown that IL8 and LTB4 (Mikami et al, 1998) are the major chemoattractants in patients with bronchial disease and indeed both correlate with the MPO activity as indicated here. The source of these 2 chemoattractants, however, is unknown. For instance, endotoxin and TNF- α (both of which are likely to be present in the airway) are known to increase IL8 production by epithelial cells (Khair et al, 1994). In addition, IL8 is stored within the specific granules of a neutrophil and is also released from this cell on activation (Takahashi et al, 1993). Finally, it has been suggested that elastase itself when released from the neutrophil can lead to IL8 production by bronchial epithelial cells (Nakamura et al, 1992). Whatever the mechanism it would therefore be predicted that

IL8 levels should correlate not only with neutrophils (as indicated by the MPO concentration) but also with the elastase activity. Although the IL8 levels measured here do correlate with MPO (as suggested by previous workers (Riise et al, 1995)(Pesci et al, 1998)(Yamamoto et al, 1997)), the relationship is less robust than that for MPO and elastase activity, suggesting that the relationship is not a simple cause and effect but probably reflects multiple sources of IL8. However, even though there is a highly statistical relationship between IL8 and MPO and elastase, examination of the raw data suggests this may not be a simple linear relationship. Indeed the data would suggest that the relationship maybe curvilinear with a threshold effect leading to rapidly increasing concentration of neutrophil MPO once the IL8 level exceeds approximately 10 nM (Figure 3-2).

The relationship with LTB4 on the other hand seems to be much more straightforward, although there was a wide scatter of values for both MPO and LTB4, the relationship tends to follow a general linear trend (Figure 3-3). However the dose relationship between MPO and LTB4 suggests either that the neutrophil itself may be the source of both proteins or that LTB4 is the major factor determining neutrophil recruitment in these samples.

However it has been shown that LTB4 and IL8 are additive in their effect on neutrophil recruitment in vitro (Mikami et al, 1998). It may well be therefore that at low levels of IL8, recruitment of small numbers of neutrophils leads to release of LTB4 from the activated cell. This may thereafter add to the chemoattractant gradient recruiting more cells in a linear fashion. However, once IL8 levels exceed 10 nM the inflammatory

process may become excessive leading to much greater neutrophil recruitment (as indicated by MPO) than would be expected for LTB₄ alone. This concept would be consistent with the potential curvilinear relationship between IL8 and MPO as well as the data points for MPO displaced from the LTB₄ curve (see Figure 3-3). Again this concept would be consistent with a hierarchical organisation of chemoattractants (Foxman et al, 1997).

Alternatively it is possible that other chemoattractants are involved in samples where neutrophil recruitment is excessive. The solid symbols in figures 3-2 and 3-3 represent samples where MPO exceeds 10 Units/ml. These samples appear displaced from the relationship of both LTB₄ and IL8 to MPO and this complex relationship needs further comment. Preliminary data indicated that of the 10 samples colonised with *Pseudomonas aeruginosa*, 7 were in this high MPO group of 17 samples. It therefore remains possible that the colonising organism may also influence cell migration and this point will be covered in the next chapter.

Studies of the relationships of the inflammatory process to elastase are worthy of further comment. When released from the neutrophil this enzyme would be rapidly inhibited by both alpha-1-antitrypsin and, in the airway, more particularly by SLPI. This study showed that there were low levels of SLPI associated with high levels of elastase activity. In vitro studies have indicated that elastase can adversely affect SLPI production by epithelial cells (Sallenave et al, 1994) thus it would be expected that an inverse relationship should exist. However, the data as presented here indicates that SLPI concentration does not decrease until the elastase activity of the samples is in

excess of 50 nM. The relationship between SLPI and elastase therefore is not a simple linear one and the data presented here may indicate a threshold at which significant interference with epithelial cell metabolism or the development of epithelial damage occurs resulting in a reduction in SLPI secretion. At present whether this relationship reflects cause or effect is not known and interpretation will have to await intervention studies using specific anti-elastases. If the reduction in SLPI production is merely a reflection of the elastase activity in the airway, the introduction of an effective anti-elastase would be expected to lead to an acute rise in SLPI concentration. However if significant airway cell damage is responsible for the reduction in SLPI, a longer period of repair would be necessary before SLPI concentrations return to normal.

There is, however, a similar relationship between the elastase activity and inflammation in the airway as reflected in leakage of serum albumin. Protein leakage increased only when free elastase activity was >50 nM, and increased further when the elastase activity exceeded 100 nM. The exact mechanism for protein leakage is currently unknown. Previous studies in patients with bronchiectasis have shown antibiotic therapy reducing the bacterial load and hence neutrophil influx, results in a rapid reduction in protein leakage as sputum purulence and elastase activity disappear (Stockley et al, 1979). The rapidity of this response would suggest that airway leakage is not a direct effect of epithelial cell damage. The leakage may therefore reflect an effect of elastase on the tight junction between epithelial cells (Peterson et al, 1995), the simultaneous release of vasoactive mediators in the airway, and cleavage of cadherins that link endothelial cells (Carden et al, 1998). Once again interpretation of the associations will await studies with effective anti-elastases.

The relationship between elastase and IL8 is of further interest. As indicated above, studies have suggested that IL8 release by epithelial cells can be induced by the presence of free elastase (Nakamura et al, 1992). However, since both mediators can also be derived from the neutrophil (although from different cell granules) it would be expected that the two concentrations would be related. The data provided here shows that the relationship is present although weak and it is therefore unlikely that these 2 proteins directly represent a cause and effect. Also, as indicated above, the relationship between IL8 and myeloperoxidase does not appear to be linear across a wide range of inflammatory situations unlike results of previous more limited studies (Riise et al, 1995)(Pesci et al, 1998). However, it is also possible that the presence of additional (as yet uncharacterised) factors may play a role when inflammation is excessive.

Finally Keatings and colleagues showed that increased neutrophil influx was associated with worse lung function in patients with chronic bronchitis (Keatings et al, 1997) although separation of cause or effect has also not been possible. Similarly, the current study showed that both increased neutrophil influx (as assessed by the concentration of MPO), elastase activity, chemoattractants IL8 and LTB₄, and protein leak were associated with worse lung function in patients with chronic bronchitis without alpha-1-antitrypsin deficiency. The relevance of this is currently uncertain as these correlations are weak and this study does not separate cause or effect. In addition there was no association in the other two patient groups (those with alpha-1-antitrypsin deficiency or bronchiectasis) although it should be noted that all patients with alpha-1-antitrypsin deficiency had an FEV₁ < 56% predicted which would tend to minimise the range of inflammation that might potentially be predicted. Thus in this group there was a limited

spectrum of disease being assessed which might make any relationship more difficult to determine. The association is thus complex and further studies are required to determine whether the observation in chronic bronchitis without alpha-1-antitrypsin deficiency represents a true cause and effect.

In summary the interrelationship between the neutrophil and some of its products and the inflammatory process has been assessed in patients with different causes of bronchial disease to provide a wide spectrum of neutrophilic inflammation. Although relationships can be demonstrated it is clear that these are complex and understanding the interplay of various mediators will require the development of specific antagonists and appropriately designed intervention studies.

**4. AIRWAY BACTERIAL LOAD IN STABLE OBSTRUCTIVE
AIRWAYS DISEASE:
A POTENT STIMULUS FOR AIRWAY INFLAMMATION**

4.1 INTRODUCTION

As described in the general introduction chapter, the defence system of the lungs in patients with chronic bronchitis often fails to maintain sterility in the airways, leading to persistent lower airway colonisation with bacteria (Monso et al, 1995)(Murphy et al, 1992)(Stockley, 1998)(Pela et al, 1998).

In animal models, small numbers of bacteria instilled into the airways can be cleared efficiently by the primary host defence mechanisms alone (Onofrio et al, 1983). However, as increasing numbers of organisms are instilled, secondary host defences are activated (Onofrio et al, 1983)(Vial et al, 1984) and this is likely to reflect the release of pro inflammatory mediators, resulting in an acute inflammatory response with predominant recruitment of neutrophils (Vial et al, 1984)(Toews et al, 1984). It is possible that persistent bacterial airway colonisation in humans with chronic airways diseases contributes in important ways to the morbidity of the disease. In particular, the chronic airway bacterial load may stimulate secondary host defences and lead directly to persistent airway inflammation even in the stable clinical state (Stockley, 1998)(Hill et al, 1986). This possibility has received little attention in clinical studies in man. For this reason, quantitative sputum bacteriology in patients with "stable" chronic bronchitis and its relationship of the acute inflammatory response in the lung was studied. This included the measurement of sputum colour assessed macroscopically, neutrophil influx (MPO), the neutrophil chemoattractants IL8 and LTB₄, neutrophil elastase and its inhibitor SLPI, and finally airway protein leakage.

4.2 METHODS

4.2.1 Study Population

Three groups of patients with established bronchial disease and a history of chronic bronchitis, as defined by the Medical Research Council (Medical Research Council, 1965), were studied in the stable clinical state. These included; 64 patients with chronic bronchitis with alpha-1-antitrypsin (AAT) deficiency, who had AAT levels $< 11 \mu\text{M}$ (Silverman et al, 1989), and had the phenotype PiZ; 55 subjects with chronic bronchitis (without AAT deficiency); and 43 patients with idiopathic bronchiectasis diagnosed by high resolution computed tomography or bronchogram. None of the patients reported any change in respiratory symptoms (including sputum characteristics) within the eight weeks prior to the initial visit, and none were on oral steroids or antibiotics during the study.

4.2.2 Lung Function Testing

All subjects underwent standardised lung function testing (Society et al, 1994) at the time of the study.

4.2.3 Processing of samples

The sputum and serum processing, and assessment of sputum colour, MPO, elastase activity, IL8, LTB₄, SLPI, and protein leakage (sputum/serum albumin ratio (%)) is described in the general methods (Chapter 2). In patients who provided more than one sample, the results from all sputum samples for the subject were averaged, and the mean result was used.

4.2.4 Quantitative Bacteriology

Quantitative bacteriology was carried out on all spontaneously expectorated sputum samples as described in the general methods chapter. For data analysis, the bacterial load is expressed as the sum of all bacterial species present in that sample.

4.2.5 Statistical Analysis

Values are reported as mean \pm SE. The Kruskal-Wallis test was used to compare results at different bacterial loads, and if significant the Mann-Whitney U test for non paired data (one tailed) was used to compare individual groups. Correlations between the inflammatory markers were measured with the Spearman correlation coefficient (2 tailed). A p value < 0.05 was considered to be statistically significant.

4.3 RESULTS

The demographic information, smoking histories, and lung function measurements for the study population are shown in Table 4-1. The ages of the patients with chronic bronchitis without AAT deficiency and bronchiectasis were similar, while the mean age of the patients with AAT deficiency was lower ($p < 0.005$). Nearly half of the patients with bronchiectasis, but none of the patients with chronic obstructive bronchitis or AAT deficiency, were lifelong non smokers. As in other reports (Brantly et al, 1988), few of the patients with AAT deficiency were current smokers.

Characteristic	Chronic bronchitis	AAT deficiency	Bronchiectasis
Age (yr.)	67.4 ± 1.4	49.3 ± 1.7	62.7 ± 2.6
range	(51-78)	(33-66)	(36-82)
Gender (M/F)	38/17	49/15	15/28
Current smoker	30	9	4
Ex- smoker	25	55	18
Never smoker	0	0	21
FEV ₁ (% Predicted)	36.0 ± 4.5	26.7 ± 3.1	63.8 ± 4.9
FEV ₁ /FVC (% Predicted)	49.3 ± 2.9	38.4 ± 3.1	70.5 ± 3.3

Table 4-1: Mean ± SE demographics of the study group

4.3.1 Sputum samples analysed

The subjects provided 336 sputum samples for analysis. These included 72 samples from 55 subjects with chronic bronchitis without AAT deficiency, 179 samples from 64 subjects with AAT deficiency, and 85 samples from 43 patients with bronchiectasis. Individual subjects provided up to 4 samples over as many as 12 weeks, whereas 74 subjects provided a single sample only.

4.3.2 Bacterial Isolates

Among the 336 samples, 26% were classified as mixed normal flora (MNF) where no viable bacteria were cultured or yielded only a sparse culture ($< 10^5$ cfu/ml) of normal oral commensals. Of the remaining samples, 5% yielded between 10^5 and 10^6 cfu/ml, 8% yielded 10^6 and 10^7 cfu/ml, 13% yielded 10^7 and 10^8 cfu/ml, and 48% yielded $\geq 10^8$ cfu/ml.

The overwhelming majority of the 'primary' (predominant) pathogens isolated were non typeable *Haemophilus influenzae* or *Haemophilus parainfluenzae* (grouped as *Haemophilus* species). *Haemophilus* species were often isolated in pure culture, particularly when the airway bacterial load was relatively low. Among the 45 samples with $\leq 10^7$ cfu/ml, 98% yielded *Haemophilus* species alone and the remaining sample yielded *Streptococcus pneumoniae*.

In the 42 sputum samples with 10^7 - 10^8 cfu/ml, the dominant organisms were *Haemophilus* species in 71%. In the remaining 12 samples, 4 grew coliforms, 4

Staphylococcus aureus, 2 *Proteus mirabilis*, and one each of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*.

Among the 160 samples with the heaviest bacterial loads ($\geq 10^8$ cfu/ml), 69% contained *Haemophilus species* as the predominant organism. Among the remaining samples, 15% yielded *Moraxella catarrhalis* and 13% yielded *Pseudomonas aeruginosa*. Less common organisms included coliforms in 2, *Streptococcus pneumoniae* in 2, and one each of *Acinetobacter lwoffii* and a *Corynebacterium* species.

In 20% of the samples, a secondary organism was isolated; however, in most of these samples (92%), the predominant isolate was already present at $>10^8$ cfu/ml. These secondary organisms were present in minimally lower numbers in 48% of samples in which they were present, while their concentration was at least one order of magnitude lower in the remainder. The secondary pathogens were *Haemophilus influenzae* and *Streptococcus pneumoniae* in 69% and 15% of the samples, respectively. For data analysis, the bacterial load is expressed as the sum of all bacterial species present in that sample.

4.3.3 Airway neutrophils

MPO concentration, as a measure of the number of airway neutrophils, correlated with airway bacterial load ($r= 0.58$; $p< 0.0005$). In samples with a low bacterial load, little MPO was present (Figure 4-1). However, samples that contained $>10^6$ and $<10^7$ cfu/ml had a highly significant increase in MPO ($p < 0.01$). Samples with $\geq 10^8$ cfu/ml had an 18 fold increase in MPO when compared with samples containing only MNF ($p< 10^{-5}$).

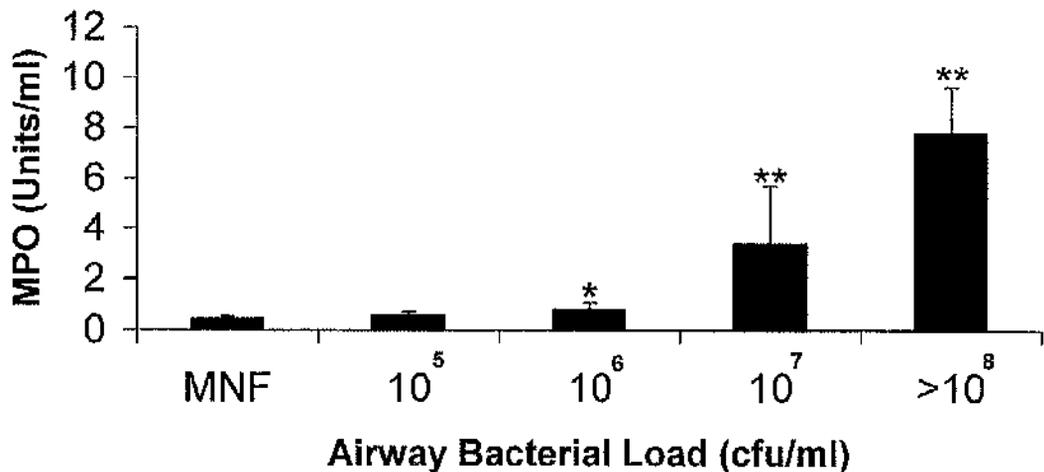


Figure 4-1: Relationship between sputum MPO (a measure of airway neutrophils) and airway bacterial load. MNF = mixed normal flora. Note the progressive increase in MPO with increasing bacterial load (from 10^6 cfu/ml). The asterisks indicate significant differences when compared to samples containing only MNF (* $p< 0.01$ and ** $p< 0.005$). Histograms represent mean data \pm SE bar lines.

Sputum colour, as an easily assessed visible surrogate for MPO, also correlated with airway bacterial load ($r= 0.52$; $p< 0.0005$). Using the nine-point scale, the average colour for samples containing only MNF was 2.8 ± 0.2 , corresponding to a barely

detectable yellow colour. In samples containing $\geq 10^8$ cfu/ml, sputum colour was increased to an average of 4.2 ± 0.1 ($p < 0.0001$ when compared with samples containing only MNF) (Figure 4-2).

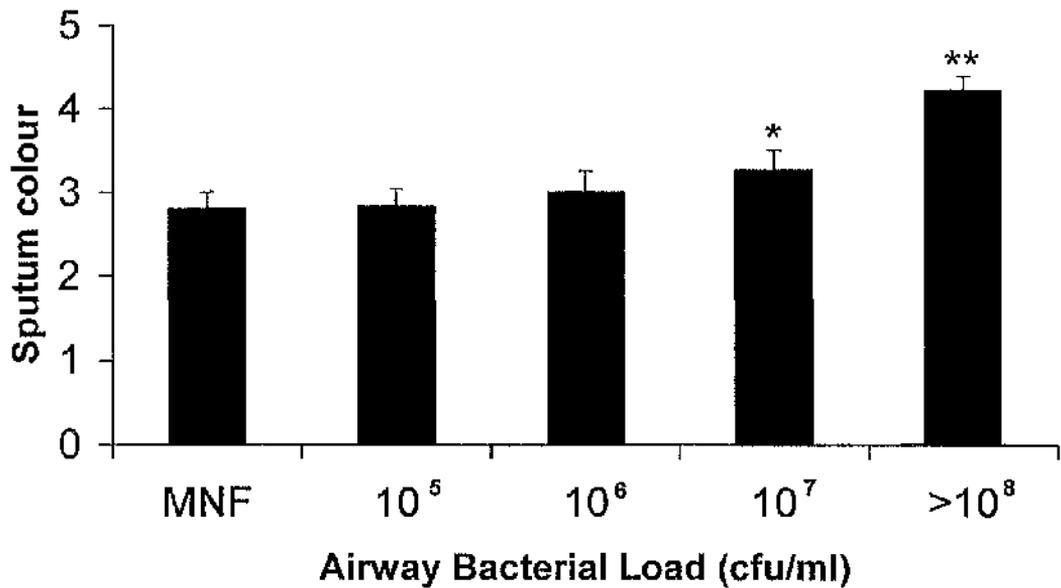


Figure 4-2: Relationship between sputum colour and airway bacterial load. Note the progressive increase in colour with increasing bacterial load (from 10^7 cfu/ml). The asterisks indicate significant differences when compared to samples containing only MNF (* $p < 0.05$ and ** $p < 0.005$). Histograms represent mean data \pm SE bar lines.

