Factors influencing airway inflammation in chronic obstructive pulmonary disease

Adam Hill, Simon Gompertz, Robert Stockley

Airway inflammation in chronic obstructive pulmonary disease (COPD) is currently the subject of rapidly increasing research interest, investigating both the nature of the inflammatory cells and the cytokines present. The data are being used to define and assess the severity, cause, prognosis, and response to treatment of this disease. The collection of spontaneous sputum or the induction of sputum has been used to study inflammation in the larger airways. It remains unclear, however, how these data relate to the pathological processes at the site of airflow obstruction (the small airways and alveolar region). Nevertheless, even the interpretation of the results from the large airways depends on the many factors that will influence the inflammation.

The initial process of sputum collection, sample processing, and the performance of assays in the biological fluids being tested may all influence the results. In addition, airways inflammation may be altered by the patient's clinical state, current treatment, and the nature of the disease (asthma, COPD, or bronchiectasis). Other factors including smoking, α_1 -antitrypsin (AAT) deficiency, and bacterial colonisation may also affect airways inflammation. Despite these many factors that may influence airways inflammation, many papers currently in the literature do not address them. The purpose of this review is to indicate the influence of these factors on airways inflammation to assist interpretation of the data currently in the literature.

Pathophysiology

EOSINOPHIL

Airway inflammation in asthma is classically related to the eosinophil. Eosinophil granules contain a variety of mediators capable of causing inflammatory damage to the airway epithelium including eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), metalloproteinases, platelet activating factor, and cysteinyl leukotrienes.¹ In addition, the immunological basis of asthma is distinct and reflected in the nature of the cytokines released into the airway.

However, there is some overlap with COPD which may reflect the fact that asthma can lead to fixed airflow obstruction or that patients with COPD may have coincidental asthma.² Indeed, some recent studies have indicated that sputum eosinophilia is a predictor of steroid

responsive airflow obstruction^{3,4} and thus may identify the chronic asthma component or a subtype of the COPD syndrome.

NEUTROPHIL

In contrast, however, the neutrophil is thought to be the major cell involved in the airways of patients with COPD and those with bronchiectasis, although it has also been implicated in the later irreversible phase of asthma. In neutrophilic inflammation in the bronchial tree there is a complex interaction of many mediators including chemoattractants and cytokines that regulate gene transcription, adhesion molecule expression, the processes of cell migration, their activation and degranulation.

PROTEASES

The recruited neutrophil releases proteolytic enzymes including neutrophil elastase, cathepsin G, and matrix metalloproteinases. The importance of these enzymes—and, specifically, neutrophil elastase—has been demonstrated by both in vitro and in vivo studies. Neutrophil elastase can produce most of the pathological features of chronic bronchitis and emphysema, and promote bacterial colonisation as seen in patients with bronchiectasis. More specifically, neutrophil elastase can cause epithelial damage,⁵ reduce ciliary beat frequency,⁶ produce mucous gland hyperplasia,⁷ stimulate mucus secretion,⁸ inactivate many critical lung host defences,^{9,10} and damage interstitial tissue¹¹ leading to pathological changes that cause human alveolar destruction.

ANTI-PROTEINASES

In order for neutrophil elastase to have these effects, the elastase released from the activated neutrophil has to overcome the naturally occurring inhibitors such as secretory leukoprotease inhibitor (SLPI) and AAT. SLPI is generally thought to be the main inhibitor in the airways¹² and hence protects against bronchial disease, whereas AAT is thought to be of importance predominantly in the lower airways¹³ and is thus more relevant to alveolar disease.

CHEMOATTRACTANTS

The major neutrophil chemoattractants in patients with COPD and bronchiectasis are believed to be interleukin 8 (IL-8) and leuko-

Department of
Respiratory Medicine,
Queen Elizabeth
Hospital, Birmingham
B15 2TH, UK
A T Hill
S Gompertz
R A Stockley

Correspondence to:
Professor R Stockley
r.a.stockley@bham.ac.uk

triene B₄ (LTB₄).^{14 15} IL-8 is a 16 kDa protein that is a member of the C-X-C family of cytokines. It is made and released by bronchial epithelial cells,¹⁶ monocytes/macrophages,¹⁷ and even the neutrophils themselves.¹⁸ Regulation of IL-8 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 IL-8 gene,¹⁹ and lung secretions from patients with COPD and bronchiectasis contain TNF- α .^{20 21} Furthermore, recent work has indicated that cigarette smoke can stimulate epithelial cells to release IL-8²² via the transcription factor NF- κ B^{23 24} and this may be the mechanism that leads to neutrophil recruitment to the lungs of smokers. In addition, bacteria can also induce IL-8 expression by epithelial cells^{19 25} but at present it is uncertain whether the effect of bacterial products on IL-8 expression is direct or the result of initial TNF- α secretion. IL-8 levels are raised in patients with COPD and patients with bronchiectasis^{26–29} and correlate with myeloperoxidase (MPO) released from activated neutrophils.^{26 27 29} This relationship between IL-8 and a neutrophil marker supports the concept that IL-8 is an important lung chemoattractant for neutrophils.

