Sputum chemotactic activity in chronic obstructive pulmonary disease: effect of α₁-antitrypsin deficiency and the role of leukotriene B₄ and interleukin 8

I S Woolhouse, D L Bayley, R A Stockley

Background: Neutrophil recruitment to the airway is thought to be an important component of continuing inflammation and progression of chronic obstructive pulmonary disease (COPD), particularly in the presence of severe α₁-antitrypsin (α₁-AT) deficiency. However, the chemoattractant nature of secretions from these patients has yet to be clarified.

Methods: The chemotactic activity of spontaneous sputum from patients with stable COPD, with (n=11) and without (n=11) α₁-AT deficiency (P/2), was assessed using the under-agarose assay. The contribution of leukotriene B₄ (LTB₄) and interleukin 8 (IL-8) to the chemotactic activity was examined using an LTB₄ receptor antagonist (B12 315 2W) and an IL-8 monoclonal antibody, respectively.

Results: Neutrophil chemotactic activity (expressed as % fMLP control) was significantly higher in patients with α₁-AT deficiency (mean (SE) 63.4 (8.9)% v 36.7 (5.5)%; mean difference 26.7% (95% CI 4.9 to 48.4), p<0.05). The mean (SE) contribution of both LTB₄ and IL-8 (expressed as % fMLP control) was also significantly higher in α₁-AT deficient patients than in patients with COPD with normal levels of α₁-AT (LTB₄, 31.9 (6.3)% v 18.0 (3.7)%); mean difference 13.9% (95% CI 1.4 to 29.1), p<0.05; IL-8: 24.1 (5.2)% v 8.1 (1.2)%; mean difference 15.9% (95% CI 4.7 to 27.2), p<0.05. When all the subjects were considered together the mean (SE) contribution of LTB₄ (expressed as % total chemotactic activity) was significantly higher than IL-8 (46.8 (3.5)% v 30.8 (4.6)%; mean difference 16.0% (95% CI 2.9 to 29.2), p<0.05). This difference was not significantly influenced by α₁-AT phenotype (p=0.606).

Conclusions: These results suggest that the bronchial secretions of COPD patients with α₁-AT deficiency have increased neutrophil chemotactic activity. This relates to the increased levels of IL-8 and, in particular LTB₄, which accounted most of the sputum chemotactic activity in the patients with COPD as a whole. Increased chemotactic activity, together with inhibitor deficiency, may contribute to the more rapid disease progression seen in α₁-AT deficiency via increased neutrophil recruitment and release of neutrophil elastase.

Increased numbers of neutrophils are found in bronchial lavage samples¹⁾ and bronchial biopsy specimens from subjects with chronic obstructive pulmonary disease (COPD), even when they are clinically stable, and there is evidence that the decline in forced expiratory volume in 1 second (FEV₁) is related to airway neutrophilia.¹3,4⁾ For these reasons, and the fact that neutrophil enzymes can cause all the pathological features of COPD, it has long been thought that neutrophils play a central role in the pathogenesis and progression of COPD. In the lower airways α₁-antitrypsin (α₁-AT) is thought to be the major inhibitor of neutrophil elastase,³ and subjects with severe deficiency of α₁-AT (P/2 phenotype; serum α₁-AT concentration <11 μM) develop early onset and rapidly progressive pulmonary emphysema.⁴ ¹¹-¹³ Although the major function of α₁-AT is considered to be an anti-elastase defending the lower respiratory tract from elastolytic destruction, previous studies have also shown increased numbers of neutrophils and a greater degree of inflammation in both the lower airways¹⁴ and the larger airways of deficient subjects with chronic bronchitis compared with non-deficient subjects.¹⁵ ¹⁶ This increased neutrophil recruitment is thought to contribute further to the development of the rapidly progressive lung destruction seen in α₁-AT deficiency.