4.3.4 Sputum chemoattractants

Immunoreactive IL8 and LTB4 were detectable in all samples, and both correlated with airway bacterial load ($r = 0.67$; $p < 10^{-5}$ and $r = 0.48$; $p < 0.0005$ for IL8 and LTB4, respectively). Figure 4-3 demonstrates the relationship between IL8 and bacterial load and shows that sputum IL8 concentration increased significantly ($p < 0.05$) for samples containing $>10^6$ and $<10^7$ cfu/ml when compared to the results for MNF. Samples with $\geq 10^8$ cfu/ml had a 5.3-fold higher IL8 concentration when compared to samples containing only MNF ($p < 10^{-5}$). LTB4 was also significantly higher in samples containing $>10^6$ and $<10^7$ cfu/ml than in samples containing only MNF (Figure 4-4). Samples with $\geq 10^8$ cfu/ml had a 5-fold higher LTB4 concentration when compared to samples containing only MNF ($p < 10^{-5}$).

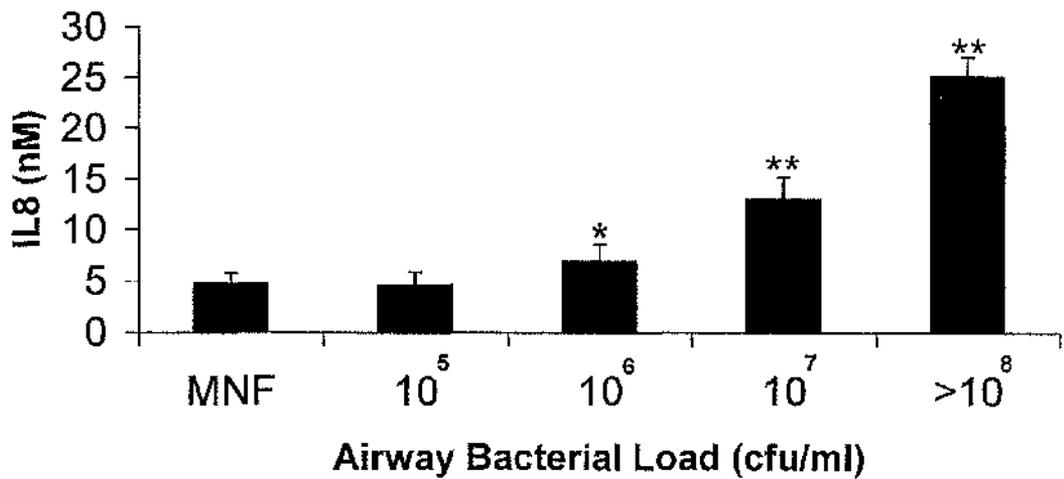


Figure 4-3: Relationship between sputum IL8 (nM) and bacterial load. Note that concentrations of IL8 increased progressively with increasing bacterial load (from 10^6 cfu/ml). The asterisks indicate significant differences when compared to samples containing only MNF (* $p < 0.05$ and ** $p < 0.005$). Histograms represent mean data \pm SE bar lines.

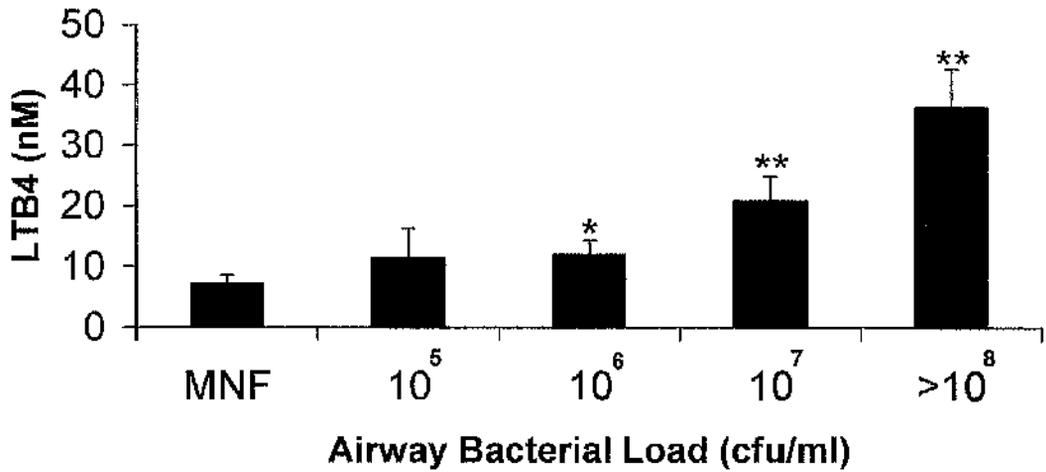


Figure 4-4: Relationship between sputum LTB4 (nM) and airway bacterial load. Note that concentrations of LTB4 increased progressively with increasing bacterial load (from 10^6 cfu/ml). The asterisks indicate significant differences when compared to samples containing only MNF (* $p < 0.05$ and ** $p < 0.005$). Histograms represent mean data \pm SE bar lines.

4.3.5 Sputum leukocyte elastase activity

Leukocyte elastase activity in the samples also increased with increasing bacterial load ($r= 0.43$; $p< 0.0005$). Among 89 samples that contained only MNF, 29% contained detectable, but invariably low concentrations of leukocyte elastase activity (Figure 4-5). Elastase activity was present in 78% of the 27 samples containing 10^6 - 10^7 cfu/ml, with a significantly greater mean value when compared with samples containing only MNF (Figure 4-5). However 87% of the 160 samples with $\geq 10^8$ cfu/ml had detectable elastase activity, and the mean value was 86-fold higher than samples containing MNF alone ($p< 10^{-5}$).

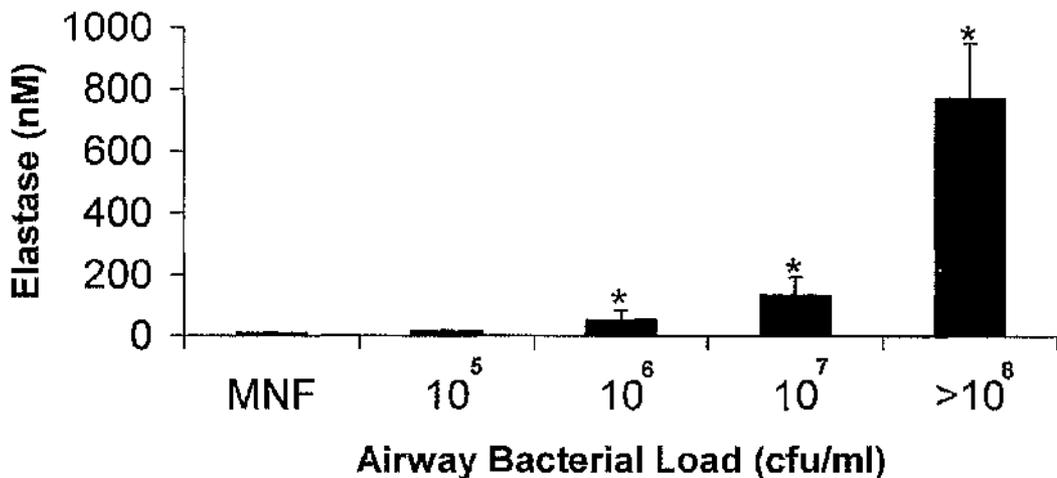


Figure 4-5: Relationship between sputum leukocyte elastase activity (nM) and airway bacterial load. Note the progressive increase in leukocyte elastase activity with increasing bacterial load (from 10^6 cfu/ml). The asterisk indicates significant differences when compared to samples containing only MNF (* $p< 0.005$). Histograms represent mean data \pm SE bar lines.

4.3.6 Sputum secretory leukocyte proteinase inhibitor

Levels of SLPI correlated negatively with bacterial load ($r = -0.52$, $p < 0.0001$). Further examination of the data, however, revealed that the mean SLPI concentrations only fell at high bacterial loads ($>10^8$ cfu/ml). In samples that contained only MNF, the mean SLPI concentration was $4.0 \pm 0.6 \mu\text{M}$ whereas in samples with $\geq 10^8$ cfu/ml, SLPI concentrations were reduced to $1.4 \pm 0.2 \mu\text{M}$ ($p < 0.0001$ when compared with the MNF samples) (Figure 4-6).

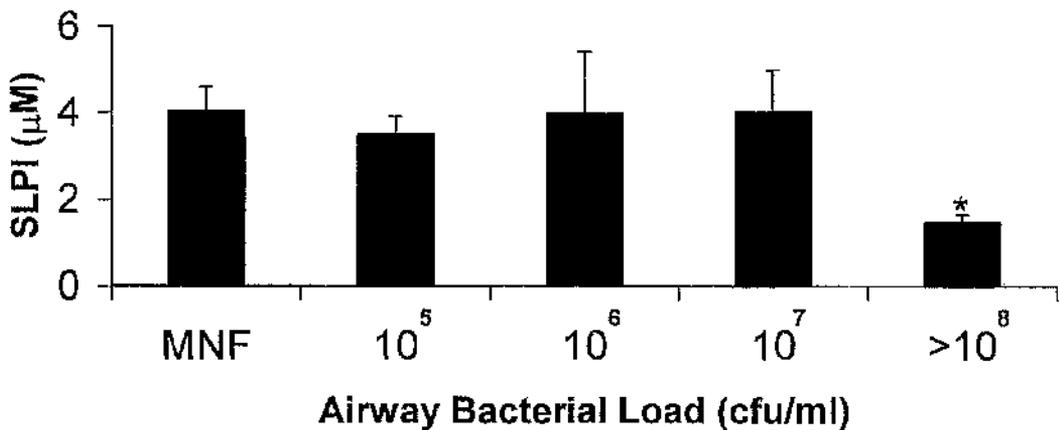


Figure 4-6: Relationship between sputum secretory leukoprotease inhibitor (μM) and airway bacterial load. Note the mean levels of SLPI do not change until bacterial loads $>10^8$ cfu/ml. The asterisk indicates a significant difference compared to samples containing only MNF ($p < 0.001$). Histograms represent mean data \pm SE bar lines.

4.3.7 Airway protein leakage

The sputum/serum albumin ratio showed a positive correlation with airway bacterial load ($r= 0.44$, $p< 0.0001$). Samples colonised with 10^7 and $\geq 10^8$ cfu/ml showed a greater sputum/serum albumin ratio ($p< 0.005$) compared to samples containing MNF alone Figure 4-7.

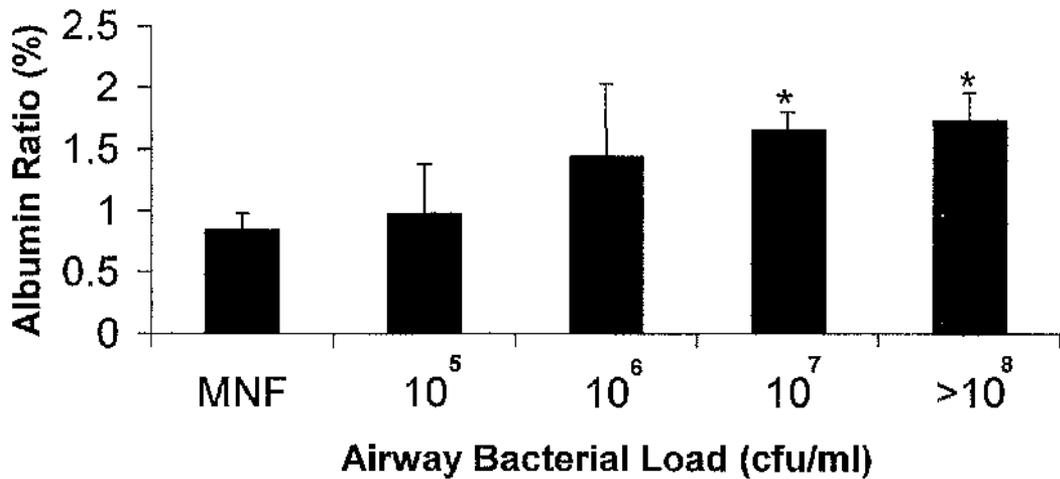


Figure 4-7: Relationship of airway protein leakage (sputum/serum albumin ratio (%)) to airway bacterial load. Note the significant increase in samples containing 10^7 or more cfu/ml. Asterisks indicate significant differences compared to samples containing only MNF ($p< 0.005$). Histograms represent mean data \pm SE bar lines.

4.3.8 Effect of Type and Severity of Airflow Obstruction

Among the 43 patients with bronchiectasis, the airway bacterial load was $> 10^8$ in 70%, while only one patient was in the MNF group. The airway bacterial load in the other two patient groups was less intense than in the bronchiectasis group and there was no clear relationship between the airway bacterial load and the severity of airflow obstruction. For example even in the chronic bronchitis group, the FEV₁ (% predicted) was 28.2 ± 3.2 for those with MNF (n = 19) and 29.1 ± 3.3 for those with $> 10^8$ cfu/ml (n=17). Among the patients with AAT deficiency, those with MNF (n = 15) had a mean FEV₁ of $28.7 (\pm 4.6)\%$ of predicted, and those with $\geq 10^8$ cfu/ml (n = 24) had an FEV₁ of $26.3 (\pm 3.7)\%$ of predicted.

4.3.9 Effect of bacterial species

When *Pseudomonas aeruginosa* was found, the bacterial load was always $> 10^8$ cfu/ml (except one sample 10^7 - 10^8 cfu/ml). To determine the effect(s) of bacterial species alone, further analysis was confined to samples which: 1) contained only a single isolate; 2) contained $\geq 10^8$ cfu/ml of that organism; and 3) were derived from subjects with bronchiectasis alone. This resulted in 5 samples colonised by *Pseudomonas aeruginosa*, 17 colonised by *Haemophilus sp.*, and 3 colonised by *Moraxella catarrhalis*.

Table 4-2 shows the results of the analysis of these 25 samples. Samples from which *Pseudomonas aeruginosa* had been isolated differed significantly from each of the other two groups in that: 1) there was a higher concentration of MPO; 2) a higher concentrations of active elastase; and 3) a greater airway protein leakage. There were

however no significant differences in IL8, LTB4, or SLPI concentrations between the groups.

Organism	<i>Pseudomonas Aeruginosa</i>	<i>Haemophilus Sp.</i>	<i>Moraxella catarrhalis</i>
No. Samples	5	17	3
MPO (Units/ml)	38.76 ± 11.56	8.1 ± 2.21**	4.59 ± 2.93*
Leukocyte elastase (µM)	4.18 ± 0.60	0.75 ± 0.23**	0.11 ± 0.06*
IL8 (nM)	21.87 ± 4.41	30.99 ± 4.41	32.39 ± 24.00
LTB4 (nM)	14.71 ± 3.20	33.09 ± 7.21	19.35 ± 12.08
SLPI (µM)	0.78 ± 0.53	1.47 ± 0.41	1.91 ± 1.01
Albumin ratio (%)	3.36 ± 0.40	1.71 ± 0.45*	0.82 ± 0.17*

Table 4-2: Effects of bacterial species on airway inflammation. Results are means ± S.E. *p < 0.025 and **p < 0.005 when compared with *Pseudomonas aeruginosa*.

4.4 DISCUSSION

Quantitative bacterial culture has been used to demonstrate a wide range of airway bacterial load among patients with daily productive cough in the stable clinical state. This study included three groups of patients to cover a wide range of airways colonisation with adequate numbers in each quintile of colonising load. Exclusion of patients with AAT deficiency or bronchiectasis did not influence the overall relationship between colonising load and airways inflammation (Hill et al, 2000). Increasing airway bacterial load was strongly related to: 1) the number of neutrophils (sputum colour and MPO); 2) the concentration of neutrophil chemoattractants IL8 and LTB₄; 3) the concentration of active leukocyte elastase; and 4) the sputum/serum albumin ratio. *Pseudomonas aeruginosa* provoked a more intense inflammatory response than *Haemophilus sp.* and *Moraxella catarrhalis*, when present in comparable numbers and was associated with higher concentrations of active leukocyte elastase and more airway protein leakage.

Patients with current or recent exacerbations of lung disease were excluded, to avoid a potential dynamic state of both bacterial load (Monso et al, 1995) and airway inflammation (Anthonisen et al, 1987). These results challenge conventional thinking about bacterial "colonisation" of the airways in chronic bronchitis. Organisms such as non typeable *Haemophilus influenzae* are common colonisers of the normal upper respiratory tract, being present in up to 75% of healthy adults (Murphy et al, 1987). The lower airways of patients with chronic bronchitis are also commonly colonised with such bacteria although the subjects do not appear to be acutely ill (Murphy et al, 1992).

Thus, the role of this airway bacterial load has been uncertain. The results presented here, however, indicate that the concentration of viable organisms, regardless of the bacterial species, can have an important influence on airway inflammation in "stable" patients.

It is plausible that airway bacterial load promotes airway inflammation because bacterial products may directly initiate and perpetuate lung inflammation through induction of the local secretion of IL8 (Bedard et al, 1993)(Khair et al, 1994). In addition alveolar macrophages activated by phagocytosis may be a source of the observed LTB4 (Bigby et al, 1987)(Hubbard et al, 1991). Other indirect mechanisms may also amplify and perpetuate neutrophil influx, including: 1) chemoattractants and leukocyte elastase released from activated neutrophils in the airways (Takahashi et al, 1993)(Doerfler et al, 1989)(Nakamura et al, 1992); 2) leukocyte elastase-inhibitor complexes (Banda et al, 1988); and 3) products of local extracellular matrix degradation (Senior et al, 1980).

Concentrations of the local proteinase inhibitor, SLPI, decreased when airway bacterial load $\geq 10^8$ cfu/ml. This may be a direct consequence of the effect of leukocyte elastase on the bronchial epithelium (Sallenave et al, 1994), or the result of bronchial epithelial injury (Amitani et al, 1991). Changes in SLPI are noteworthy because elastase-mediated acute airway injury would be enhanced, possibly increasing inflammation, when levels of SLPI are reduced. The increase in inflammation may be due, at least in part, by increased serine proteinase generation of the chemoattractants C5a (Brozna et al, 1977) and perhaps IL8 (Nakamura et al, 1992).

Taken together, the data reported herein indicate that persistent bacterial colonisation of the airways may not be a benign process in patients with chronic bronchitis, even in the absence of an acute deterioration of symptoms.

It is of special interest that the concentration of leukocyte elastase varied by more than 80-fold with changes in airway bacterial load. This enzyme has been shown to have important adverse effects the airways, including inducing mucus hypersecretion (Sommerhoff et al, 1990), impairment of ciliary function (Smallman et al, 1984), damage to immunoglobulins (Solomon, 1978), reduction in opsonophagocytosis (Tosi et al, 1990), and damage to epithelial tissue (Amitani et al, 1991). Thus, active leukocyte elastase may both cause airway pathology and also promote continued bacterial colonisation.