LTB₄ is a biologically active fatty acid synthesised in myeloid cells from arachidonic acid. It was isolated and purified from neutrophils in 1978³⁰ but was subsequently chemically synthesised and its ability to activate neutrophils was shown in 1980.³¹ LTB₄ is now known to be a major chemotactic product of activated neutrophils³¹ and macrophages.³² LTB₄ induces recruitment and activation of neutrophils as well as monocytes, eosinophils, and lymphocytes. It promotes chemotaxis by increasing neutrophil adherence to venule walls via the integrin Mac-1 and its receptor ICAM-1.³³

The cytokine TNF- α has been found to be increased in the airways of patients with COPD²⁰ and bronchiectasis.²¹ It is produced by activated macrophages, mast cells, and other inflammatory cells^{34–37} as well as by airway epithelial cells.³⁸ TNF- α induces airway hyperresponsiveness,³⁹ may promote IL-8 production by epithelial cells⁴⁰ and neutrophils,⁴¹ is chemotactic for neutrophils and eosinophils both directly and by activation of adhesion molecules on endothelial cells,^{42 43} and promotes vascular leakage.⁴⁴ However, in vivo it has been shown to inhibit neutrophil chemotaxis⁴⁵ and therefore its role in lung inflammation is complex and requires further study.

There are a number of other chemoattractants that could play a role in the airways including C5a generated from complement activation⁴⁶; modified AAT⁴⁷; leucocyte elastase-inhibitor complexes⁴⁸; protein and peptide components of damaged extracellular matrix such as collagen,⁴⁹ elastin⁵⁰ and laminin⁵¹; antigen-antibody complexes⁵²; bacterial factors including the formyl peptides such as fMLP⁵³ and lipopolysaccharide or endotoxin

which can prime neutrophil functions including the chemotactic responses.⁵⁴

A significant proportion, however, of the chemotactic activity of lung secretions in patients with COPD and bronchiectasis can be attributed to IL-8 and LTB₄. Studies by Mikami *et al*¹⁴ have shown that up to 43% of the chemotactic activity was dependent on IL-8 and 27% on LTB₄. It was further shown that the chemoattractants worked in an additive way for neutrophil recruitment.¹⁴ However, these workers also emphasised that some of the chemotactic activity of the lung secretion was due to a protein or proteins that remain to be identified. 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 play a role and cells migrating into tissues may thus encounter multiple chemoattractant signals. Foxman *et al*⁵⁵ have shown that neutrophils can even 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 the initial attractant. These mechanisms of navigation from one gradient to another in complex chemoattractant fields are likely to be necessary for successful localisation and phagocytosis within sites of inflammation.⁵⁵

The relationship between chemoattractants and neutrophils may be further complicated since neutrophil activation can lead to the release of both IL-8¹⁸ and LTB₄³¹ that may enhance further neutrophil recruitment. Release of elastase from the neutrophil may also stimulate epithelial cells to produce more IL-8¹⁶ and macrophages to release LTB₄,³² while at the same time reducing production of its own natural inhibitor, SLPI,⁵⁶ thereby perpetuating its function. 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 IL-8 by epithelial cells.¹⁹ The understanding of these complex interrelationships and the role of each component awaits in vivo studies with specific agonists and antagonists.

RELATIONSHIP BETWEEN ELASTASE ACTIVITY AND OTHER INFLAMMATORY MARKERS

Neutrophil elastase is thought to be of major importance in the pathogenesis of airways disease in COPD and bronchiectasis. Studies from spontaneous sputum have shown relationships between neutrophil elastase activity and several features of airways disease including the following: neutrophil influx; chemoattractants (IL-8 and LTB₄); SLPI; and protein leakage (as determined by sputum/serum albumin ratio).⁵⁷ The close correlation between neutrophil elastase and neutrophil influx would be expected as it is stored within the primary granules of the neutrophil and would be released simultaneously with other granule

Figure 1 Concentrations of IL-8 (nM) in patients with COPD with (PiZ) and without (PiM) α_1 -antitrypsin deficiency, and in patients with bronchiectasis in the stable state (S) and during an exacerbation (E). Both inpatient and outpatient exacerbations are shown. The histograms represent mean (SE) values derived from Crooks *et al.*,¹⁵ Hill *et al.*,⁷⁰ and Gompertz *et al.*⁷¹ * $p < 0.05$ versus patients in the stable clinical state.

constituents such as MPO following degranulation. Similarly, sputum elastase activity relates closely to the chemoattractants IL-8 and LTB₄, probably because these cytokines are responsible for neutrophil (and hence elastase) recruitment. However, as indicated above, elastase can result in IL-8 production by epithelial cells and LTB₄ release by macrophages which may partly explain the relationship. Elastase is related inversely to SLPI concentration, probably by directly suppressing its release,⁵⁶ although a low SLPI concentration would, of course, facilitate continued activity of the enzyme. Finally, as elastase activity is increased above 50 nM, protein leakage also increases, although the exact mechanism is currently unknown. However, previous studies in patients with bronchiectasis have shown that antibiotic treatment reduces the bacterial load and neutrophil influx leading to a rapid reduction in protein leakage as sputum purulence and elastase activity disappear.⁵⁸ The rapidity of this response would suggest that airway leakage is not a direct effect of major airways damage or epithelial cell destruction. The leakage may therefore reflect an effect on the tight junction between epithelial cells which can be mediated by elastase⁵⁹ or the simultaneous release of vasoactive mediators such as NK-1⁶⁰ in the airway during inflammation.