The bacterial species isolated from samples reported here are similar to those from previous studies of sputum lower airway bacteriology. Since *Haemophilus species*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* are normal oropharyngeal commensals, non-quantitative sputum cultures may lead to confusion concerning their role because they cannot confirm a lower respiratory source for the isolates (Murphy et al, 1992)(Monso et al, 1995). In the study reported here, quantitative cultures of expectorated sputum provides greater certainty of their source. Bacteria that were present at $\geq 10^6$ cfu/ml were strongly associated with evidence of airway inflammation suggesting a cause and effect. In previous work by others, quantitative culture of the lower airways by means of the protected specimen brush (Monso et al, 1995) also demonstrated that lower respiratory colonisation was common in "stable" COPD (25%

of cases had significant growth of $\geq 1,000$ cfu/ml and 5% had $\geq 10,000$ cfu/ml). However, airway inflammation was not studied, and this technique is not applicable to routine clinical testing.

Future studies of antibacterial intervention in stable patients with high airway bacterial loads, both at baseline and during exacerbations, can be expected to provide insights into the potential benefits and complications of reducing the bacterial load in these patients. Indeed, published studies of stable patients with bronchiectasis who produce purulent sputum have indicated that clinical benefits and a reduction in airways inflammation can be obtained from antibiotic therapy both in the short and long term (Hill et al, 1986). It should be noted that, for infections of the uroepithelium, it has long been standard clinical practice to base therapeutic decisions upon both quantitative bacterial cultures and enumeration of inflammatory cells in urine specimens. The present results suggest that a similar strategy with respiratory secretions may improve the management of infection of the respiratory tract and provide more logical indicators of the need for antibiotic therapy.

**5. AIRWAYS INFLAMMATION IN CHRONIC BRONCHITIS:
THE EFFECTS OF SMOKING AND ALPHA-1-ANTITRYPSIN
DEFICIENCY**

5.1 INTRODUCTION

Chronic bronchitis was first recognised as a disabling disorder in 1808 (Badham, 1808). It is often a feature of COPD, and recent studies have shown that airway neutrophils are increased in patients with chronic bronchitis (Riise et al, 1995) and that the degree of neutrophil recruitment is related to the severity of airflow obstruction (Keatings et al, 1997)(Di Stefano et al, 1998). Furthermore increased neutrophils in the airways are related to the rate of progression of airflow obstruction (Stanescu et al, 1996). Smoking (the major risk factor in COPD) increases neutrophil recruitment to the lung (Keatings et al, 1997)(Hunninghake et al, 1983) possibly by inducing the bronchial epithelium to secrete the important neutrophil chemoattractant IL8 (Mio et al, 1997).

Neutrophils contain a serine elastase which has been shown to reproduce many of the features of chronic bronchitis and emphysema (Introduction chapter). For neutrophil elastase to have these effects, it has to overcome the anti-elastases that protect the tissues. SLPI is thought to be the most critical anti-elastase protecting the airways (Morrison et al, 1987), whereas alpha-1-antitrypsin (AAT) is thought to be less important at this site, although critical at the alveolar level protecting against the development of emphysema (Gadek et al, 1981).

Subjects with AAT deficiency (PiZ phenotype) have decreased circulating (about 15-20%) (Lieberman et al, 1972) and alveolar concentrations of AAT (Gadek et al, 1981) which facilitates the development of early onset and rapidly progressive emphysema (Brantly et al, 1988). 30-40% of these patients also have chronic bronchitis (Eriksson,

1965)(Brantly et al, 1988) although the nature of the upper airways inflammation has not been studied in these patients.

The purposes of the study were to assess upper airways inflammation using sputum from patients with chronic bronchitis and airflow obstruction to determine the effect of continued smoking and, in particular, investigate the role of AAT in the bronchi by studying subjects with AAT deficiency.

5.2 METHODS

5.2.1 Study population

The following patients were studied: 42 patients with chronic obstructive bronchitis ($FEV_1 < 70\%$ Predicted) with normal AAT (PiM) referred as COB and 39 patients with a similar degree of airflow obstruction who had AAT deficiency of the PiZ phenotype. All patients had chronic bronchitis (Medical Research Council, 1965) and were studied in the stable clinical state at least 8 weeks after a clinical exacerbation and none had received oral steroids or antibiotics within that time period. More patients with AAT deficiency (26) received regular inhaled steroids than the COB group (18, $p < 0.05$) although all other therapy was similar.

5.2.2 Processing of samples and lung function testing

The sputum and serum processing, and assessment of sputum MPO, elastase activity, IL8, LTB₄, SLPI, and protein leakage (sputum/serum AAT ratio (%)) is described in the general methods (Chapter 2). All subjects underwent standardised lung function testing (Society et al, 1994) at the time of the study.

5.2.3 Statistical Analysis

Values are reported as mean (\pm standard error). The Mann-Whitney U test for non paired data was used to compare different groups (AAT deficiency with COB, subgroups of patients colonised with an identifiable pathogen compared to patients not colonised, and finally COB patients who were current or ex smokers). A p value less than 0.05 was considered to be statistically significant.

5.3 RESULTS

The mean age for the patients with COB was 67.1 years (SE \pm 1.2), range 44-79 years and 15 were female. The patients with AAT deficiency were younger with a mean age of 50 (\pm 1.5), range 33-66 years ($p < 0.005$) and 9 were female ($p > 0.1$). Average lung function for both groups is summarised in Table 5-1. Twenty of the patients in the COB group were ex-smokers and the remaining 22 were current smokers, whereas the majority of patients in the AAT deficiency group (34) were ex smokers with 5 current smokers.

	COB		PiZ AAT deficiency	
	Observed	%Predicted	Observed	% Predicted
FEV ₁ (L)	0.8 (0.1)	30.1 (3.1)	0.9 (0.1)	27.4 (3.0)
FVC (L)	2.0 (0.2)	64.3 (4.4)	2.9 (0.2)	70.7 (4.6)
FEV ₁ /FVC (%)	36.4 (2.2)	46.3 (2.8)	29.9 (2.1)	36.5 (2.5)
RV/TLC (%)	61.3 (1.7)	159.8 (3.9)	41.6 (2.4)	130.8 (7.4)
kCO (mmol/m/kPa/L)	0.8 (0.1)	51.4 (5.1)	1.0 (0.1)	53.3 (6.2)

Table 5-1: Average lung function data (\pm SE) is shown for the patients with COB and those with PiZ AAT deficiency. Values are also expressed as a percentage of that predicted for the patients age, height and sex (Society et al, 1994).

Table 5-2 summarises the average sputum values for all the patients in each group, as well as data for the current and ex-smokers in the COB group. The current smokers had slightly better lung function with an average FEV₁ (% predicted) for the patients age and height (Society et al, 1994) of 35.1 % (\pm 3.4) than the ex smokers (25.0 % \pm 1.8, $p < 0.05$) although the ages were similar for both groups (current smokers 66.0 \pm 1.9 years and 68.8 \pm 1.5 years for ex smokers).

Mean \pm SE	COB n=42	COB		PiZ AAT def. n=39
		Current smokers n= 22	Ex- smokers n= 20	
		Sputum	0.4 \pm 0.1	
MPO (Units/ml)	****	**** λ	****	
Sputum	0.03 \pm 0.01	0.05 \pm 0.03	0.02 \pm 0.01	0.18 \pm 0.09
Elastase (μ M)	**	*	*	
Sputum	8.2 \pm 1.2	10.4 \pm 1.9	6.9 \pm 1.7	11.2 \pm 2.6
IL8 (nM)		ϕ		
Sputum	7.9 \pm 1.4	8.3 \pm 2.4	7.8 \pm 1.7	32.0 \pm 8.7
LTB4 (nM)	***	*	*	
Sputum	3.1 \pm 0.5	2.1 \pm 0.4	4.2 \pm 0.9	2.4 \pm 0.4
SI,PI (μ M)	*			
Sputum/Serum	1.1 \pm 0.2	1.2 \pm 0.3	0.6 \pm 0.1	2.2 \pm 0.3
AAT Ratio (%)	****	****	*	
Sputum	0.3 \pm 0.1	0.4 \pm 0.10	0.2 \pm 0.03	0.1 \pm 0.01
AAT (μ M)	****	****	****	

Table 5-2: Data for the COB patients, and subset into COB current and ex-smokers, and those with PiZ AAT deficiency. PiZ AAT def. = PiZ alpha-1-antitrypsin deficient patients. Values are the mean (standard error). Significant differences between the COB group and the group with PiZ AAT deficiency are indicated (* = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.005$, **** = $p < 0.001$). Significant difference between the COB current and ex- smokers (λ = $p = 0.06$ and ϕ = $p < 0.05$).**

All samples from the COB group contained measurable quantities of all proteins with the exception of elastase activity which was present in only 14 of the 42 patients. Subgroup analysis showed that the II.8 and MPO concentrations were lower in the ex smokers although the latter just failed to reach significance (Table 5-2).

The AAT deficiency group showed evidence of greater degree of bronchial inflammation compared to the COB group. The LTB₄ and MPO concentrations were higher and elastase activity was detected in most samples (25/39) but the concentrations of its inhibitors SLPI and AAT were lower despite increased protein leakage of AAT. These differences were maintained even when the AAT deficient group was compared to the subgroups of patients with COB who were current or ex smokers (Table 5-2).

Quantitative bacterial culture indicated that some of the patients in both the COB and AAT deficient groups were colonised by bacteria despite being in a stable clinical state. Nineteen of the patients with COB had either no bacterial growth in their sputum or low numbers ($<10^5$ colony forming units/ml [cfu/ml]) of mixed normal flora. However for the remainder the median bacterial load was 3×10^8 cfu/ml (range = 1×10^7 - 5×10^9). Of these patients 17 cultured *Haemophilus sp.*, 4 *Moraxella catarrhalis* and 2 *Streptococcus sp.* Sputum from thirteen patients with AAT deficiency grew no recognised organism or mixed normal flora. Twelve patients had $<10^7$ cfu/ml of a single viable organisms in their sputum (all were *Haemophilus sp.*). The remaining 14 patients had organisms at $\geq 10^7$ cfu/ml in their sputum with a median bacterial load of 8×10^8

(range = 2×10^7 - 2×10^9). Of the organisms grown, 11 were *Haemophilus sp.*, 1 *Staphylococcus aureus*, 1 *coliform*, and 1 *Pseudomonas aeruginosa*.

The presence of bacterial colonisation may influence the degree of bronchial inflammation (Hill et al, 1998) as discussed in the previous chapter. In view of this both the COB and AAT deficient groups were subdivided according to the results of bacterial culture. However in the COB group the identified pathogen load was $> 10^7$ cfu/ml whereas the patients with AAT deficiency had a wider range of concentration of colonising organisms (10^5 - 10^9). Therefore the groups were only compared for those subjects where either no pathogen was present or where the bacterial load was $> 10^7$ cfu/ml.

5.3.1 Mixed normal flora

The sputum MPO (Figure 5-1) and LTB₄ concentration (Figure 5-2) were greater in subjects with AAT deficiency (there was no significant difference in IL8 concentration [Figure 5-2]). Samples from 9 of the 13 patients with AAT deficiency had low but detectable concentrations of elastase activity (mean = $0.02 \mu\text{M} \pm 0.004$) whereas eighteen of the 19 samples from the COB patients had no detectable elastase activity (in the remaining subject the value was $0.03 \mu\text{M}$, $p < 0.0005$) (Figure 5-3). Average protein leakage in the AAT deficient group ($1.8\% \pm 0.5$) was higher ($p < 0.05$) than the COB group ($0.8\% \pm 0.1$) (Figure 5-4), whereas sputum AAT concentrations were lower in the AAT deficient group ($0.08 \mu\text{M} \pm 0.02$, $p < 0.005$) than the COB group ($0.24 \mu\text{M} \pm 0.06$) which is consistent with the serum deficiency of this inhibitor (Figure 5-3). The

concentrations of secretory leukoprotease inhibitor however were similar in both the AAT deficiency ($4.0 \pm 0.7 \mu\text{M}$) and COB patients ($4.4 \pm 1.0 \mu\text{M}$) (Figure 5-3).

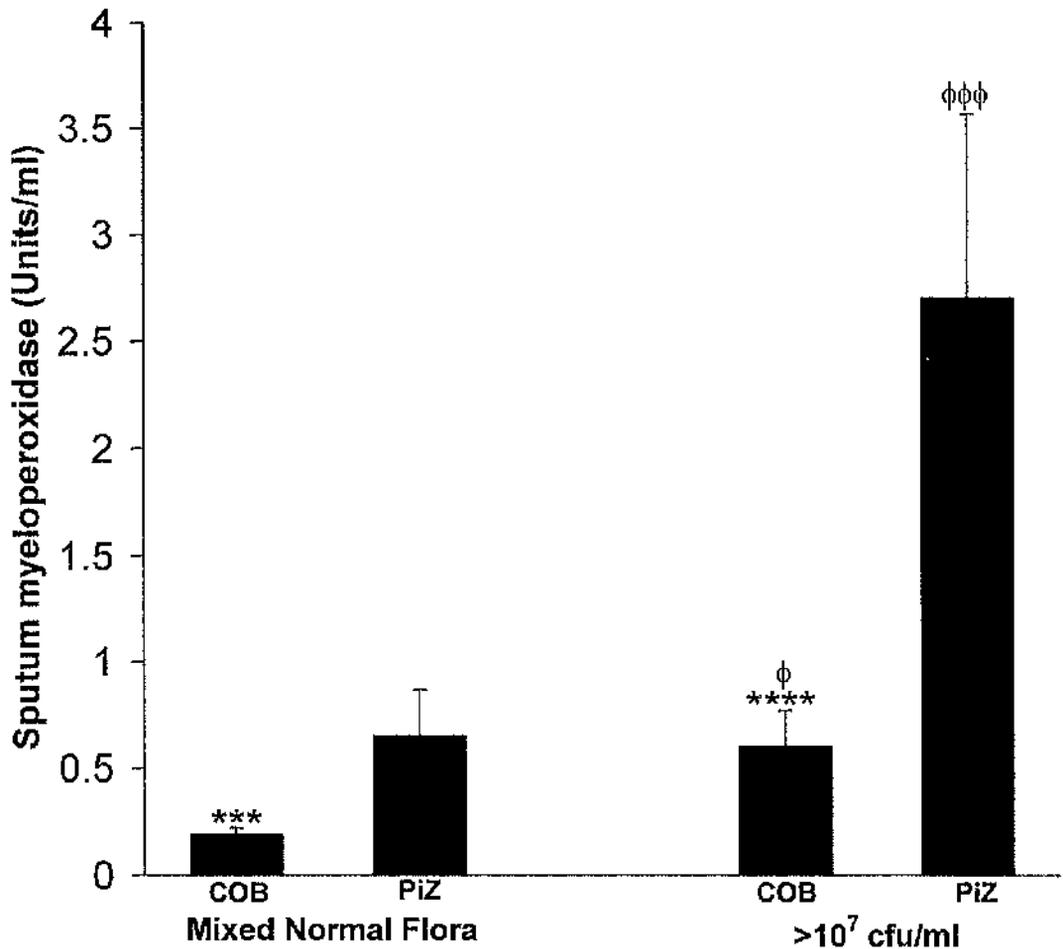


Figure 5-1: Sputum Myeloperoxidase (Units/ml) is shown for patients with COB and PiZ AAT deficiency. The data is separated into patients colonised ($>10^7$ cfu/ml) or not colonised (mixed normal flora) with bacteria in the stable clinical state. The histograms represent mean results for the subgroup together with the standard error bar. The asterisks indicate a significant difference between COB and PiZ AAT deficiency (*) = $p < 0.005$; **** = $p < 0.001$), and the symbol (ϕ) significant differences within the COB and PiZ AAT deficiency patient groups related to bacterial load ($\phi = p < 0.05$; $\phi\phi\phi = p < 0.005$).**

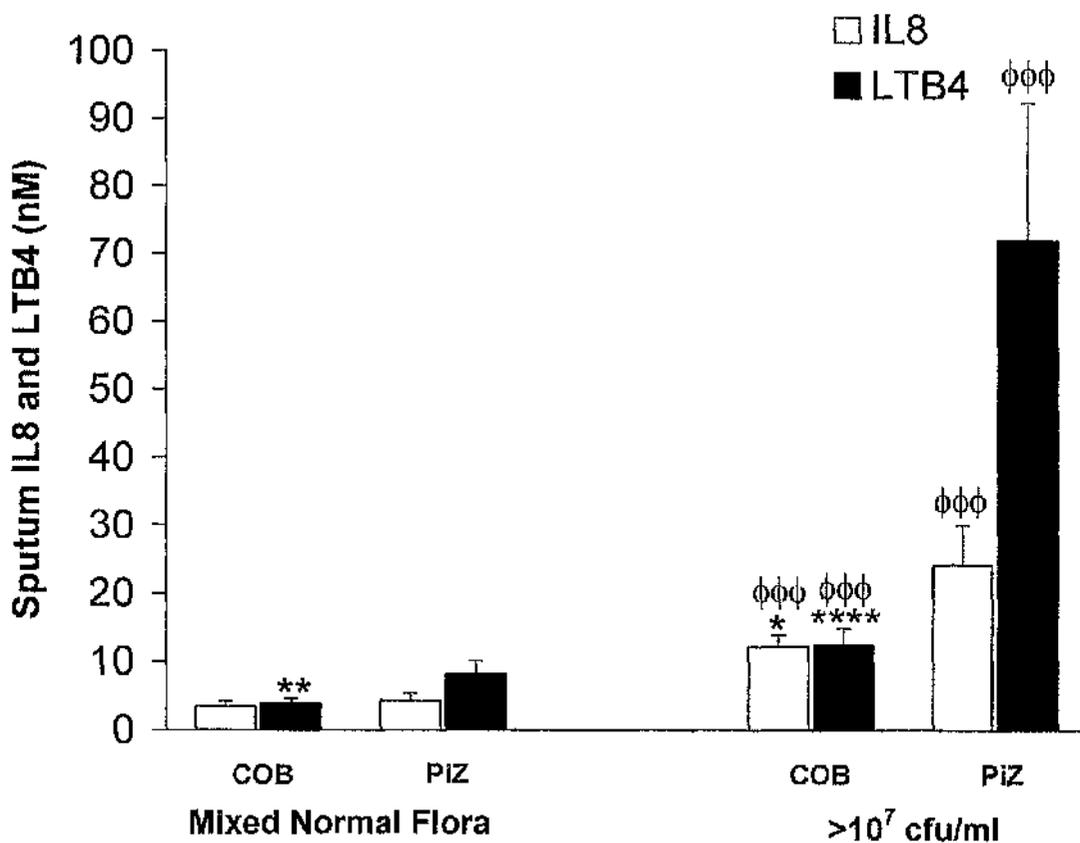


Figure 5-2: The sputum concentrations of IL8 and LTB4 (nM) are shown for patients with COB and PiZ AAT deficiency. The data is separated into patients colonised (>10⁷ cfu/ml) or not colonised (mixed normal flora) with bacteria in the stable clinical state. The histograms represent mean results for the subgroup together with the standard error bar. The asterisks indicate a significant difference between COB and PiZ AAT deficiency (* = p< 0.05; ** = p< 0.01; **** = p< 0.001), and the symbol (φ) significant differences within the COB and PiZ AAT deficiency patient groups related to bacterial load (φφφ = p< 0.005).