Disease process

Induced sputum samples have confirmed the presence of increased concentrations of products derived from neutrophil granules (MPO, human neutrophil lipocalin (HNL)) and from eosinophils (EPO, ECP) in patients with asthma or COPD compared with normal individuals.⁶¹ Although MPO and HNL were significantly higher in patients with COPD than in those with asthma, the concentrations of ECP and EPO were similar in the two groups of patients indicating a degree of eosinophil influx in addition to neutrophil influx in COPD. The cytokines TNF- α and IL-8 were also increased in both conditions, although only IL-8 was significantly higher in COPD than in asthma.²⁰ Other inflammatory

mediators have also been found to be increased in induced sputum, including the tachykinin substance P in individuals with asthma and chronic bronchitis.⁶²

Patients with bronchiectasis are characterised by major airways inflammation thought to be driven by persistent bacterial colonisation.⁶³ Such patients have been shown to have significant neutrophil influx with raised MPO⁶³ and readily detectable elastase activity,⁶⁴ cathepsin B,⁶⁵ proteinase 3,⁶⁶ increased chemoattractants (IL-8, TNF- α , IL-1 β , and LTB₄),²¹ increased endothelin-1 (a potent bronchoconstrictor with mitogenic activity for airway smooth muscle),⁶⁷ and increased albumin protein leakage.⁶⁴ Patients with COPD also have airways inflammation, but to a lesser degree.^{68–69} This is illustrated in fig 1 which compares IL-8 levels in patients with COPD with and without AAT deficiency, and in patients with bronchiectasis in the stable state and during an exacerbation.^{15 70 71}

Technical factors

INDUCED SPUTUM

Not all patients with asthma or COPD regularly expectorate sputum in the stable clinical state. There has therefore been much interest in assessing airway inflammation using the technique of sputum induction in patients with either asthma or COPD, even when patients also expectorate spontaneously. There are several aspects that may influence the concentrations of airway inflammatory mediators including the process itself (whole sample versus selected plugs), the effect of the varying concentrations of hypertonic saline used (3–5%), the length of the induction process, the effects of dithiothreitol (DTT), and the effects of repetitive induction.

The effect of the concentration of hypertonic saline is not currently known and requires further study as it may potentially disrupt ionic binding of proteins and mediators. In addition, little is known about whether the length of sputum induction influences airways inflammation, although a study by Bhowmik *et al* found that sputum induction for seven or 14 minutes had no influence on sputum cell counts, cell viability, or cytokine measurement.⁷² However, induction does influence airway inflammation and, after a 24 hour period, a repeated induction identifies increased neutrophil counts.^{73 74}

The main methods of processing induced sputum involve either selection of sputum plugs or use of the entire sputum sample. This has been compared in healthy and asthmatic subjects and both methods have been found to have the same diagnostic value in distinguishing asthmatics from healthy subjects, although selected plugs had a higher proportion of eosinophils, a slight increase in viable non-squamous cells, and higher concentrations of ECP than the whole sample.⁷⁵

Comparison of induced and spontaneous sputum samples in patients with COPD⁷² and asthma⁷⁶ has demonstrated higher cell viability in induced samples, less squamous cell contamination, and better quality cytopins.⁷⁶

Figure 2 Individual IL-8 concentrations (nM) in current and ex-smokers. Median concentrations are indicated by the horizontal bars. * $p < 0.05$ versus ex-smokers. Data derived from Hill *et al.*⁸⁷

Whether this relates to lysis of less viable cells as a result of hypertonic saline required to induce expectoration remains to be determined. Total and differential cell counts are similar in both types of sample but other measurements can be altered—for example, lower fibrinogen levels have been found in induced samples.⁷⁶ Bayley *et al* found that sputum induction (whole sample) produced approximately a 50% dilution in a number of inflammatory parameters including MPO, elastase activity, IL-8, LTB₄, and albumin leakage⁷⁷ compared with levels in spontaneous sputum from the same patients.

The authors routinely use and have validated assays to measure inflammatory mediators in sputum sol samples obtained by ultracentrifugation of untreated whole spontaneous sputum.⁷⁸ Many other workers use the reducing agent DTT to hydrolyse disulphide bonds and to render sputum samples less viscous before processing and cell counting.^{61 72 76} However, DTT may have effects on the disulphide bonds within inflammatory molecules themselves (such as IL-8) or may alter their release from treated sputum macromolecules. Efthimiadis *et al* found that DTT affected both cell numbers and viability and increased the levels of ECP in induced sputum from asthmatic patients⁷⁹ but had no effect on the levels of fibrinogen, IL-5, or IL-8. Other authors have found a significant reduction in EPO and MPO levels in DTT treated induced sputum from asthmatic patients but no effect on ECP.⁸⁰ It is probably wisest to retain an aliquot of sample without DTT for cytokine assays unless the assays have been formally validated for samples treated with DTT.

Smoking

Prevalence studies in at least 10 countries have confirmed the association between smoking and the development of chronic bronchitis.⁸¹ Cigarette smoke recruits inflammatory cells to the lungs and studies have shown increased numbers of neutrophils in bronchoalveolar lavage fluid from smokers.^{82 83} In addition, the lungs of smokers with airflow obstruction con-

tain more neutrophils than smokers without airflow obstruction^{84 85} and the number of neutrophils recovered is related to the amount smoked.⁸⁶

Continued smoking seems to be the major stimulus to airways inflammation in patients with chronic bronchitis and emphysema, whereas smoking cessation is believed to be beneficial in reducing the rate of lung function decline. In studies of patients with chronic obstructive bronchitis, current smokers had higher sputum (induced and spontaneous) IL-8 concentrations.^{20 87} One hypothesis is that cessation of smoking in patients with COPD leads to a reduction in airways IL-8 concentration (fig 2) which in turn reduces neutrophil recruitment, thereby providing a mechanism to explain the beneficial effect of smoking cessation on progression of neutrophil mediated lung disease.⁸⁷ The reason for this effect is not clear at present but may relate to a reduction in epithelial production of IL-8 which can be induced by cigarette smoke.²²

AAT deficiency

Subjects with severe AAT deficiency (PiZ phenotype) have decreased (about 15–20% normal) circulating and alveolar concentrations of AAT which facilitates the development of early onset and rapidly progressive emphysema.⁸⁸ About 30–40% of these patients have chronic bronchitis,^{88 89} although the nature of the airway inflammation measured in spontaneous sputum has only recently been studied.