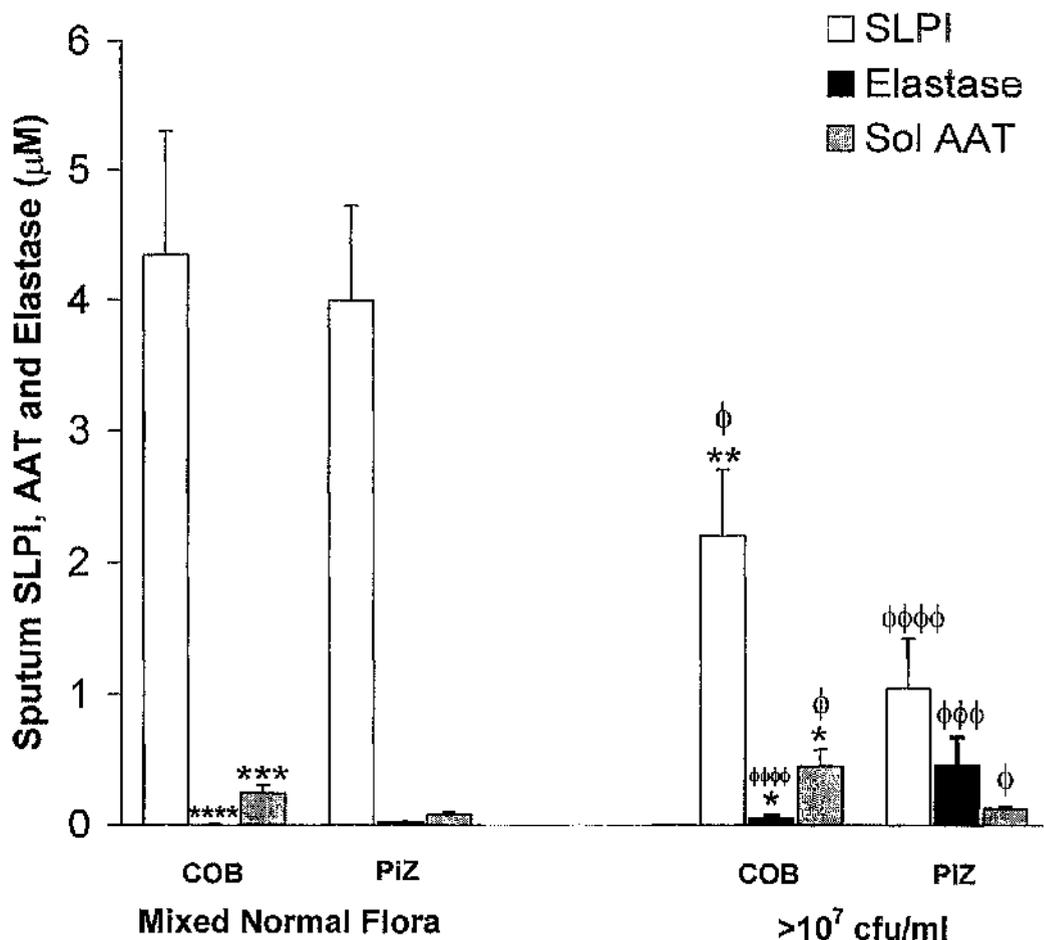


Figure 5-3: The sputum concentrations of SLPI, AAT, and Elastase (μM) are shown for patients with COB and PiZ AAT deficiency. The data is separated into patients colonised ($>10^7$ cfu/ml) or not colonised (mixed normal flora) with bacteria in the stable clinical state. The histograms represent mean results for the subgroup together with the standard error bar. The asterisks indicate a significant difference between COB and PiZ AAT deficiency (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$; **** = $p < 0.001$), and the symbol (ϕ) significant differences within the COB and PiZ AAT deficiency patient groups related to bacterial load (ϕ = $p < 0.05$; $\phi\phi\phi$ = $p < 0.005$; $\phi\phi\phi\phi$ = $p < 0.001$).

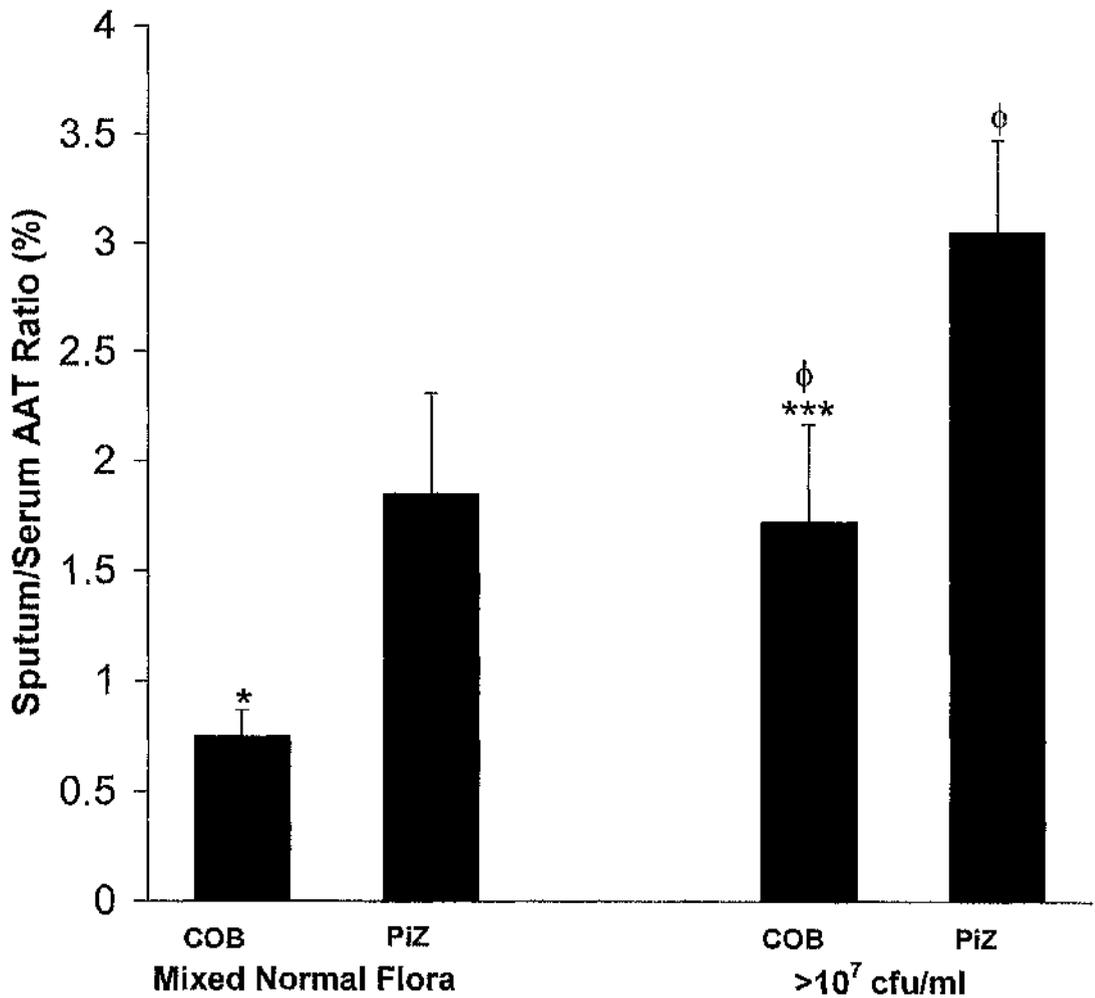


Figure 5-4: Sol to Serum AAT Ratio (%) is shown for patients with COB and PiZ AAT deficiency both with and without bacterial colonisation (mean \pm SE). The asterisks indicate a significant difference between COB and PiZ AAT deficiency (***) $p < 0.005$, and the symbol (ϕ) significant differences within the COB and AAT deficiency patients related to bacterial load (ϕ $p < 0.05$).

5.3.2 $>10^7$ colony forming units/ml

The sputum MPO, LTB₄, IL8, and elastase activity concentrations were increased in both the COB and AAT deficiency group colonised by $>10^7$ cfu/ml compared to those not colonised (mixed normal flora) as shown in Figure 5-1, Figure 5-2, and Figure 5-3. Elastase activity was detectable in 7 of 23 samples from COB and 13 of 14 for AAT deficient patients (mean = $0.06 \mu\text{M} \pm 0.02$, $p < 0.001$ and mean = $0.46 \mu\text{M} \pm 0.22$, $p < 0.005$ respectively). In addition protein leakage (COB $1.7\% \pm 0.4$ and AAT deficiency $3.0\% \pm 0.4$) was greater in both patient groups (both $p < 0.05$) (Figure 5-4) and sputum AAT concentration (COB $0.45 \pm 0.13 \mu\text{M}$ and AAT deficiency $0.13 \pm 0.02 \mu\text{M}$) were increased (both $p < 0.05$) (Figure 5-3). On the other hand SLPI concentration (COB $2.2 \pm 0.5 \mu\text{M}$ and AAT deficiency $1.0 \pm 0.4 \mu\text{M}$) were reduced ($p < 0.05$ and $p < 0.0005$ respectively) (Figure 5-3).

Comparison between patient groups colonised with $>10^7$ cfu/ml showed that the AAT deficiency group had greater concentrations of MPO ($p < 0.005$) (Figure 5-1), LTB₄ ($p < 0.005$) and IL8 ($p < 0.05$) (Figure 5-2), elastase activity ($p < 0.05$) (Figure 5-3), and protein leakage ($p < 0.005$) (Figure 5-4) but lower concentration of AAT ($p < 0.05$) and SLPI ($p < 0.01$) (Figure 5-3).

5.4 DISCUSSION

Present data shows evidence of upper airways inflammation in patients with chronic obstructive bronchitis with a wide range of neutrophil influx (as reflected in the MPO concentration) in response to the chemoattractants IL8 and LTB4 which are thought to play a key role in neutrophil recruitment (Mikami et al, 1998). Elastase activity was absent or low even though these samples are known to contain the enzyme (Stockley et al, 1979) probably due to inhibition by the natural inhibitors especially SLPI.

Comparison between current and ex smokers showed that the only differences were lower IL8 and MPO concentrations in the latter group. This did not reflect differences in therapy and the ex smokers had worse lung function which would, if anything, increase neutrophil numbers (Keatings et al, 1997)(Di Stefano et al, 1998). IL8 is thought to be a major airways neutrophil chemoattractant (Richman-Eisenstat et al, 1993) and is increased in healthy smokers (Keatings et al, 1997) and subjects with COPD (Keatings et al, 1997) although the effect of smoking cessation has not been assessed previously. The current data indicates that cessation of smoking in chronic bronchitis is related to a reduction in airways IL8 concentration which in turn would reduce neutrophil recruitment thereby explaining the beneficial effect of smoking cessation on progression of lung disease. The effect may be due to loss of cigarette smoke induced epithelial production of IL8 (Mio et al, 1997).

SLPI is believed to be the major inhibitor of elastase in the airway. However the current data shows that airways inflammation is increased in the AAT deficient subjects despite

more being on inhaled steroids (which would be expected to have a beneficial effect (Llewellyn-Jones et al, 1996)) and the subjects being younger (which would also reduce bronchial inflammation) (Meyer et al, 1996)(Meyer et al, 1998). MPO concentrations were higher, as was the chemoattractant LTB₄. This suggests that LTB₄ may be the major chemoattractant responsible for the increased neutrophil migration and is consistent with previous findings in bronchoalveolar lavage (Hubbard et al, 1991). The source of the LTB₄ is uncertain although Hubbard et al. (Hubbard et al, 1991) suggested it was released from alveolar macrophages as a direct effect of uninhibited elastase due to alpha-1-antitrypsin deficiency. Indeed in the current study elastase activity was more readily detected in the alpha-1-antitrypsin deficient patients irrespective of bacterial colonisation and hence would support the suggestion that this may be responsible (Hubbard et al, 1991). The elastase activity that was more readily detected in the upper airways in alpha-1-antitrypsin deficiency is probably due to the combined effect of the lower concentrations of both AAT and SLPI. The mechanisms involved may be complex but free elastase activity can reduce secretion of SLPI (Sallenave et al, 1994) as well as increasing permeability of airway cells (Peterson et al, 1995). Despite the latter effect which accounts for an increase in AAT "leak" into the lung, the low AAT concentrations in these subjects may be critical in determining the overall changes suggesting that this inhibitor also has a major role in the airways.

Bacterial colonisation is clearly related to the degree of airways inflammation suggesting this may not always be a benign situation even though the patients were "clinically" stable. Controlling for airways colonisation, indicated the AAT deficient group still had increased airways inflammation which is likely to be responsible for or reflect the

development of severe airflow limitation at an earlier age. It is of importance to note that these changes were present in ex smokers with AAT deficiency and would suggest that measures other than smoking cessation may be critical in stabilising lung disease in these patients, such as alpha-1-antitrypsin augmentation therapy, but this requires further studies.

In conclusion patients with chronic bronchitis have evidence of bronchial inflammation. The chemoattractant interleukin 8 plays a key role in neutrophil recruitment and the beneficial effects of smoking cessation may be mediated by reducing concentration of this cytokine. Patients with AAT deficiency have greater inflammation (probably related to increased LTB₄ production) and this suggests AAT has an important role in protecting the airways from the inflammatory effects of elastase. Bacterial colonisation may play a major role in determining airways inflammation even in the stable state as shown in Chapter 4.

**6. BRONCHIAL INFLAMMATION DURING AN
ACUTE EXACERBATION IN PATIENTS WITH
PiZ ALPHA-1-ANTITRYPSIN DEFICIENCY**

6.1 INTRODUCTION

As discussed in the general introduction chapter, the lower respiratory tract is usually kept sterile by effective local host defences. However in the presence of established airways disease bacteria are often isolated from expectorated mucus even when the patient appears clinically stable. This presents problems in the determination of the cause of an acute exacerbation of airways disease and hence rationalisation of therapy. In a recent review these problems were highlighted although it was emphasised that when the bacterial load was low, the local host defences may be adequate (Stockley, 1998). However as the bacterial load increases, recruitment of the secondary host defences would result in increased airways inflammation and neutrophil influx (Stockley, 1998). The resolution of such episodes requires a combination of effective therapy resulting in the loss of the inflammatory stimulus and an appropriate acute phase response (Stockley, 1998). In this respect AAT may play a critical role by inactivating elastase activity released by the activated airway neutrophils, both down-regulating inflammation and protecting airways tissue from enzyme induced damage (Stockley, 1998).

Subjects with AAT deficiency often have airways disease (Eriksson, 1965)(Brantly et al, 1988) and hence will be susceptible to bacterial colonisation and acute bacterial infective exacerbations. However because of their deficiency such subjects will not have an appropriate AAT acute phase response which may therefore influence the degree of airways damage caused by such infections. The nature of the airway inflammation and its response to bacterial exacerbations have yet to be studied in AAT deficiency.

The purposes of this study were two-fold. Firstly to assess the inflammatory nature of acute exacerbations in subjects with AAT deficiency and compare this to chronic bronchitic patients without deficiency. Secondly to monitor the inflammatory process and its resolution in patients with AAT deficiency following appropriate antibacterial therapy. Monitoring the inflammatory response included the following measurements: the recruitment of neutrophils (as reflected by MPO activity); the concentration of chemoattractants in the bronchial secretions (IL8 and LTB₄); the concentration of active neutrophil elastase and its natural inhibitors (AAT and SLPI); protein leakage (sputum/serum albumin ratio) as a measure of airways inflammation. Finally the acute phase response of both C-reactive protein and AAT were measured in the serum.

6.2 METHODS

11 patients with chronic bronchitis and homozygous AAT deficiency (PiZ) were studied. None of the patients had ever received AAT replacement therapy. The patients made contact and were seen in the clinic within 48 hours of the onset of new or worsening symptoms suggestive of an acute exacerbation of their disease. The presence of an exacerbation was based on the criteria suggested by Anthonisen (Anthonisen et al, 1987), and included an increase in all 3 of the symptoms of breathlessness, sputum volume and sputum purulence.

6.2.1 Processing of samples

On the day of clinic visit the patients collected sputum over 4 hours from rising. The sputum characteristics, sputum and serum processing, assessment of sputum colour, MPO, elastase activity, IL8, LTB4, SLPI, protein leakage (sputum/serum albumin ratio (%)), and the acute phase response of serum AAT and CRP, was carried out as described in Chapter 2. All subjects underwent standardised lung function testing within 6 months of the study when patients were in a stable clinical state.

6.2.2 Treatment

After sample collection the patients were started on 14 days antibiotics (no patients received treatment before presenting with their exacerbation). They were commenced routinely on Amoxicillin 500mg 3 times daily unless *Moraxella catarrhalis* was suspected on Gram stain, and such patients were started on Cefuroxime axetil 500mg twice daily. These antibiotics were unchanged except in 1 patient where Cefuroxime was changed to Ciprofloxacin 750mg twice daily the following day as *Pseudomonas*

aeruginosa was cultured in addition to *Moraxella catarrhalis* (the dominant organism). No patient received oral steroid therapy, and all other treatment (including inhaled steroids) remained constant throughout the study. The patients returned 1, 3, 5, 7, 14 and 28 days after commencement of therapy and at these visits samples were collected and assessed as above except for follow up day 1 when serum alone was collected. Day 14 was the end of antibiotic treatment and day 28 was 2 weeks later when patients were stable. All patients filled in a daily diary card recording morning and evening peak expiratory flow rates, as well as their symptoms. Finally the patients indicated the day they felt back to their stable clinical state (diary card at end of thesis).

6.2.3 Comparison with patients with chronic bronchitis without AAT deficiency

The data obtained on the day of presentation was compared to that from 11 control patients with chronic bronchitis who had normal AAT. These patients were chosen from a database of outpatients presenting with a similar acute exacerbation (increasing breathlessness, increasing sputum volume and purulence) and were matched with the AAT deficient patients for stable state FEV₁ (% predicted) to ensure a comparable degree of lung function impairment. However complete samples for the control patients were only available for the day of presentation, day 5, and day 28 (stable clinical state) for comparison with the AAT deficient patients.

6.2.4 Statistical analysis

Values are reported as mean (\pm standard error). The Friedman test was used to compare the inflammatory markers throughout the exacerbation, and, where significant, the Wilcoxon Rank test for paired data was used to compare results from the start of the

exacerbation to different points during the exacerbation. The Mann Whitney U test for unpaired data was used to compare data between patients with and without AAT deficiency. A *P* value <0.05 was considered to be statistically significant.

6.3 RESULTS

Demographic details for the AAT deficient and control patients are shown in Table 6-1. Both groups were receiving similar therapy and, in particular, 8 of the AAT deficient group and 9 of the control group were on long term inhaled corticosteroids. No patients were on oral steroid therapy and neither group had evidence of bronchiectasis either clinically or visible on high resolution computed tomography of the chest. The results are shown for post bronchodilator FEV₁, FEV₁/VC ratio, TLC and gas transfer (kCO) expressed as a % of the value predicted for the patients age, height, and sex (Society et al, 1994). Neither group had significant reversibility (>12% increase) to nebulised β_2 agonist (salbutamol 5mg). The average rise in FEV₁ was 55.0 mls (\pm 10.8) for the AAT deficient group and 65.0 mls (\pm 36.4) for the control group.

Mean \pm SE	Patients with AAT deficiency	Control patients without AAT deficiency
Age (years) (range)	48.3 \pm 2.9* (37-66)	67.6 \pm 2.0 (58-79)
Sex (Male:Female)	8:3	7:4
Current Smokers	3	4
Ex Smokers	7	7
Non Smoker	1	0
Pack Years	32.0 \pm 5.9	48.1 \pm 8.9
FEV ₁ (L) (% Predicted)	1.3 \pm 0.3 (36.3 \pm 7.4)	1.4 \pm 0.3 (38.0 \pm 5.7)
FEV ₁ /VC (% Predicted)	44.3 \pm 7.0	45.4 \pm 4.7
TLC (% Predicted)	125.0 \pm 5.0	117.2 \pm 3.6
kCO (% Predicted)	60.2 \pm 9.9	68.6 \pm 4.9

Table 6-1: Demographics and lung function tests for the study. Mean values are shown (\pm SE) for patient demographics features including lung function expressed as a % predicted for sex, age, and height. The asterisk indicates the only statistical difference between the two groups ($p < 0.001$).

6.3.1 Bacteriology (Table 6-2)

All samples obtained at presentation had greater than 25 neutrophils (with < 10 epithelial cells) per high power field ($\times 100$) on Gram stain. All 11 samples from the AAT deficient patients subsequently grew one or two bacterial pathogens (Table 6-2) with a median bacterial load of 6.6×10^8 cfu/ml (range 2.7×10^7 - 5.4×10^9). The sputum samples from the non AAT deficient patients also contained one or two bacterial organisms on quantitative culture. The predominant species included non-typeable *Haemophilus influenzae* (n=6), *Streptococcus pneumoniae* (n=3), and *Moraxella catarrhalis* (n=2). The bacterial load was similar to that for the AAT deficient group (median 2.3×10^8 cfu/ml; range 1.2×10^7 - 9.0×10^9).

Patient	Day 0		Day 14	Day 28 (Stable)
1	S. pneumoniae* 7.9×10^7 cfu/ml		Mixed normal flora	NTHI 8.0×10^6 cfu/ml
2	NTHI 9.1×10^3 cfu/ml		NTHI 2.8×10^8 cfu/ml	NTHI 3.2×10^8 cfu/ml
3	NTHI 6.6×10^3 cfu/ml		NTHI 2.1×10^8 cfu/ml	NTHI 3.2×10^8 cfu/ml
4	H. para 2.7×10^7 cfu/ml		Mixed normal flora	H. para 5.0×10^6 cfu/ml
5	NTHI 6.3×10^8 cfu/ml		Mixed normal flora	Mixed normal flora
6	M. cat 1.1×10^9 cfu/ml	Ps. Aer 6.4×10^8 cfu/ml	Mixed normal flora	S. viridans 8.4×10^7 cfu/ml
7	M. cat 2.6×10^9 cfu/ml	NTHI 5.4×10^8 cfu/ml	Mixed normal flora	Mixed normal flora
8	NTHI 5.8×10^8 cfu/ml		Mixed normal flora	NTHI 1.5×10^7 cfu/ml
9	M. cat 2.4×10^8 cfu/ml		Mixed normal flora	Mixed normal flora
10	M. cat 4.8×10^9 cfu/ml	NTHI 6.2×10^8 cfu/ml	NTHI 3.9×10^8 cfu/ml	NTHI 4.2×10^9 cfu/ml
11	S. pneumoniae 2.2×10^9 cfu/ml		Mixed normal flora	Mixed normal flora

Table 6-2: Sputum bacteriology in patients with AAT deficiency at the start of the exacerbation, end of treatment, and 2 weeks after treatment finished. *The organism is indicated together with the viable number in sputum (cfu/ml). S. Pneumoniae = *Streptococcus pneumoniae*; NTHI = Non typeable *Haemophilus influenzae*; M. Cat = *Moraxella catarrhalis*; Ps. Aer = *Pseudomonas aeruginosa*; H. para = *Haemophilus parainfluenzae*; S. viridans = *Streptococcus viridans*.

6.3.2 Sputum analysis at presentation (Table 6-3)

When first seen all patients with AAT deficiency had purulent sputum (see Methods) with a mean sputum colour value of 4.6 (SE \pm 0.3) which is consistent with the high sputum MPO level. In addition the sputum concentration of both the chemoattractants IL8 and LTB4 were high, and free elastase activity was present in 10 of the 11 sputum samples (range; 5-1,631 nM). Although the sputum SLPI concentration was low, the sputum AAT concentration was relatively high for this group consistent with increased protein leakage from serum. This increased leakage was supported by the high sputum/serum albumin ratio.

The control patients without AAT deficiency showed differences compared to the patients with AAT deficiency at presentation (Table 6-3). Although the average sputum MPO value was similar to that seen in the AAT deficient group ($p= 0.17$), the sputum elastase activity (detectable in 8/11 patients) was lower ($p= 0.02$) with a range from 0-166 nM in the non-deficient patients. In addition the average sputum concentrations of both IL8 and LTB4 were lower in the non deficient group ($p= 0.01$ and $p= 0.02$ respectively) and the protein leakage was lower although this just failed to reach statistical significance ($p= 0.06$). The sputum concentrations of both SLPI and AAT were higher in the non-deficient group ($p= 0.02$ and $p< 0.001$ respectively).

	Controls without AAT deficiency	AAT Deficiency	P value
Sputum MPO (units/ml)	1.0 ± 0.3	1.4 ± 0.4	0.17
Sputum Elastase (nM)	30.1 ± 15.4	254.3 ± 147.2	0.02
Sputum IL8 (nM)	10.4 ± 2.6	30.9 ± 9.2	0.01
Sputum LTB4 (nM)	15.9 ± 4.9	33.2 ± 8.3	0.02
Sputum SLPI (µM)	1.9 ± 0.6	1.0 ± 0.4	0.02
Sputum AAT (µM)	0.91 ± 0.14	0.25 ± 0.09	<0.001
Sputum/Serum Albumin Ratio (%)	1.5 ± 0.4	3.8 ± 1.8	0.06
Serum AAT (µM)	31.3 ± 3.4	4.8 ± 0.7	<0.001
Serum CRP (mg/L)	42.9 ± 19.3	34.0 ± 18.5	0.9

Table 6-3: Bronchial inflammation at the start of the exacerbation. *Average results (± standard error) are shown for 11 patients with AAT deficiency and 11 controls at the start of an exacerbation. The significance of any difference between the groups (p) is shown.

6.3.3 The effect of antibiotic therapy in patients with AAT deficiency

After 14 days of therapy the pathogen had been cleared in 8 of the 11 AAT deficient patients and the bacterial load was lower in the remaining three (Table 6-2). All patients improved clinically (as assessed by the symptom diary card) during antibiotic therapy. There was a reduction in sputum colour ($p < 0.01$), MPO ($p < 0.005$), IL8 ($p < 0.01$), LTB4 ($p < 0.005$), and sputum elastase activity ($p < 0.01$) which fell in the 10 subjects where it was present at the start of the exacerbation becoming undetectable in 2. The sputum AAT fell ($0.11 \pm 0.04 \mu\text{M}$, $p < 0.005$) and the SLPI concentration rose ($p < 0.01$). Finally there was a reduction in protein leakage in the airway as the sputum/serum albumin ratio fell ($p < 0.01$). These results are summarised in Figures 6-1 - Figure 6-7.

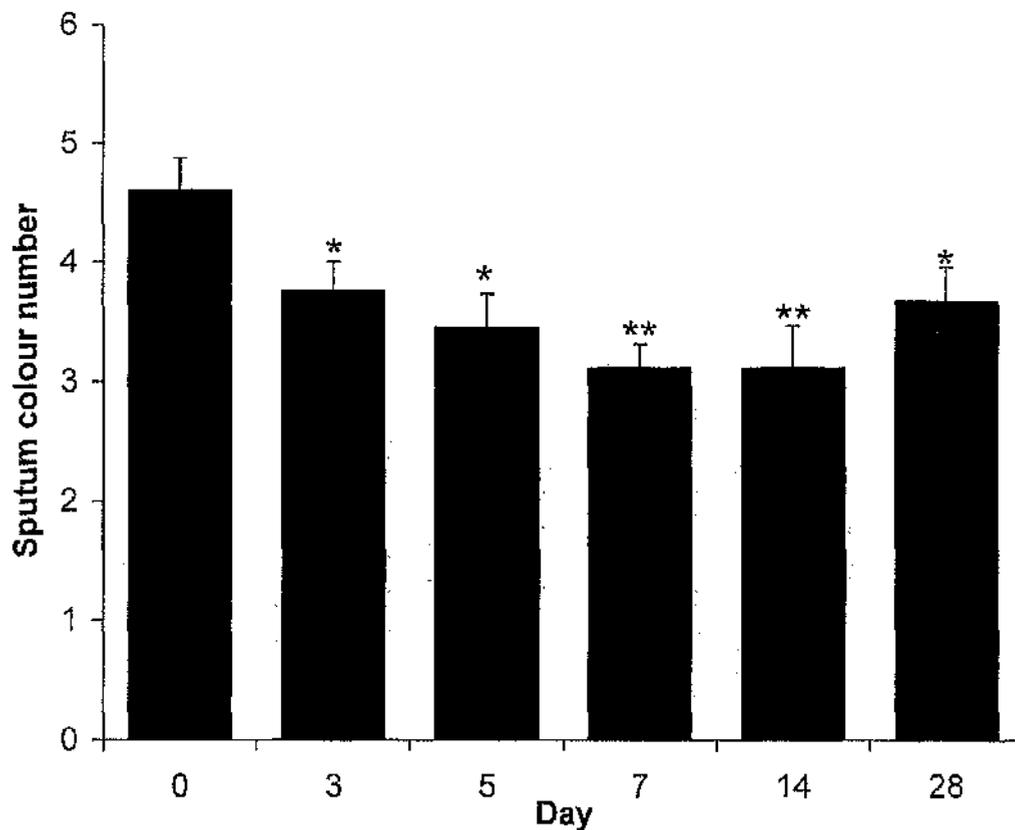


Figure 6-1: Time course of response of sputum colour number following treatment. The average data \pm standard error is shown for samples at presentation, at different times during therapy up to day 14 and in the stable state 2 weeks after cessation of therapy. The asterisks indicate values that are significantly reduced compared to those at presentation (* $p < 0.05$; ** $p < 0.01$).

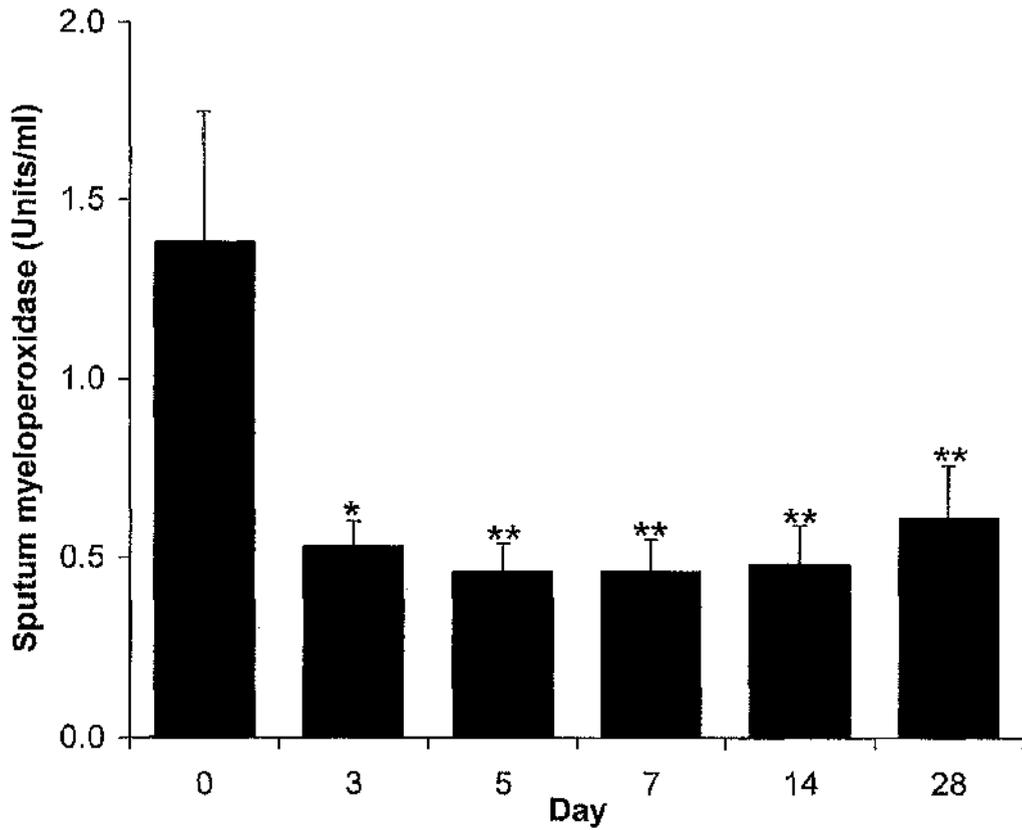


Figure 6-2: Time course of response of sputum myeloperoxidase following treatment. The average data \pm standard error is shown for samples at presentation, at different times during therapy up to day 14 and in the stable state 2 weeks after cessation of therapy. The asterisks indicate values that are significantly reduced compared to those at presentation (* $p < 0.01$; ** $p < 0.005$).

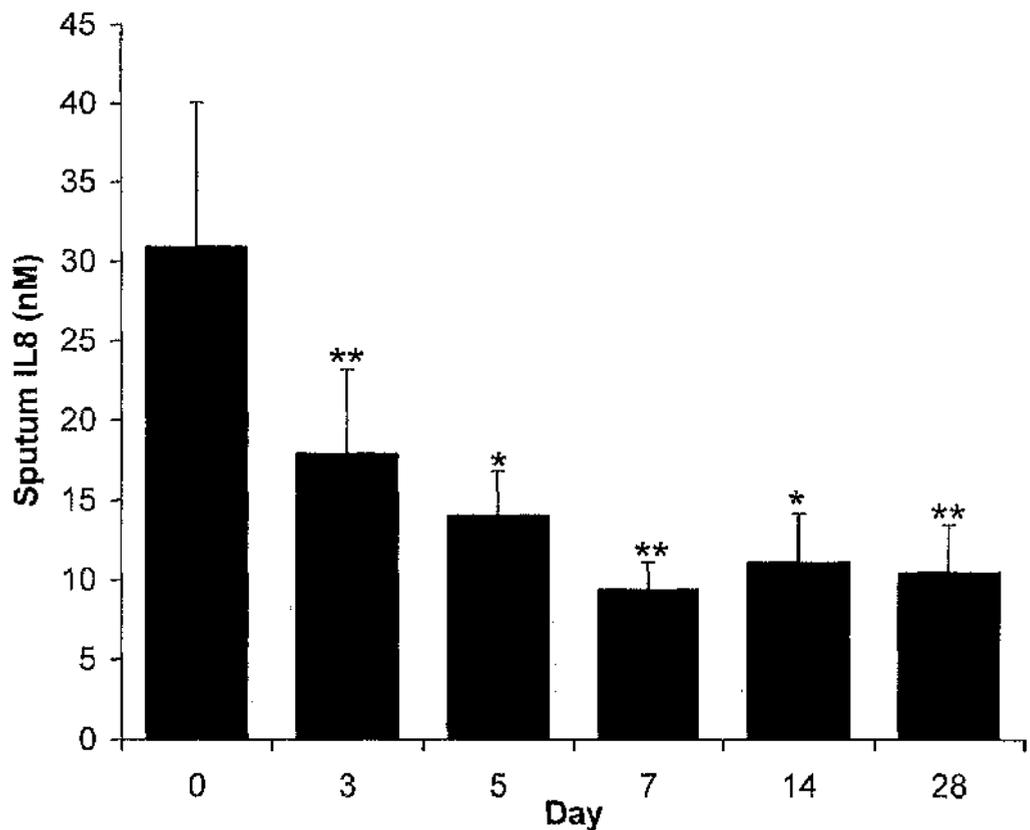


Figure 6-3: Time course of response of sputum interleukin 8 following treatment. The average data \pm standard error is shown for samples at presentation, at different times during therapy up to day 14 and in the stable state 2 weeks after cessation of therapy. The asterisks indicate values that are significantly reduced compared to those at presentation (* $p < 0.01$; ** $p < 0.005$).

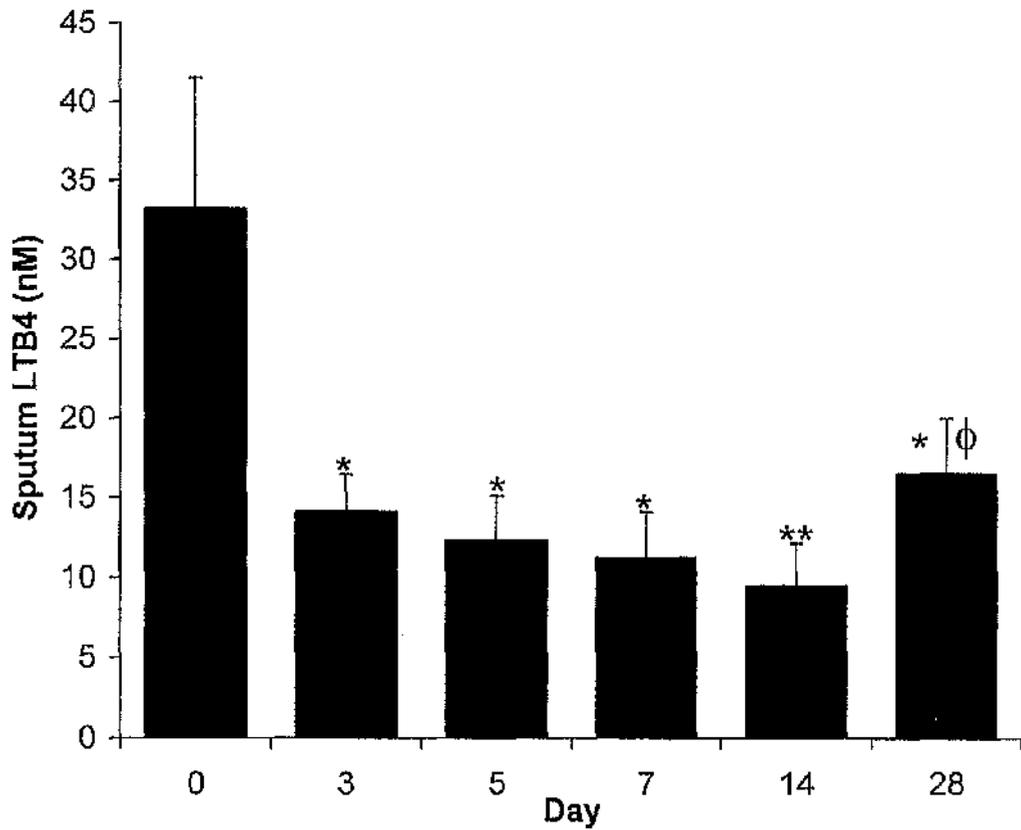


Figure 6-4: Time course of response of sputum leukotriene B4 following treatment. The average data \pm standard error is shown for samples at presentation, at different times during therapy up to day 14 and in the stable state 2 weeks after cessation of therapy. The asterisks indicate values that are significantly reduced compared to those at presentation (* $p < 0.05$; ** $p < 0.005$). Values for leukotriene B4 in the stable clinical state are significantly higher than at the end of treatment - day 14 ($\phi p < 0.05$).