The pathogenesis of emphysema in AAT deficient patients is becoming clearer. Initial lavage studies revealed that PiZ patients have increased numbers of neutrophils in the lower airways, suggesting a greater potential elastase burden than in smokers with emphysema who have normal levels of AAT.⁹⁰ In addition, patients with PiZ AAT deficiency have reduced concentrations of immunoreactive AAT in both sputum¹² and lavage fluids.^{13 90} The pathogenic mechanism for the development of lung disease is thought to result from the low AAT concentrations in the presence of an increased neutrophil burden, leading to inadequate control of neutrophil elastase activity. In addition, extensive connective tissue destruction by migrating neutrophils may be amplified in AAT deficient patients as the Z type AAT has a slightly lower association rate constant for elastase.⁹¹ Finally, the low AAT levels result in an inability to control elastase activity in a wide area around the activated neutrophil resulting in even more connective tissue destruction.^{92 93}

In the airways, however, SLPI is believed to be the major inhibitor of elastase.¹² Studies with spontaneous sputum have shown that inflammation is increased in the larger airways of patients with chronic obstructive bronchitis and AAT deficiency.^{69 87} Sputum concentrations of MPO and LTB₄ are higher which suggests that LTB₄ is the major chemoattractant responsible for the increased neutrophil migration, as suggested by previous studies using bronchoalveolar lavage.³² The source of the LTB₄ is uncertain, although Hubbard *et al*⁹² suggested that it was released from alveolar

Table 1 Airway inflammation at the start of an exacerbation in outpatients with COPD, with and without severe α_1 -antitrypsin (AAT) deficiency

Sputum measurements at start of the exacerbation compared with stable state	COPD	AAT deficiency	AAT deficiency vs COPD at start of the exacerbation
Myeloperoxidase	↑↑	↑↑	↔
Elastase activity	↑↑	↑↑	↑↑
Interleukin 8	↔	↑↑	↑↑
Leukotriene B ₄	↑↑	↑↑	↑↑
Secretory leukoprotease inhibitor	↓	↓	↓
Sputum α_1 -antitrypsin	↑	↑	↑
Albumin leakage (serum → sputum)	↑	↑	↑

macrophages as a direct effect of uninhibited elastase. Sputum elastase activity is also increased in patients with AAT deficiency and could have the same effect in the larger airways as that seen in bronchoalveolar lavage, stimulating LTB₄ production by airway macrophages.

The elastase activity detected in the larger airways in patients with AAT deficiency is probably due to the combined effect of lower sputum concentrations of both AAT and SLPI. Indeed, the low SLPI concentration may be a more important determinant of elastase activity in the larger airway where it is the major inhibitor. The mechanisms involved in disturbing the elastase/anti-elastase balance are likely to be complex in the larger airways. For instance, although elastase has been shown to decrease SLPI secretion from epithelial cells,⁵⁶ it will also increase epithelial cell permeability, resulting in greater AAT leakage from plasma. In subjects with normal AAT levels these two effects may balance each other, retaining the anti-elastase screen. However, in patients with AAT deficiency the low serum and hence secretion AAT level may be less effective as SLPI falls, resulting in a continued deficient screen in the larger airways and hence uncontrolled elastase activity and local damage.

Bacteriology

In animal models low numbers of bacteria instilled into the airways are efficiently cleared by the primary host defence mechanisms alone.⁹⁴ However, as increasing numbers of organisms are instilled, secondary host defences are activated including neutrophil migration.^{94–95} This is likely to reflect the release of pro-inflammatory mediators resulting in an acute inflammatory response with recruitment of neutrophils.^{95–96} Some patients with COPD have persistent bacterial colonisation and it is likely that this contributes to the morbidity of the disease. In particular, the chronic bacterial load may stimulate secondary host defences and lead directly to persistent airway inflammation even in the stable clinical state,^{97–98} although this has received little clinical attention.

In a recent study by Hill *et al* a wide range of airway bacterial load was found among patients with chronic bronchitis while “clinically stable”. Increasing airway bacterial load was strongly correlated with the following in sputum: (1) increasing numbers of neutrophils (MPO); (2) increasing concentrations of the neutrophil chemoattractants IL-8 (fig 3) and LTB₄; (3) increasing concentrations of active leucocyte elastase; and (4) increasing evidence

Figure 3 Relationship between sputum IL-8 concentrations (nM) and airway bacterial load (colony forming units/ml). MNF = mixed normal flora. Histograms represent mean (SE). Data derived from Hill *et al*.⁹⁹ **p*<0.05, ***p*<0.01 versus MNF.

of airway inflammation leading to albumin leakage.⁹⁹ This has been supported by bronchoalveolar lavage data which showed that patients with stable COPD and potentially pathogenic organisms had a higher proportion of neutrophils and a higher TNF- α concentration.¹⁰⁰ Thus, chronic bacterial colonisation can also influence airways inflammation significantly even when patients are “clinically stable” and needs to be taken into account when interpreting secretion data obtained from different patient groups.