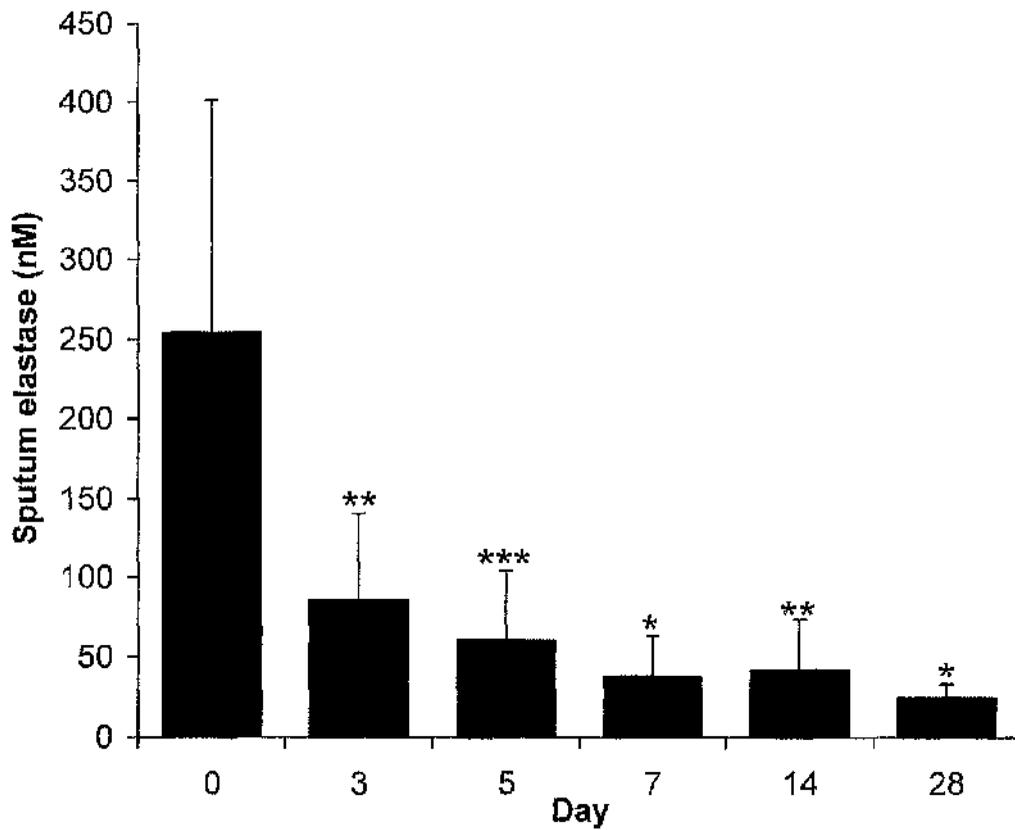


Figure 6-5: Time course of changes in sputum elastase activity. The asterisk indicates values for elastase that were significantly different from presentation (* $p < 0.05$; ** $p < 0.01$; * $p < 0.005$).**

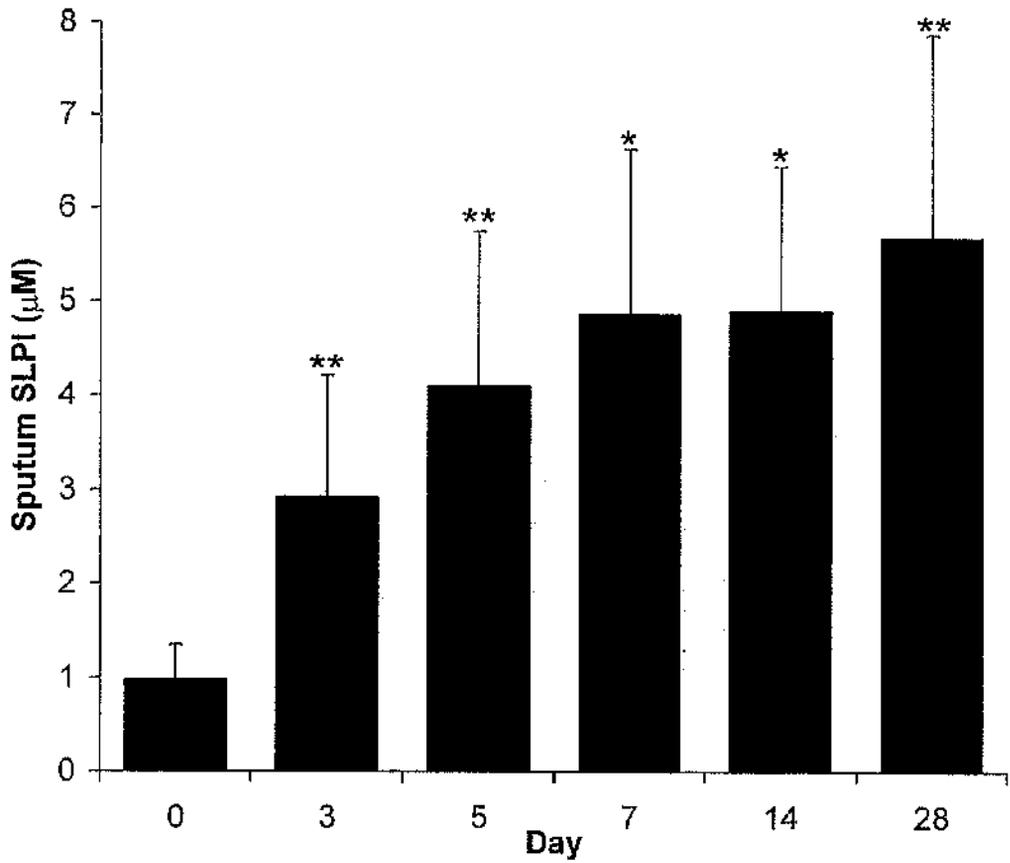


Figure 6-6: Time course of changes in sputum secretory leukoprotease inhibitor. The asterisks indicate values for SLPI that were significantly different from those at presentation (* $p < 0.01$; ** $p < 0.005$).

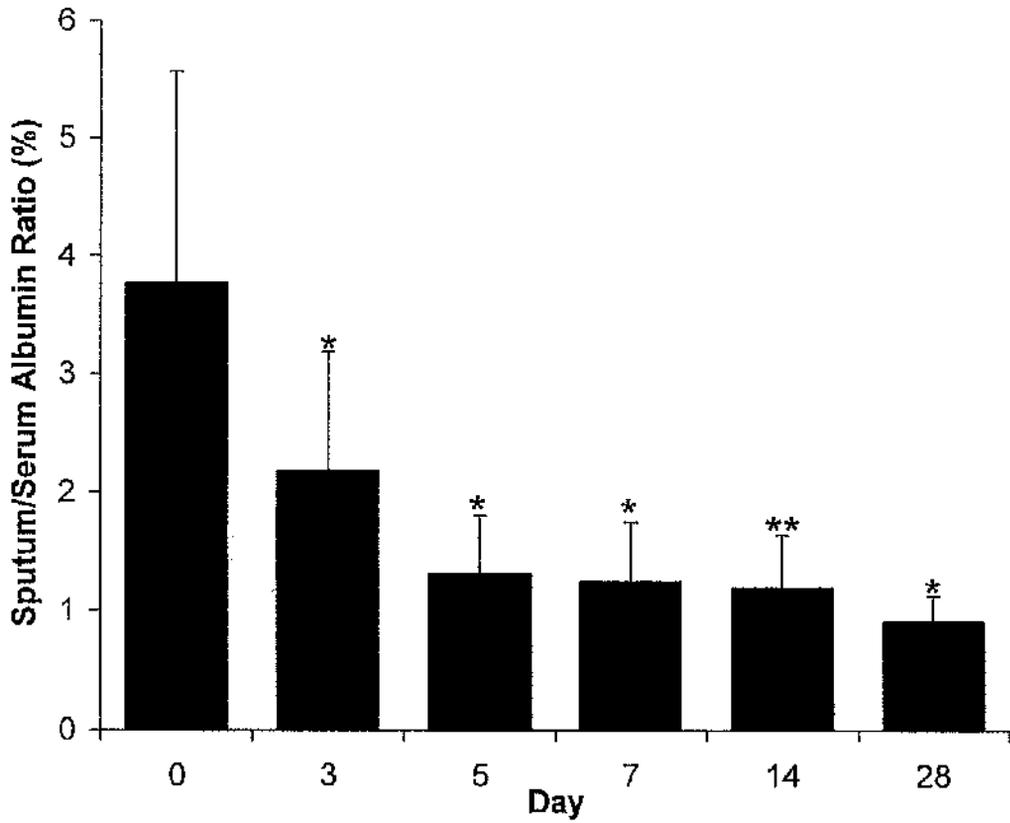


Figure 6-7: Sputum/serum albumin ratio is shown following presentation with the exacerbation (\pm standard error). The asterisks indicate values significantly lower than at presentation (* $p < 0.05$; ** $p < 0.01$).

6.3.4 Acute phase response

At presentation the serum CRP and AAT concentrations were raised to average values of 34.0 mg/L (\pm 18.5) and 4.8 μ M (\pm 0.7) respectively in patients with AAT deficiency (Figure 6-8 & Figure 6-9). The results for CRP were similar to that for the non deficient subjects (42.9 ± 19.3 , $p= 0.9$). On the other hand the control subjects had greater ($p < 0.001$) serum concentrations of AAT ($31.3 \pm 3.4 \mu$ M) at the start of the exacerbation as would be expected.

By the end of therapy (day 14) the serum values for the AAT deficient subjects had fallen significantly (CRP: $8.9 \text{ mg/l} \pm 3.9$, $p= 0.02$; AAT $4.1 \pm 0.5 \mu$ M, $p= 0.03$) and remained low when the patients were reviewed on day 28 whilst still clinically stable (CRP: $7.0 \text{ mg/l} \pm 2.9$ and AAT $3.6 \pm 0.5 \mu$ M). Similarly both the serum values of CRP and AAT were reduced at this stage in the non deficient subjects (CRP $7.0 \text{ mg/l} \pm 2.6$ and AAT $23.8 \pm 2.2 \mu$ M, both $p < 0.05$).

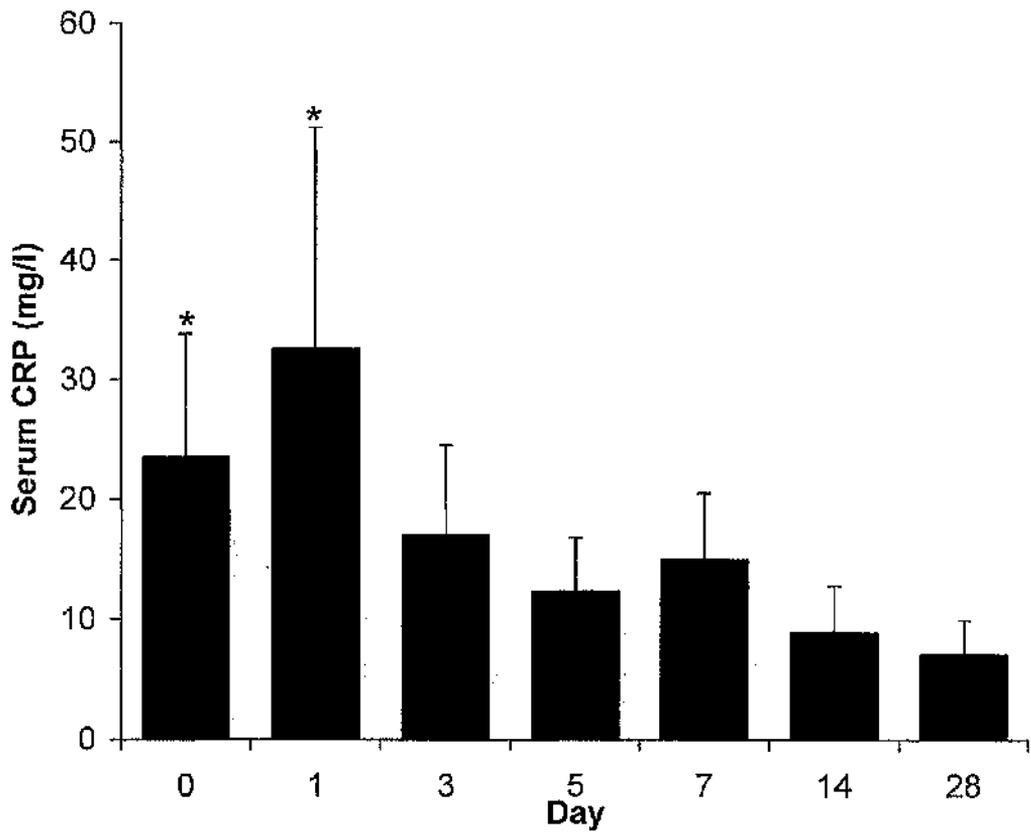


Figure 6-8: The average value for C-reactive protein is shown (\pm standard error). Asterisk indicate values that are higher than in the stable clinical state (day 28) (* $p < 0.01$).

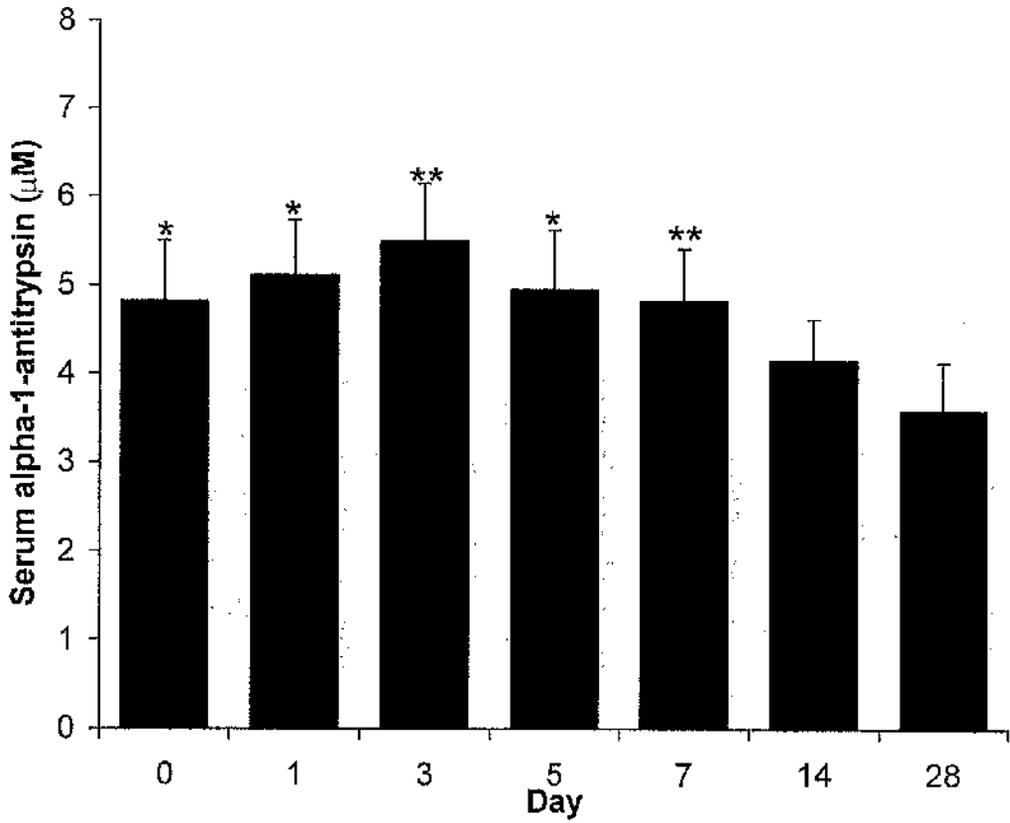


Figure 6-9: The average value for AAT are shown (\pm standard error). Asterisks indicate values that are higher than in the stable clinical state (day 28) (* $p < 0.05$; ** $p < 0.01$).

6.3.5 Time course of the response in patients with AAT deficiency

The patients showed a gradual improvement in symptoms and indicated a return to their stable clinical state (on the diary card), on average 13.2 (\pm 2.1) days after the start of therapy. The biochemical response to antibiotic therapy, however, was generally rapid with a significant decrease in sputum colour number ($p < 0.05$) to 3.8 ± 0.2 by the third day of treatment. This was associated with a significant decrease in sputum MPO ($p < 0.01$), IL8 ($p < 0.005$) and LTB4 concentrations ($p = 0.02$) by the third day of treatment. The lowest average concentrations of MPO, IL8 and LTB4 were found by the 5th, 7th and 14th day of therapy respectively (these results are summarised in Figure 6-2, Figure 6-3, & Figure 6-4). By the third day of treatment sputum elastase activity had decreased ($p < 0.01$) (Figure 6-5) and the sputum AAT concentration had fallen ($p < 0.005$) to $0.17 \mu\text{M}$ (\pm 0.06). This latter effect was related to a rapid fall in protein leakage ($p = 0.02$) as shown in Figure 6-7. However, the sputum SLPI concentration rose ($p < 0.005$) to $2.9 \pm 1.3 \mu\text{M}$ by the third day of treatment (Figure 6-6).

On the fifth day, the MPO, elastase activity, IL8, LTB4, and protein leakage remained low in the deficient subjects (Figures 6-2 - Figure 6-7). These results were similar to those observed in the non-deficient patients at this time with the exception that patients with AAT deficiency still had higher IL8 concentration and elastase activity (AAT deficient patients:- IL8= $14.1 \pm 2.8 \text{ nM}$ and elastase activity = $60.9 \pm 44.0 \text{ nM}$: non-deficient patients:- IL8= $4.9 \pm 1.9 \text{ nM}$ and elastase activity = $2.6 \pm 1.7 \text{ nM}$: both $p < 0.005$) and the sputum AAT concentration remained lower ($p < 0.001$) in the deficient patients compared to the controls ($0.12 \mu\text{M} \pm 0.04$ and $0.48 \mu\text{M} \pm 0.11$ respectively).