Exacerbations

Airway inflammation would be expected to increase during exacerbations of COPD, in particular those that meet Anthonisen’s criteria of increasing sputum volume and sputum purulence.¹⁰¹ Earlier work with spontaneous sputum found an increased proteinase burden during such exacerbations with increased protein leakage.⁵⁸ Further work by Balbi and colleagues demonstrated a higher number of neutrophils and eosinophils in bronchoalveolar lavage specimens during exacerbations of chronic bronchitis,¹⁰² and bronchial biopsy specimens taken during such episodes have a marked eosinophilia and a milder increase in the number of neutrophils, activated CD3+ T lymphocytes, and TNF- α positive cells.¹⁰³

A recent study by Hill *et al* used spontaneous sputum to investigate the nature of an exacerbation in patients with chronic bronchitis with and without AAT deficiency.⁷⁰ The exacerbations filled all three Anthonisen criteria with increased sputum volume, purulence, and worsening breathlessness.¹⁰¹ At the start of the exacerbation there was increased airway inflammation reflected by a high sputum level of MPO, elastase activity, chemoattractant (LTB₄), and evidence of protein leakage in patients with chronic obstructive bronchitis without AAT deficiency (table 1). Subjects with AAT deficiency also had increased airways inflammation at the start of their exacerbation and this was greater than in matched patients without AAT deficiency (table 1).⁷⁰ Acute bacterial exacerbations in patients with chronic obstructive bronchitis that required hospital admission were associated with even greater airways inflammation (fig 1).¹⁵

Treatment

The Isolde, Euroscop, and Copenhagen Lung studies have investigated whether inhaled corticosteroids reduce the rate of decline in forced expiratory volume in one second (FEV₁) over a two year period in patients with COPD but have not directly measured airway inflammation. During two weeks of treatment with inhaled or oral corticosteroids Keatings *et al* could not demonstrate any effect on airway inflammation assessed with induced sputum.¹⁰⁴ Similarly, Culpitt *et al* showed that inhaled steroids given over four weeks had no anti-inflammatory effect.¹⁰⁵ However, two other longer studies have shown that treatment for eight weeks with inhaled steroids reduced chemotactic activity and increased elastase inhibitory capacity in spontaneous sputum¹⁰⁶ and reduced the number of neutrophils in induced sputum.¹⁰⁷ In addition, other investigators have shown that oral steroids can reduce eosinophils and ECP levels in patients with COPD with eosinophilia⁴ and that oral steroids reduced vascular protein leakage.¹⁰⁸ On balance therefore it is likely that steroids do alter airway inflammation and again this should be borne in mind when assessing the results of patient studies.

Conclusion

A number of markers can be measured in sputum to assess airway inflammation. However, the process is complex in vivo and many factors including the disease itself, clinical state, current smoking history, colonisation, and treatment may influence the results. In addition, the processing of samples can influence the results and assay validation in the biological fluid therefore becomes critical. Many of the studies published in the literature involve small numbers of patients and many (if not all) of the factors have not been noted or stratified for. Future studies therefore need to bear this in mind and should ensure validation of all assays. The interpretation of the complex interaction between various mediators will probably only be clarified by the use of specific intervention strategies.

- Walsh GM, Wardlaw AJ. Eosinophils. In: Stockley RA, ed. *Pulmonary defences*. Chichester: John Wiley & Sons, 1997:127–47.
- American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. *Am J Respir Crit Care Med* 1995;152:S77–121.
- Chanez P, Vignola AM, O' Shaughnessy T, *et al*. Corticosteroid reversibility in COPD is related to features of asthma. *Am J Respir Crit Care Med* 1997;155:1529–34.
- Pizzichini E, Pizzichini MM, Gibson P, *et al*. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. *Am J Respir Crit Care Med* 1998;158:1511–7.
- Amitani R, Wilson R, Rutman A, *et al*. Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol* 1991;4:26–32.
- Smallman LA, Hill SL, Stockley RA. Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect. *Thorax* 1984;39:663–7.
- Snider GL, Stone PJ, Lucey EC, *et al*. Eglin-c, a polypeptide derived from the medicinal leech, prevents human neutrophil elastase-induced emphysema and bronchial secretory cell metaplasia in the hamster. *Am Rev Respir Dis* 1985;132:1155–61.
- Sommerhoff CP, Nadel JA, Basbaum CB, *et al*. Neutrophil elastase and cathepsin G stimulate secretion from cultured bovine airway gland serous cells. *J Clin Invest* 1990;85:682–9.
- Solomon A. Possible role of PMN proteinases in immunoglobulin degradation and amyloid formation. In: Havemann K, Janoff A, eds. *Neutrophil proteinases of human polymorphonuclear leukocytes*. Baltimore: Urban and Schwarzenberg, 1978:423–38.
- Tosi MF, Zakem H, Berger M. Neutrophil elastase cleaves C3bi on opsonized *Pseudomonas* as well as CR1 on neutrophils to create a functionally important opsonin receptor mismatch. *J Clin Invest* 1990;86:300–8.
- Snider GL, Lucey EC, Christensen TG, *et al*. Emphysema and bronchial secretory cell metaplasia induced in hamsters by human neutrophil products. *Am Rev Respir Dis* 1984;129:155–60.
- Morrison HM, Kramps JA, Afford SC, *et al*. Elastase inhibitors in sputum from bronchitic patients with and without alpha 1-proteinase inhibitor deficiency: partial characterization of a hitherto unquantified inhibitor of neutrophil elastase. *Clin Sci* 1987;73:19–28.
- Gadek JE, Fells GA, Zimmerman RL, *et al*. Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphysema. *J Clin Invest* 1981;68:889–98.
- Mikami M, Llewellyn-Jones CG, Bayley D, *et al*. The chemotactic activity of sputum from patients with bronchiectasis. *Am J Respir Crit Care Med* 1998;157:723–8.
- Crooks SW, Bayley DL, Hill SL, *et al*. Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. *Eur Respir J* 2000;15:274–80.
- Nakamura H, Yoshimura K, McElvaney NG, *et al*. Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest* 1992;89:1478–84.
- Standiford TJ, Kunkel SL, Rolfe MW, *et al*. Regulation of human alveolar macrophage- and blood monocyte-derived interleukin-8 by prostaglandin E2 and dexamethasone. *Am J Respir Cell Mol Biol* 1992;6:75–81.
- McCain RW, Holden EP, Blackwell TR, *et al*. Leukotriene B4 stimulates human polymorphonuclear leukocytes to synthesize and release interleukin-8 in vitro. *Am J Respir Cell Mol Biol* 1994;10:651–7.
- Khair OA, Devalia JL, Abdelaziz MM, *et al*. Effect of *Haemophilus influenzae* endotoxin on the synthesis of IL-6, IL-8, TNF-alpha and expression of ICAM-1 in cultured human bronchial epithelial cells. *Eur Respir J* 1994;7:2109–16.
- Keatings VM, Collins PD, Scott DM, *et al*. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530–4.
- Tsang KW, Ho PL, Lam WK, *et al*. Inhaled fluticasone reduces sputum inflammatory indices in severe bronchiectasis. *Am J Respir Crit Care Med* 1998;158:723–7.
- Mio T, Romberger DJ, Thompson AB, *et al*. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med* 1997;155:1770–6.
- Sugano N, Shimada K, Ito K, *et al*. Nicotine inhibits the production of inflammatory mediators in U937 cells through modulation of nuclear factor-kappaB activation. *Biochem Biophys Res Commun* 1998;252:25–8.
- Nishikawa M, Kakemizu N, Ito T, *et al*. Superoxide mediates cigarette smoke-induced infiltration of neutrophils into the airways through nuclear factor-kappaB activation and IL-8 mRNA expression in guinea pigs in vivo. *Am J Respir Cell Mol Biol* 1999;20:189–98.
- Inoue H, Massion PP, Ueki IF, *et al*. *Pseudomonas* stimulates interleukin-8 mRNA expression selectively in airway epithelium, in gland ducts, and in recruited neutrophils. *Am J Respir Cell Mol Biol* 1994;11:651–63.
- Riise GC, Ahlstedt S, Larsson S, *et al*. Bronchial inflammation in chronic bronchitis assessed by measurement of cell products in bronchial lavage fluid. *Thorax* 1995;50:360–5.
- Pesci A, Balbi B, Majori M, *et al*. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* 1998;12:380–6.
- Richman-Eisenstat JB, Jorens PG, Hebert CA, *et al*. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Am J Physiol* 1993;264:L413–8.
- Yamamoto C, Yoneda T, Yoshikawa M, *et al*. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 1997;112:505–10.
- Borgeat P, Samuelsson B. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. *J Biol Chem* 1979;254:856–79.
- Ford-Hutchinson AW, Bray MA, Doig MV, *et al*. Leukotriene B4, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 1980;286:264–5.
- Hubbard RC, Fells G, Gadek J, *et al*. Neutrophil accumulation in the lung in alpha-1-antitrypsin deficiency. Spontaneous release of leukotriene B4 by alveolar macrophages. *J Clin Invest* 1991;88:891–7.
- Crooks SW, Stockley RA. Leukotriene B4. *Int J Biochem Cell Biol* 1998;30:173–8.
- Gosset P, Tsiopoulos A, Wallaert B, *et al*. Increased secretion of tumor necrosis factor alpha and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J Allergy Clin Immunol* 1991;88:561–71.