The serum acute phase response in patients with AAT deficiency was most obvious with CRP which peaked on the first day after presentation and fell significantly ($p < 0.05$) by the fifth day of treatment (Figure 6-8). The AAT response was small but peaked at day 3, falling thereafter (average presentation = $4.8 \pm 0.7 \mu\text{M}$; day 3 = $5.5 \pm 0.7 \mu\text{M}$; day 14 = $4.1 \pm 0.5 \mu\text{M}$; and day 28 = $3.6 \pm 0.5 \mu\text{M}$) (Figure 6-9).

6.3.6 Stable clinical state

When the AAT deficient patients were restudied 28 days after presentation (14 days after cessation of therapy), 7 were colonised with an identifiable organism (Table 6-2) although the viable bacterial load in these 7 subjects (median value on day 28 = 8.4×10^7 ; range = 5.0×10^6 - 4.2×10^9) was lower than that at presentation (median = 6.6×10^8 organisms/ml; range = 2.7×10^7 - 5.4×10^9 ; $p < 0.05$).

The continued well being of the patients was reflected in a persistently lower sputum colour compared to that at presentation ($p < 0.01$). The average sputum MPO and IL8 concentration remained low (Table 6-4) although the sputum LTB4 concentration was higher ($p = 0.02$) than at the end of therapy ($9.5 \pm 2.6 \text{ nM}$) as summarised in Figure 6-4. Elastase activity became detectable in all but 1 patient by day 28 although the average value remained low (Figure 6-5). Finally sputum SLPI concentration remained high (Figure 6-6) and the sputum AAT concentration remained low ($0.10 \mu\text{M} \pm 0.03$) consistent with a persistent reduction in protein leakage (Figure 6-7).

The results obtained on day 28 were not significantly different to those obtained in the AAT deficient patients when they had been studied in the stable clinical state more than 8 weeks prior to their exacerbation (Table 6-4).

	Baseline	Exacerbation Onset	Resolution (day 28)	P value Baseline- Exacerbation
Sputum MPO (units/ml)	0.5 ± 0.2	1.4 ± 0.4	0.6 ± 0.2	< 0.005
Sputum Elastase (nM)	28.0 ± 16.0	254.3 ± 147.2	25.0 ± 8.0	< 0.005
Sputum IL8 (nM)	12.6 ± 4.2	30.9 ± 9.2	10.4 ± 3.0	0.01
Sputum LTB4 (nM)	17.2 ± 8.2	33.2 ± 8.3	16.5 ± 3.5	< 0.005
Sputum SLPI (µM)	4.0 ± 1.4	1.0 ± 0.4	5.7 ± 2.2	< 0.005
Sputum AAT (µM)	0.07 ± 0.02	0.25 ± 0.09	0.10 ± 0.03	0.01
Sputum/Serum Albumin Ratio (%)	0.6 ± 0.2	3.8 ± 1.8	0.9 ± 0.2	< 0.01
Serum CRP (mg/L)	6.3 ± 4.3	34.0 ± 18.5	7.0 ± 2.9	< 0.01

Table 6-4: Average results of biochemical measurements (± SE) are shown for the AAT deficient patients. Results are shown for baseline (at least 8 weeks prior to the initial exacerbation), at the start of the exacerbation and 2 weeks after the cessation of therapy (Resolution- day 28). Significant differences are shown comparing baseline values with the start of the exacerbation.

All non deficient patients improved with significant reductions in MPO (0.4 ± 0.1 Units/ml, $p= 0.02$), elastase activity (1.7 ± 1.1 nM, $p= 0.02$), LTB₄ (4.9 ± 1.3 nM, $p= 0.01$), sputum AAT ($0.49 \mu\text{M} \pm 0.10$, $p= 0.02$), albumin leakage (0.6 ± 0.1 %, $p < 0.01$) and C-reactive protein ($7.0 \text{ mg/L} \pm 2.6$, $p= 0.04$) by day 28 and the SLPI levels rose by day 28 ($11.8 \mu\text{M} \pm 6.5$, $p= 0.02$). Comparison with the results obtained in the AAT deficient subjects on day 28 showed that some differences still existed as the MPO, LTB₄, and elastase activity were all lower in the non deficient group ($p < 0.001$, $p < 0.01$ and $p < 0.001$ respectively) whereas sputum AAT was higher ($p < 0.001$). However the IL8 ($8.8 \text{ nM} \pm 2.9$), SLPI, albumin ratio, C-reactive protein and the bacterial load (6 were colonised with a median bacterial load of 5.5×10^7 cfu/ml (range= 3.0×10^6 - 1.7×10^9 cfu/ml)) was similar to the AAT deficient subjects.

6.3.7 Peak expiratory flow rates

Peak flow rates were recorded throughout the exacerbation. In patients with AAT deficiency, the mean morning pre-bronchodilator peak expiratory flow rates at presentation with the exacerbation was $236.4 \text{ L/min} \pm 41.4$, which increased ($p < 0.005$) by day 14 to $261.8 \text{ L/min} \pm 45.4$ (11.9%). The mean evening post-bronchodilator peak expiratory flow rates at the start of the exacerbation was $260.0 \text{ L/min} \pm 40.5$ which also increased ($p < 0.005$) by day 14 to $278.6 \text{ L/min} \pm 40.8$ (10.2%). The mean morning and evening peak expiratory flow rates on day 28 were not significantly different from day 14 (day 28: mean morning $265.5 \text{ L/min} \pm 44.6$; mean evening $285.5 \text{ L/min} \pm 43.6$).

The peak flow rates in the non deficient patients were not significantly different to the AAT deficient group throughout the study and followed a similar pattern. The mean morning pre-bronchodilator peak expiratory flow rates at presentation with the exacerbation was 205.0 L/min \pm 32.7, which also increased ($p < 0.005$) by day 14 to 225.5 L/min \pm 32.2 (12.1%). The mean evening post-bronchodilator peak expiratory flow rates at the start of the exacerbation was 216.8 L/min \pm 30.6 which increased ($p < 0.005$) by day 14 to 236.4 L/min \pm 31.7 (9.6%). The mean morning and evening peak expiratory flow rates on day 28 were not significantly different from day 14 (day 28: mean morning 223.6 L/min \pm 31.7; mean evening 237.3 L/min \pm 31.0).

6.4 DISCUSSION

In the present study all AAT deficient patients presented with an increase in symptoms and purulent sputum production associated with the presence of a high bacterial load of a single or (in 3 cases) 2 bacterial pathogens. The exacerbations were characterised by high sputum colour and MPO concentration consistent with the presence of a significant number of neutrophils which is probably a result (at least in part) of the high concentration of the two chemoattractants IL8 and LTB₄.

Elastase activity was present in most of the samples at presentation and may be expected to have a detrimental effect on airways host defences. The enzyme activity probably reflects several factors including increased neutrophil recruitment (as indicated by MPO), reduced SLPI concentration probably as a result of suppression of inhibitor release by elastase itself (Sallenave et al, 1994) and low AAT concentration (see results), despite a small acute phase response and increased protein leakage into the airways.

This latter concept is supported by comparison with subjects who had normal AAT. In these patients the severity of the exacerbation indicated by the acute phase protein CRP, influx of neutrophils (as assessed by MPO), and bacterial load was similar. However the activity of elastase in the sputum was much lower which probably reflects the greater influx of AAT. This inactivation of elastase would in turn, prevent the effect on SLPI release accounting for the higher concentration of this protein which, in turn, would also facilitate elastase inactivation.

Of interest the subjects with AAT deficiency also had higher levels of the chemoattractants LTB₄ and IL8 than the non deficient subjects at presentation. The exact source of these chemoattractants is uncertain since both can be derived from the activated neutrophils (Borgeat et al, 1979)(Takahashi et al, 1993). In addition the infection could stimulate epithelial cells to release IL8 (Khair et al, 1994). However both patient groups had a similar influx of neutrophils (MPO) and bacterial load suggesting this was not the case. Previous studies have suggested that macrophage release of LTB₄ is increased in AAT deficiency as a result of free elastase activity (Hubbard et al, 1991). In addition elastase has been reported to increase IL8 release from epithelial cells (Nakamura et al, 1992). Thus the greater elastase activity may explain the increases in both chemoattractants seen in AAT deficiency. Nevertheless it might be predicted that the greater concentration of chemoattractants should increase neutrophil recruitment (MPO) in the AAT deficient group and yet this was not the case (see results). However as seen in the interrelationships chapter the relationship between both IL8 and LTB₄ and MPO is shallow. The differences in chemoattractant concentrations between these 2 patient groups would only produce a predicted change in MPO equivalent to 0.5 Units/ml which is unlikely to be detected in the relatively small group of patients studied here.

Following initiation of therapy in the AAT deficient group the inflammatory response reversed briskly in the group as a whole. Sputum colour, MPO and the chemoattractants all fell, as did the elastase activity. This latter effect was probably enhanced by the rise in SLPI concentration (approximately 2 μ M), despite the slight fall in average sputum

AAT concentration of 0.08 μM by day 3 (due mainly to decreased protein leakage). The sputum became sterile in the majority of subjects and the systemic acute phase response (both CRP and AAT) settled. These results provide an objective measure to support the patients subjective feeling of improvement and a return to their normal clinical state and is consistent with a resolution of the exacerbation and the associated inflammation.

Two weeks after the cessation of therapy the sputum remained sterile in 4/11 patients and in the other patients the bacterial load was less than at presentation. Inflammation remained low as indicated by the sputum colour, MPO, IL8, elastase activity (although the majority of samples had become positive), protein leakage and acute phase response, although the LTB₄ concentration had risen (though less than at presentation). These results are however consistent with the patient's usual clinical state, as confirmed by comparison with samples from the 11 patients studied at least 8 weeks prior to the clinical exacerbation (Table 6-4). In addition this extra data confirms that the change at the start of the exacerbation was major and indicated a clear pathological process associated with their clinical deterioration.

Of interest, however, the LTB₄ concentration had risen in the whole group studied here 2 weeks after treatment had ceased although the reasons are currently uncertain. It is unlikely that this change represents the early stages of relapse after antibiotic therapy since the results are similar to those obtained in the stable clinical state at least 8 weeks prior to the exacerbation (Table 6-4). Previous studies in AAT deficiency had identified high LTB₄ concentrations in bronchoalveolar lavage. The authors argued that the source

could be macrophages stimulated by neutrophil elastase as a result of the defective AAT inhibitory screen (Hubbard et al, 1991). Whether this mechanism is correct and applicable to the airways, where SLPI is the major antielastase in the stable state (Morrison et al, 1987), remains to be clarified.

However it should be noted that the elastase activity in these samples was not significantly greater than at the end of treatment (day 14) although more samples were positive. In addition more samples had become colonised with bacteria and this could also have an effect by stimulating LTB₄ release from airway phagocytes. Clearly this complex interrelationship requires further study.

Some differences were still observed between the AAT deficient and non deficient subjects by day 28. The non deficient patients had higher sputum AAT (as would be expected) but lower MPO, elastase activity, and LTB₄. AAT deficiency is associated, therefore, not only with increased inflammation at the start of the exacerbation but also in the stable state. However several factors including lung function, smoking habit, airway bacterial load, and treatment such as inhaled corticosteroids can influence inflammation. For this reason both groups were matched for all these potential confounding factors as well as the severity of their exacerbation as indicated by the objective measurement of C-reactive protein. It is likely, therefore, that the results reported here reflect, predominantly, the AAT deficiency itself and increased upper airways inflammation as an ongoing phenomenon. Clearly further studies are indicated to clarify whether this alone determines the more rapid rate of progression of disease in AAT deficiency.

Finally it is worth commenting on the acute phase response of serum AAT in the deficient subjects. Although basal levels are low in AAT deficiency, acute phase responses can be induced by Danazol (Wewers et al, 1986) and Tamoxifen (Wewers et al, 1987). This is the first report to my knowledge of the acute phase response in a naturally occurring infection in AAT deficiency. Unlike CRP where the response is early and marked, and resolution is rapid, the AAT response is minimal, slower (peaking on day 3) and falls slowly. The average increase is approximately 40% but it should be emphasised that the highest concentration (5.5 μM) is still well below normal plasma concentrations in the absence of an acute phase response or even the putative lung protective threshold of 11 μM (Society, 1995). Thus despite the increase in protein leakage into the airway the ability of AAT to modulate the inflammatory process is still markedly impaired in AAT deficient subjects.

In conclusion acute exacerbations of chronic bronchitis in subjects with AAT deficiency, in the presence of bacteria in secretions, are associated with marked neutrophil influx. Although the specific nature of these episodes can only remain speculative the poor AAT acute phase response and the brisk response following commencement of antibiotic therapy suggests that such episodes are due to the bacteria and should be promptly treated to protect the airways tissues. Whether AAT deficiency makes patients more susceptible to bacterial colonisation of the airways bacteria remains to be determined. However the current study suggests that AAT replacement may play a role in modulation of elastase activity in the airway during acute exacerbations in deficient subjects.

7. GENERAL DISCUSSION

7.1 The interrelationship of sputum inflammatory markers in patients with chronic bronchitis

The neutrophil and in particular its product neutrophil elastase has been implicated in the pathogenesis of chronic bronchitis. In vitro and in vivo work have demonstrated that neutrophil elastase can cause epithelial damage (Amitani et al, 1991), reduce ciliary beat frequency (Smallman et al, 1984), cause mucus gland hyperplasia (Snider et al, 1985), cause mucus secretion (Sommerhoff et al, 1990), and has been shown to inactivate many of the critical lung host defences (Solomon, 1978)(Tosi et al, 1990). Thus neutrophil elastase can cause the typical features of chronic bronchitis including facilitating bacterial colonisation, another feature often seen in such patients.

The inflammatory process in the bronchial tree is complex and involves the release of many mediators including cytokines and chemoattractants that regulate adhesion molecules, the processes of cell migration, and their activation and degranulation. The summation of these processes in chronic bronchitis is predominantly the recruitment of neutrophils and release of the neutrophil proteinase elastase. In order for the neutrophil elastase to have the effects in the airways, the elastase released from the activated neutrophil has to overcome the naturally occurring inhibitors such as secretory leukoprotease inhibitor and alpha-1-antitrypsin (secretory leukoprotease inhibitor being thought to be the major anti-proteinase in the airways).

There have been many studies of the effects of elastase in isolation in vivo and, in addition, a multitude of in vitro experiments exploring the role of this mediator.

However, there have been very few studies assessing the complex interplay of inflammatory cells and appropriate mediators in patients with chronic bronchitis.

Recent limited studies have indicated that neutrophils in the airway of patients with chronic bronchitis are increased (Riise et al, 1995)(Pesci et al, 1998) and that these cells or their product myeloperoxidase is related to the presence of a single chemoattractant, interleukin 8 (Riise et al, 1995)(Pesci et al, 1998)(Yamamoto et al, 1997). Other chemoattractants however may be of importance. For instance studies by Mikami et al (Mikami et al, 1998) revealed that both interleukin 8 and leukotriene B4 play a significant role in neutrophil chemotaxis (demonstrating that up to 43% of the chemotactic activity was due to interleukin 8 and 27% due to leukotriene B4), and that these chemoattractants work in an additive way for neutrophil recruitment. In addition these workers found that some of the chemotactic activity was not due to these two chemoattractants suggesting that others may also be involved. This is of importance since *in vitro* studies have indicated that chemoattractants may interact in an additive way (Mikami et al, 1998) and in addition there may be a hierarchical response influencing the cell and its migration pattern (Foxman et al, 1997). Thus simple associations between cell numbers and single chemoattractants do not indicate effect and cause.

Furthermore *in vitro* studies have shown the relationship between chemoattractants and neutrophils may be even more complex. Neutrophil activation can also lead to the release of both interleukin 8 (McCain et al, 1994) and leukotriene B4 (Ford-Hutchinson et al, 1980) that may result in further neutrophil recruitment. However this could also

account, at least in part, for any correlation between neutrophils and the chemoattractants. Release of elastase from the neutrophil may have several effects possibly stimulating epithelial cells to produce more interleukin 8 (Nakamura et al, 1992) and macrophages to release leukotriene B4 (Hubbard et al, 1991) whilst at the same time reducing production of its own natural inhibitor, secretory leukoprotease inhibitor (Sallenave et al, 1994) thereby perpetuating its own activity. Furthermore impairment of host defences by elastase could facilitate bacterial colonisation and endotoxin release from bacteria can also provide a further potential mechanism for neutrophil recruitment by stimulating production of interleukin 8 by epithelial cells (Khair et al, 1994).

Previous studies have provided some insight into the complex mechanisms involved in the bronchial inflammatory response, but have been limited by a small numbers of patients and the study of single and isolated interactions. The study in Chapter 3 investigated a large cohort of patients with a wide spectrum of bronchial inflammation to study the complex interrelationships of the inflammatory markers in more detail: neutrophil influx (as reflected by sputum myeloperoxidase concentration) and the chemoattractants interleukin 8 and leukotriene B4; active neutrophil elastase and the chemoattractants (interleukin 8 and leukotriene B4), its own inhibitor secretory leukoprotease inhibitor, and bronchial protein leak (leakage of albumin from serum into the airways); and finally FEV₁ (% predicted) and myeloperoxidase, interleukin 8 and leukotriene B4, secretory leukoprotease inhibitor, and bronchial protein leak.

The degree of neutrophil influx as assessed by myeloperoxidase correlated well with elastase activity, although this was less apparent at lower concentration where many samples with detectable myeloperoxidase activity had no detectable elastase activity. This is to be expected since the presence of bronchial inhibitors such as secretory leukoprotease inhibitor and alpha-1-antitrypsin would be expected to inhibit the low concentrations of elastase released when neutrophil numbers are low.

Neutrophil recruitment is dependent upon the chemoattractants being released and studies have suggested that interleukin 8 and leukotriene B4 are the most important chemoattractants in patients with bronchial disease. In the current studies both interleukin 8 and leukotriene B4 correlated with neutrophil recruitment and elastase activity. Previous studies have suggested that the relationship between interleukin 8 and myeloperoxidase is linear but examination of the raw data in the current study suggests this may not be the case and the relationship appears curvilinear. The relationship with leukotriene B4 on the other hand does appear to follow a linear trend. It has been shown that leukotriene B4 and interleukin 8 are additive in their effect on neutrophil recruitment. It may well be therefore that at low levels of interleukin 8, recruitment of small numbers of neutrophils leads to release of leukotriene B4 from the activated cell. This may thereafter add to the chemoattractant gradient recruiting more cells in a direct manner. However, once interleukin 8 levels exceed 10 nM the inflammatory process may become excessive leading to much greater neutrophil recruitment than would be expected for the leukotriene B4 concentration (Chapter 3, Figure 3-2 and Figure 3-3). This concept would be consistent with the potential curvilinear relationship between interleukin 8 and myeloperoxidase and would be consistent with a hierarchical

organisation of chemoattractants with one superseding the other as inflammation increases. Alternatively it is possible that other chemoattractants are involved in samples where neutrophil recruitment is excessive and this requires further study.