- 35 Galli SJ, Gordon JR, Wershil BK. Cytokine production by mast cells and basophils. *Current Opin Immunol* 1991;3: 865-72.
- 36 Gordon JR, Galli SJ. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J Exp Med* 1991;174:103-7.
- 37 Dubravac DB, Spriggs DR, Mannick JA, et al. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. *Proc Natl Acad Sci USA* 1990;87:6758-61.
- 38 Arnold R, Humbert B, Werchau H, et al. Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. *Immunology* 1994;82:126-33.
- 39 Kips JC, Tavernier J, Pauwels RA. Tumor necrosis factor causes bronchial hyperresponsiveness in rats. *Am Rev Respir Dis* 1992;145:332-6.
- 40 Kwon OJ, Au BT, Collins PD, et al. Tumor necrosis factor-induced interleukin-8 expression in cultured human airway epithelial cells. *Am J Physiol* 1994;267:L398-405.
- 41 Cassatella MA, Meda L, Bonora S, et al. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide. *J Exp Med* 1993;178:2207-11.
- 42 Casale TB, Carolan EJ. Combination of IL-8 plus TNF alpha induces additive neutrophil migration. *Allergy Asthma Proc* 1999;20:361-3.
- 43 Pober JS, Gimbrone MA, Jr., Lapierre LA, et al. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J Immunol* 1986;137:1893-6.
- 44 Vassalli P. The pathophysiology of tumor necrosis factors. *Ann Rev Immunol* 1992;10:411-52.
- 45 Otsuka Y, Nagano K, Nagano K, et al. Inhibition of neutrophil migration by tumor necrosis factor. Ex vivo and in vivo studies in comparison with in vitro effect. *J Immunol* 1990;145:2639-43.
- 46 Fick RB, Jr., Robbins RA, Squier SU, et al. Complement activation in cystic fibrosis respiratory fluids: in vivo and in vitro generation of C5a and chemotactic activity. *Pediatr Res* 1986;20:1258-68.
- 47 Stockley RA, Shaw J, Afford SC, et al. Effect of alpha-1-proteinase inhibitor on neutrophil chemotaxis. *Am J Respir Cell Mol Biol* 1990;2:163-70.
- 48 Banda MJ, Rice AG, Griffin GL, et al. The inhibitory complex of human alpha-1-proteinase inhibitor and human leukocyte elastase is a neutrophil chemoattractant. *Exp Med* 1988;167:1608-15.
- 49 Senior RM, Hinek A, Griffin GL, et al. Neutrophils show chemotaxis to type IV collagen and its 7S domain and contain a 67 kD type IV collagen binding protein with lectin properties. *Am J Respir Cell Mol Biol* 1989;1:479-87.
- 50 Senior RM, Griffin GL, Mecham RP. Chemotactic activity of elastin-derived peptides. *J Clin Invest* 1980;66:859-62.
- 51 Bryant G, Rao CN, Brentani M, et al. A role for the laminin receptor in leukocyte chemotaxis. *J Leuk Biol* 1987;41:220-7.
- 52 Boyden S. The chemotactic effect of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med* 1962;115: 453-66.
- 53 Casale TB, Abbas MK, Carolan EJ. Degree of neutrophil chemotaxis is dependent upon the chemoattractant and barrier. *Am J Respir Cell Mol Biol* 1992;7:112-7.
- 54 Young SK, Worthen GS, Haslett C, et al. Interaction between chemoattractants and bacterial lipopolysaccharide in the induction and enhancement of neutrophil adhesion. *Am J Respir Cell Mol Biol* 1990;2:523-32.
- 55 Foxman EF, Campbell JJ, Butcher EC. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J Cell Biol* 1997;139:1349-60.
- 56 Sallenave JM, Shulmann J, Crossley J, et al. Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am J Respir Cell Mol Biol* 1994;11:733-41.
- 57 Hill AT, Bayley DL, Stockley RA. The interrelationship of sputum inflammatory markers in patients with chronic bronchitis. *Am J Respir Crit Care Med* 1999;160:893-8.
- 58 Stockley RA, Burnett D. Alpha-1-antitrypsin and leukocyte elastase in infected and noninfected sputum. *Am Rev Respir Dis* 1979;120:1081-6.
- 59 Peterson MW, Walter ME, Nygaard SD. Effect of neutrophil mediators on epithelial permeability. *Am J Respir Cell Mol Biol* 1995;13:719-27.
- 60 Umeno E, McDonald DM, Nadel JA. Hypertonic saline increases vascular permeability in the rat trachea by producing neurogenic inflammation. *J Clin Invest* 1990;85: 1905-8.
- 61 Keatings VM, Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997;155:449-53.
- 62 Tomaki M, Ichinose M, Miura M, et al. Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *Am J Respir Crit Care Med* 1995;151:613-7.
- 63 Stockley RA. Bronchiectasis: new therapeutic approaches based on pathogenesis. *Clin Chest Med* 1987;8:481-94.
- 64 Stockley R, Hill S, Morrison H, et al. Elastolytic activity of sputum and its relation to purulence and to lung function in patients with bronchiectasis. *Thorax* 1984;39:408-13.
- 65 Burnett D, Stockley RA. Cathepsin B-like cysteine proteinase activity in sputum and bronchoalveolar lavage samples: relationship to inflammatory cells and effects of corticosteroids and antibiotic treatment. *Clin Sci* 1985;68:469-74.
- 66 Witko-Sarsat V, Halbwachs-Mecarelli L, Schuster A, et al. Proteinase 3, a potent secretagogue in airways, is present in cystic fibrosis sputum. *Am J Respir Cell Mol Biol* 1999;20:729-36.
- 67 Chalmers GW, Macleod KJ, Sriram S, et al. Sputum endothelin-1 is increased in cystic fibrosis and chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:1288-92.
- 68 Stockley RA. Chronic bronchitis: the antiproteinase/proteinase balance and the effect of infection and corticosteroids. *Clin Chest Med* 1988;9:643-56.
- 69 Hill A, Bayley D, Campbell E, et al. Bronchial inflammation in patients with chronic bronchitis, with and without alpha-1-antitrypsin deficiency, and patients with bronchiectasis. *Thorax* 1997;52(Suppl 6):A48.
- 70 Hill AT, Campbell EJ, Bayley DL, et al. Evidence for excessive bronchial inflammation during an acute exacerbation of COPD in patients with alpha-1-antitrypsin deficiency (PiZ). *Am J Respir Crit Care Med* 1999;160:1968-75.
- 71 Gompertz S, O'Brien C, Bayley D, et al. Comparison of stable stage bronchial inflammation in chronic bronchitis and previously unsuspected bronchiectasis. *Eur Respir J* 1999;14(Suppl 30):165s.
- 72 Bhowmik A, Seemungal TA, Sapsford RJ, et al. Comparison of spontaneous and induced sputum for investigation of airway inflammation in chronic obstructive pulmonary disease. *Thorax* 1998;53:953-6.
- 73 Holz O, Richter K, Jorres RA, et al. Changes in sputum composition between two inductions performed on consecutive days. *Thorax* 1998;53:83-6.
- 74 Nightingale JA, Rogers DF, Barnes PJ. Effect of repeated sputum induction on cell counts in normal volunteers. *Thorax* 1998;53:87-90.
- 75 Spanevello A, Beghe B, Bianchi A, et al. Comparison of two methods of processing induced sputum: selected versus entire sputum. *Am J Respir Crit Care Med* 1998;157:665-8.
- 76 Pizzichini MM, Popov TA, Efthimiadis A, et al. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:866-9.
- 77 Bayley D, Hill A, Hall G, et al. Sputum induction to assess bronchial inflammation in patients with homozygous alpha-1-antitrypsin deficiency. *Thorax* 1997;52(Suppl 6): A48.
- 78 Bayley D, Stockley R. Validation of assays for lung inflammatory markers. *Eur Respir J* 2000;15:778-81.
- 79 Efthimiadis A, Pizzichini MM, Pizzichini E, et al. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol vs phosphate-buffered saline. *Eur Respir J* 1997;10:1336-40.
- 80 Grebski E, Peterson C, Medici TC. Homogenisation with dithiothreitol (DTT) influences concentrations of inflammatory mediators in sputa of asthmatics. *Eur Respir J* 1999; 14(Suppl 30):165s.
- 81 Fletcher CM. Cigarettes and respiratory disease. In: Goodman HA, ed. *World conference on smoking and health: a summary of the proceedings*. New York: American Cancer Society, 1968:78.
- 82 Reynolds HY, Newball HH. Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. *J Lab Clin Med* 1974;84:559-73.
- 83 Hunninghake GW, Crystal RG. Cigarette smoking and lung destruction. Accumulation of neutrophils in the lungs of cigarette smokers. *Am Rev Respir Dis* 1983;128:833-8.
- 84 Stanescu D, Sanna A, Veriter C, et al. Airways obstruction, chronic expectoration, and rapid decline of FEV₁ in smokers are associated with increased levels of sputum neutrophils. *Thorax* 1996;51:267-71.
- 85 Di Stefano A, Capelli A, Lusuardi M, et al. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 1998; 158:1277-85.
- 86 Bosken CH, Hards J, Gatter K, et al. Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry. *Am Rev Respir Dis* 1992;145:911-7.
- 87 Hill AT, Bayley DL, Campbell EJ, et al. Airways inflammation in chronic bronchitis: the effects of smoking and alpha-1-antitrypsin deficiency. *Eur Respir J* 2000;15:886-90.
- 88 Brantly ML, Paul LD, Miller BH, et al. Clinical features and history of the destructive lung disease associated with alpha-1-antitrypsin deficiency of adults with pulmonary symptoms. *Am Rev Respir Dis* 1988;138:327-36.
- 89 Eriksson S. Studies in alpha-1-antitrypsin deficiency. *Acta Med Scand* 1965;432:1-85.
- 90 Morrison HM, Kramps JA, Burnett D, et al. Lung lavage fluid from patients with alpha-1-proteinase inhibitor deficiency or chronic obstructive bronchitis: anti-elastase function and cell profile. *Clin Sci* 1987;72:373-81.
- 91 Llewellyn-Jones CG, Lomas DA, Carrell RW, et al. The effect of the Z mutation on the ability of alpha-1-antitrypsin to prevent neutrophil mediated tissue damage. *Biochim Biophys Acta* 1994;1227:155-60.
- 92 Liou TG, Campbell EJ. Nonisotopic enzyme-inhibitor interactions: a novel nonoxidative mechanism for quantum

- proteolysis by human neutrophils. *Biochemistry* 1995;**34**: 16171–7.
- 93 Liou TG, Campbell EJ. Quantum proteolysis resulting from release of single granules by human neutrophils: a novel, nonoxidative mechanism of extracellular proteolytic activity. *J Immunol* 1996;**157**:2624–31.
 - 94 Onofrio JM, Toews GB, Lipscomb MF, *et al.* Granulocyte-alveolar-macrophage interaction in the pulmonary clearance of *Staphylococcus aureus*. *Am Rev Respir Dis* 1983;**127**: 335–41.
 - 95 Vial WC, Toews GB, Pierce AK. Early pulmonary granulocyte recruitment in response to *Streptococcus pneumoniae*. *Am Rev Respir Dis* 1984;**129**:87–91.
 - 96 Toews GB, Vial WC. The role of C5 in polymorphonuclear leukocyte recruitment in response to *Streptococcus pneumoniae*. *Am Rev Respir Dis* 1984;**129**:82–6.
 - 97 Stockley RA. Role of bacteria in the pathogenesis and progression of acute and chronic lung infection. *Thorax* 1998;**53**:58–62.
 - 98 Hill SL, Morrison HM, Burnett D, *et al.* Short term response of patients with bronchiectasis to treatment with amoxycillin given in standard or high doses orally or by inhalation. *Thorax* 1986;**41**:559–65.
 - 99 Hill AT, Campbell EC, Hill SL, *et al.* Airway bacterial load in stable chronic bronchitis: a potent stimulus for airway inflammation. *Am J Med* 2000 (in press).
 - 100 Soler N, Ewig S, Torres A, *et al.* Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur Respir J* 1999;**14**:1015–22.
 - 101 Anthonisen NR, Manfreda J, Warren CPW, *et al.* Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann Intern Med* 1987;**106**:196–204.
 - 102 Balbi B, Bason C, Balleari E, *et al.* Increased broncho-alveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *Eur Respir J* 1997;**10**:846–50.
 - 103 Saetta M, Di Stefano A, Maestrelli P, *et al.* Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med* 1994;**150**:1646–52.
 - 104 Keatings VM, Jatakanon A, Worsdell YM, *et al.* Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* 1997;**155**:542–8.
 - 105 Culpitt SV, Maziak W, Loukidis S, *et al.* Effect of high dose inhaled steroid on cells, cytokines, and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;**160**:1635–9.
 - 106 Llewellyn-Jones CG, Harris TA, Stockley RA. Effect of fluticasone propionate on sputum of patients with chronic bronchitis and emphysema. *Am J Respir Crit Care Med* 1996;**153**:616–21.
 - 107 Confalonieri M, Mainardi E, Della Porta R, *et al.* Inhaled corticosteroids reduce neutrophilic bronchial inflammation in patients with chronic obstructive pulmonary disease. *Thorax* 1998;**53**:583–5.
 - 108 Wiggins J, Elliott JA, Stevenson RD, *et al.* Effect of corticosteroids on sputum sol-phase protease inhibitors in chronic obstructive pulmonary disease. *Thorax* 1982;**37**:652–6.