Studies of the role of elastase in the inflammatory process are complex. When released from the neutrophil this enzyme would be rapidly inhibited by both alpha-1-antitrypsin and, in the airway, more particularly by secretory leukoprotease inhibitor. In vitro studies however have indicated that elastase can adversely affect secretory leukoprotease inhibitor release by epithelial cells and thus it would be expected that an inverse relationship should exist. However, the data indicates that secretory leukoprotease inhibitor concentration does not decrease until the elastase activity of the samples is in excess of 50 nM. The relationship between secretory leukoprotease inhibitor and elastase therefore is not a simple linear one and the data presented here may indicate a threshold at which significant interference with epithelial cell metabolism or epithelial damage occurs resulting in a reduction in secretory leukoprotease inhibitor secretion. At present whether this relationship reflects cause or effect is not known and interpretation will have to await intervention studies using specific anti-elastases. If the reduction in secretory leukoprotease inhibitor secretion is merely a reflection of the elastase activity in the airway, the introduction of an effective anti-elastase would be expected to lead to an acute rise in secretory leukoprotease inhibitor concentration. However if significant airway cell damage is responsible for the reduction in secretory leukoprotease inhibitor, a longer period of repair would be necessary before secretory leukoprotease inhibitor concentrations return to normal.

There is, however, a similar relationship between the elastase activity and inflammation in the airway as reflected by leakage of serum albumin. Protein leakage increased only when free elastase activity exceeded 50 nM, and further increased as the elastase activity rise above 100 nM. The exact mechanism for protein leakage is currently unknown. Protein leakage has been shown to be increased during exacerbations but to quickly resolve with antibiotic therapy (Stockley et al, 1979), and the rapidity of this response would suggest that airway leakage is not a direct effect of epithelial cell destruction. In vitro work has demonstrated that proteolytic enzymes and charge interactions are important in epithelial permeability, and that the leakage may reflect an effect of inflammation on the tight junction between epithelial cells (Peterson et al, 1995), as opposed to epithelial cell destruction. In addition more recent studies have shown that elastase can proteolyse endothelial cell cadherins (endothelial junction proteins) (Carden et al, 1998). The net effect could account for the increased protein leakage and would fit with the rapid reversal with treatment.

Studies have indicated that increasing neutrophil influx is associated with worse lung function in patients with chronic obstructive pulmonary disease (Keatings et al, 1997) (Di Stefano et al, 1998). The current study showed that increased neutrophil influx, elastase activity, the chemoattractants interleukin 8 and leukotriene B4, and increased protein leak were associated with worse lung function in patients with chronic bronchitis without alpha-1-antitrypsin deficiency. However there was no association in the other two patient groups (those with alpha-1-antitrypsin deficiency or bronchiectasis). It should be noted however that all patients with alpha-1-antitrypsin deficiency had an FEV₁ < 56% predicted which would tend to minimise the range of lung function

impairment which may explain the lack of association in this group. Clearly further studies are required to determine whether this observation in chronic bronchitis represents a true cause and effect.

7.2 Influence of bacterial colonisation

Organisms such as non typeable *Haemophilus influenzae* are common colonisers of the upper respiratory tract, being present in up to 75% of healthy adults (Murphy et al, 1987). The lower airways of patients with chronic obstructive pulmonary disease are also commonly colonised with such bacteria while they are not acutely ill (Murphy et al, 1992)(Monso et al, 1995). The role of this airway bacterial load, however, has been uncertain.

The study in chapter 4 examined the effect of bacterial colonisation in patients with chronic bronchitis in the stable clinical state, to determine whether the bacterial colonising load influenced the degree of bronchial inflammation, including whether there is a threshold, and whether the bacterial species influenced the degree of bronchial inflammation.

In this study, quantitative bacterial cultures have been used to demonstrate highly variable airway bacterial load among patients with chronic bronchitis. Increasing airway bacterial load was strongly correlated with the following in airway secretions: 1) increasing numbers of neutrophils (yellow to green sputum and myeloperoxidase); 2) increasing concentrations of neutrophil chemoattractants interleukin 8 and leukotriene B₄; 3) increasing concentrations of active leukocyte elastase; and 4) increasing evidence of airway injury (leakage of albumin into the airways). There appeared to be a threshold for neutrophil recruitment at $\geq 10^6$ colony forming units/ml (colonising bacterial loads below this had a similar degree of upper airways inflammation to patients that were

colonised with mixed normal flora or where no pathogen was isolated). *Pseudomonas aeruginosa* provoked a more intense inflammatory response than *Haemophilus sp.* and *Moraxella catarrhalis*, and was associated with higher concentrations of active leukocyte elastase and more severe airway injury. These results, however, indicate that the concentration of viable organisms, regardless of the bacterial species, has an important influence on airway inflammation in "stable" patients.

It is plausible that airway bacterial load promotes airway inflammation because bacterial products may directly initiate and perpetuate lung inflammation through induction of local secretion of interleukin 8 (Bedard et al, 1993)(Khair et al, 1994). In addition, alveolar macrophages activated by bacterial phagocytosis may be a source of the observed leukotriene B4 (Bigby et al, 1987)(Hubbard et al, 1991). Indirect mechanisms may also amplify and perpetuate the neutrophil influx, including: 1) chemoattractants and active leukocyte elastase released from neutrophils (Takahashi et al, 1993)(Doerfler et al, 1989)(Nakamura et al, 1992); 2) chemotaxis to leukocyte elastase-inhibitor complexes (Banda et al, 1988); and 3) chemotaxis to products of local extracellular matrix degradation (Senior et al, 1980).

Regardless of the mechanistic link between airway bacterial load and airway inflammation, this data shows that airway inflammation is associated with evidence of airway injury. Taken together, the data indicates that persistent bacterial colonisation of the airways may not be a benign process in patients with chronic bronchitis, even in the absence of an acute deterioration of symptoms.

The bacterial species that this study isolated are similar to those in other previous studies of sputum and lower airway bacteriology. Since *Haemophilus species*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* are normal oropharyngeal commensals, non-quantitative sputum cultures potentially leads to confusion because they cannot confirm a lower respiratory source for the isolates (Murphy et al, 1992)(Monso et al, 1995). In the present study, quantitative cultures of expectorated sputum removed this uncertainty as bacteria that were present at $\geq 10^6$ cfu/ml were strongly associated with evidence of airway inflammation.

Further studies of antibacterial intervention are needed in such patients. However, published studies of stable patients with bronchiectasis who produce purulent sputum have indicated that clinical benefits can be obtained from antibiotic therapy both in the short and long term (Hill et al, 1986). It is noted that, for infections of the uroepithelium, it has long been standard clinical practice to base therapeutic decisions upon quantitative bacterial cultures and enumeration of inflammatory cells in urine specimens. The present results suggest that analogous practices with respiratory secretions may improve the management of infections of the respiratory epithelium and provide improved indicators of the need for antibiotic therapy.

7.3 Role of alpha-1-antitrypsin in protection of the airways in the stable state

Secretory leukoprotease is thought to be the most critical anti-elastase in the upper airways (Morrison et al, 1987), whereas alpha-1-antitrypsin is thought to be less important, although more critical at the alveolar level protecting against the development of emphysema (Gadek et al, 1981).

The study in chapter 5 investigated the effects of continued smoking and the role of alpha-1-antitrypsin in the upper airways by comparing patients with chronic bronchitis with and without alpha-1-antitrypsin deficiency. Subjects with alpha-1-antitrypsin deficiency (PiZ phenotype) have decreased circulating (about 15-20% normal) (Lieberman et al, 1972) and alveolar concentrations of alpha-1-antitrypsin (Gadek et al, 1981) which is believed to be central to the development of early onset and rapidly progressive emphysema (Brantly et al, 1988). 30-40% of patients with alpha-1-antitrypsin deficiency have chronic bronchitis (Eriksson, 1965)(Brantly et al, 1988) although the nature of the airways inflammation has not been studied in detail.

The current study revealed evidence of upper airways inflammation in patients with chronic bronchitis. There was a wide range of neutrophil influx as reflected in the myeloperoxidase concentration as a result of the chemoattractants interleukin 8 and leukotriene B4, which are thought to play a key role in neutrophil recruitment (Mikami et al, 1998). Elastase activity was low even though these samples are known to contain the enzyme (Stockley et al, 1979) probably due to inhibition by the natural inhibitors

alpha-1-antitrypsin but particularly secretory leukoprotease inhibitor. Comparison between current and ex smokers showed that the only differences were lower interleukin 8 and myeloperoxidase concentrations in the latter group. This did not reflect differences in therapy but the ex smokers had worse lung function which would, if anything, increase neutrophil numbers (Keatings et al, 1997)(Di Stefano et al, 1998). Interleukin 8 has been suggested as the major neutrophil chemoattractant (Richman-Eisenstat et al, 1993) and airways interleukin 8 is increased in healthy smokers (Keatings et al, 1997) and subjects with COPD (Keatings et al, 1997) although the effect of smoking cessation has not been assessed previously. The current data indicates that cessation of smoking in chronic bronchitis is related to a reduction in airways interleukin 8 concentration which in turn would reduce neutrophil recruitment thereby explaining the beneficial effects of smoking cessation and progression of lung disease. The effect may be due to loss of cigarette smoke stimulation of epithelial production of interleukin 8 (Mio et al, 1997) although further studies will be necessary to confirm this.

Secretory leukoprotease inhibitor is believed to be the major inhibitor of elastase in the upper airway. However the current data shows that airways inflammation is increased in the alpha-1-antitrypsin deficient subjects despite being younger (Meyer et al, 1996)(Meyer et al, 1998), including a greater proportion of ex-smokers and more being on inhaled steroids (Llewellyn-Jones et al, 1996) (all of which would tend to limit the degree of inflammation). Myeloperoxidase concentrations were higher, as was the chemoattractant leukotriene B₄. This suggests that LTB₄ may be the major chemoattractant responsible for the increased neutrophil migration and is consistent with previous findings in bronchoalveolar lavage (Hubbard et al, 1991). The source of the

LTB₄ is uncertain although Hubbard et al. (Hubbard et al, 1991) suggested it was released from alveolar macrophages as a direct effect of uninhibited elastase due to alpha-1-antitrypsin deficiency. Indeed in the current study elastase activity was more readily detected in the alpha-1-antitrypsin deficient patients irrespective of bacterial colonisation and hence would support the suggestion that this may be responsible (Hubbard et al, 1991). The elastase activity that was more readily detected in the upper airways in alpha-1-antitrypsin deficiency is probably due to the combined effect of the lower concentrations of both alpha-1-antitrypsin and secretory leukoprotease inhibitor. The mechanisms involved may be complex but free elastase activity can reduce secretion of secretory leukoprotease inhibitor (Sallenave et al, 1994) as well as increasing permeability of airway cells (Peterson et al, 1995). Despite the latter effect which accounts for an increase in alpha-1-antitrypsin "leak" into the lung, the low alpha-1-antitrypsin concentrations in these subjects may be critical in determining the overall changes suggesting that this inhibitor also has a major role in the airways.

As demonstrated in Chapter 4, bacterial colonisation is clearly related to the degree of airways inflammation in patients with chronic bronchitis both with and without alpha-1-antitrypsin deficiency suggesting this may not always be a benign situation even though the patients were "clinically" stable. Despite airways colonisation, the alpha-1-antitrypsin deficient group still had evidence of increased airways inflammation and this is likely to be responsible for, or reflect, the mechanisms central to the development of severe airflow limitation at an earlier age. It is of importance to note that these changes were present in ex smokers and the majority of patients being on inhaled corticosteroids

and would suggest that measures other than smoking cessation and long term inhaled steroids may be critical in stabilising lung disease in these patients.

7.4 Bronchial inflammation in alpha-1-antitrypsin deficiency in exacerbations

Alpha-1-antitrypsin has been shown to be an important anti-proteinase in the bronchial airways in the stable clinical state. It would also be expected to be of particular importance during exacerbations since alpha-1-antitrypsin is also an acute phase protein, and this together with increased protein leakage into the airways due to inflammation should be important in down-regulating the inflammatory response. Therefore patients with alpha-1-antitrypsin deficiency may be susceptible to neutrophil mediated lung damage particularly during exacerbations as such patients have both a low baseline alpha-1-antitrypsin levels and fail to have an effective acute phase response.

Although basal levels are low in alpha-1-antitrypsin deficiency, acute phase responses can be induced by Danazol (Wewers et al, 1986) and Tamoxifen (Wewers et al, 1987). This is the first report of the acute phase response in a naturally occurring infection in alpha-1-antitrypsin deficiency. The alpha-1-antitrypsin acute phase response was minimal peaking on day 3, and falling slowly thereafter. The average increase was approximately 40% but the highest concentration (5.5 μM) is still well below normal plasma concentrations in the absence of an acute phase response or even the putative lung protective threshold of 11 μM (Society, 1995). Thus despite the increase in protein leakage into the airway the ability of alpha-1-antitrypsin to modulate the inflammatory process is still markedly impaired in alpha-1-antitrypsin deficient subjects.

In patients with alpha-1-antitrypsin deficiency, elastase activity was present in most of the samples at presentation with the exacerbation. The enzyme activity probably reflects several factors including increased neutrophil recruitment (as indicated by myeloperoxidase), reduced anti-proteinases secretory leukoprotease inhibitor concentration and low alpha-1-antitrypsin concentration, despite a small acute phase response and increased protein leakage into the airways. In comparison with subjects who had normal alpha-1-antitrypsin, patients with alpha-1-antitrypsin deficiency had higher mean levels of elastase activity at the start of the exacerbation, reflecting the defective anti-proteinase screen.

Within 3 days of antibiotic therapy in the alpha-1-antitrypsin deficient group there was a reduction in upper airways inflammation in the group as a whole. Sputum colour, myeloperoxidase and the chemoattractants all fell, as did the elastase activity and protein leakage. The length of time antibiotic therapy is needed and how augmentation therapy with alpha-1-antitrypsin replacement therapy modulates the bronchial inflammatory response requires further study.

Two weeks after the cessation of therapy the degree of bronchial inflammation was similar to the end of treatment with the exception of the rise in leukotriene B4. These results are however identical to the patient's usual clinical state, as confirmed by comparison with the results obtained at least 8 weeks prior to the clinical exacerbation. In addition this extra data confirms that the change at the start of the exacerbation was major and indicated a clear pathological process associated with their clinical deterioration.

Some differences were still observed between the alpha-1-antitrypsin deficient and non deficient subjects by day 28 (stable state). These included not only sputum alpha-1-antitrypsin (as would be expected) but also myeloperoxidase, elastase activity, and leukotriene B4. This suggests that alpha-1-antitrypsin deficiency is associated not only with increased inflammation at the start of the exacerbation but also in the stable state (this was studied in more detail in chapter 5) demonstrating the importance of alpha-1-antitrypsin in the upper airways both in the stable state and exacerbations.

In conclusion acute exacerbations of chronic bronchitis in subjects with alpha-1-antitrypsin deficiency, in the presence of bacteria in secretions, are associated with marked neutrophil influx. Although the specific nature of these episodes can only remain speculative the poor alpha-1-antitrypsin acute phase response and the brisk response following commencement of antibiotic therapy suggests that such episodes are due to the bacteria and should be promptly treated to protect the airways tissues. Whether alpha-1-antitrypsin deficiency makes patients more susceptible to bacterial colonisation of the airways bacteria remains to be determined. However the current study suggests that alpha-1-antitrypsin may play a role in modulation of elastase activity in the airway during acute exacerbations in deficient subjects. Further studies on the role of augmentation therapy with alpha-1-antitrypsin are required.

7.5 FUTURE STUDIES

1. Studies have highlighted the importance of neutrophils in the pathogenesis of chronic bronchitis (Riise et al, 1995) and in particular the degree of neutrophil influx has been shown to be related to the severity (Keatings et al, 1997)(Di Stefano et al, 1998) and rate of progression of airflow obstruction (Stanescu et al, 1996). The role of the eosinophils is less clear, but both eosinophils and eosinophil cationic protein have also been shown to be increased in patients with chronic bronchitis (Riise et al, 1995). Whether the eosinophil migration is a non selective migratory response to interleukin 8 (Shute, 1994) and leukotriene B4 (Oliveira et al, 1994) is not clear and further studies are indicated: assess eosinophil chemotaxis to sputum; investigate the contribution of interleukin 8 and leukotriene B4 by inhibiting their activity using a monoclonal antibody to interleukin 8 and a leukotriene B4 receptor antagonist (Mikami et al, 1998); measure the specific eosinophil chemoattractants (RANTES and Eotaxin) (Walsh et al, 1997).
2. The chemoattractants IL8 and LTB4 have been shown to be important in patients with chronic bronchitis. Other chemoattractants, for example C5a, may be important particularly where there is excess neutrophil influx and this requires further study. This could be clarified by measuring C5a in the samples and determining if chemotaxis could be reduced by a specific antibody as has been used for interleukin 8 (Mikami, 1998).

3. It has been well recognised that bacteria can colonise the bronchial tree in patients with chronic bronchitis whilst patients are apparently clinically stable. There is however little knowledge on how this influences bronchial inflammation, disease progression, frequency of exacerbations, or how it affects patients quality of life. Long term follow up of a cohort of patients comparing sputum colonisation and inflammation with decline in lung function may determine the role of bacteria in this process. The study reported in the thesis has highlighted that bacterial loads exceeding 10^5 cfu/ml is associated with significant neutrophil influx. Further studies are required to determine whether antibiotic treatment in such patients to decrease the bacterial load or eradicate the bacteria will influence bronchial inflammation, disease progression, frequency of exacerbations, or affect patients quality of life.

4. The increased bronchial inflammation in patients with alpha-1-antitrypsin deficiency has highlighted the importance of alpha-1-antitrypsin as an anti-proteinase in the airways both in the stable state and during exacerbations. The increased bronchial inflammation in the stable state was present despite patients being younger, a greater number being on inhaled steroids, and the majority being ex- smokers which would all tend to minimise the degree of bronchial inflammation.

The therapeutic strategy that may influence this process might include alpha-1-antitrypsin augmentation therapy. A controlled trial with alpha-1-antitrypsin replacement therapy is needed to determine whether such an approach can modify the progression of both the airways disease and emphysema in such patients, and whether this could also modify bacterial colonisation. The increase in alpha-1-

antitrypsin following therapy may decrease or remove elastase activity, which would in turn down regulate the inflammatory response (less interleukin 8 release from epithelial cells and less leukotriene B4 release from activated macrophages). In addition there may be less elastase to damage the primary host defences and therefore bacterial colonisation may be modified by replacement therapy. A study monitoring these factors before, during, and after augmentation therapy would determine whether this concept is true.

Alpha-1-antitrypsin may be particularly important in exacerbations as alpha-1-antitrypsin is an acute phase protein. However in patients with PiZ alpha-1-antitrypsin deficiency, such subjects will not have an appropriate alpha-1-antitrypsin acute phase response, which likely accounts for the excessive bronchial inflammation seen in such patients. Again a controlled trial with alpha-1-antitrypsin replacement therapy is needed to determine whether alpha-1-antitrypsin replacement therapy can reduce the number of exacerbations or speed the recovery of exacerbations.

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9. PUBLICATIONS ARISING FROM THIS THESIS

Abstracts

1. Out-patient management of α -1 antitrypsin deficiency- current practice in the West Midlands 1996. AT Hill and RA Stockley. *Thorax* 1996;51(Supplement 3):P172.
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