Neutrophil influx from the blood stream into the lungs of patients with COPD implies the presence of sensitised neutrophils and/or increased chemotactic signals, but few data have been published on these mechanisms. Leukotriene B₄ (LTB₄) and interleukin 8 (IL-8) are both potent chemotactic agents capable of promoting neutrophil transendotelial migration,¹⁷ ¹⁸ and increased levels of these two chemoattractants have been found in secretions from patients with COPD, particularly those with severe α₁-AT deficiency.¹⁹ ²⁰ Hubbard et al also reported higher levels of LTB₄ and chemotactic activity in alveolar macrophage supernatant from α₁-AT deficient patients compared with non-deficient subjects.²¹ These workers concluded that LTB₄ was the important chemoattractant and showed that uninhibited elastase activity was the likely reason for its production. However, the chemotactic activity of bronchial secretions from patients with COPD and α₁-AT deficiency has not previously been assessed in detail, so it is not known whether the higher levels of LTB₄ and/or IL-8 account for the increased neutrophil recruitment seen in these patients. Furthermore, little is known about the relative contributions of these two chemoattractants in COPD in general. In a previous study of a small number of patients with severe COPD and normal α₁-AT levels, LTB₄, accounted for approximately 30% of the total chemotactic activity at presentation of an exacerbation,²² but there are no previous studies of the contribution of IL-8 in these patients.

Understanding the chemoattractant nature of secretions in COPD in general, and α₁-AT deficiency in particular, is of critical importance for the development of new therapeutic strategies. The aims of the present study were (1) to compare the chemotactic activity of sputum from matched COPD patients with and without α₁-AT deficiency and (2) to assess the contribution of both LTB₄ and IL-8 to this chemotactic
activity using a specific LTB4 receptor antagonist and a monoclonal IL-8 antibody.

**METHODS**

**Patients and sputum collection**

For verification of the methodology a pool of mucopurulent and mucoid spontaneous sputum (characterised according to a 9 point colour chart17) sol phase was obtained from six patients with COPD with α1-AT deficiency (PiZ) and six patients with normal α1-AT (PiM), as described previously.18 For subsequent studies a sample was collected over 4 hours (from rising) from 11 patients with α1-AT deficiency (PiZ) and 11 matched patients with normal α1-AT (PiM) at least 2 months after the most recent acute exacerbation. All patients had a history of chronic bronchitis, as defined by daily sputum production for at least 3 months in 2 consecutive years.20

**Isolation of blood neutrophils**

Polymorphonuclear neutrophils (PMNs) were isolated from the whole blood of healthy volunteers as described previously.19 The PMNs (>96% pure, >98% viable, by exclusion of trypan blue) were resuspended at required concentrations in RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 2 mg/ml bovine serum albumin.

**PMN chemotaxis**

The chemotaxis assay was performed using the under-agarose method as described previously.21 The major advantage of this method is that it allows assessment of both chemotaxis and spontaneous movement (chemokinesis), whereas membrane filter chamber methods, such as the Boyden method, only allow directed movement to be assessed. The optimal dilution of sputum for the assay was determined using the sputum sol phase pools. Subsequent chemotactic studies were performed in triplicate at the optimal dilution and averaged to obtain the result for that sample. A simultaneous chemotaxis assay was performed to 100 nM n-formylmethionyl leucylphenylalanine (fMLP) and the results were then expressed as a percentage of this fMLP control.

**Validation of the methodology to assess the contribution of LTB4 and IL-8**

Increasing concentrations of the LTB4 receptor antagonist (BIIL 315 ZW) and the IL-8 antibody (anti-IL8 monoclonal antibody; R&D Systems, Abingdon, UK) were used to assess their effect on the chemotactic response to optimal concentrations of pure LTB4 (Sigma Chemicals, Poole, UK), IL-8 (R&D Systems), and a mixture of the two. For each set of experiments the LTB4 receptor antagonist was preincubated with normal PMNs and the IL-8 antibody was preincubated with the chemotacticant(s) for 1 hour before the chemotaxis assay. The effect of the LTB4 receptor antagonist and the IL-8 antibody on PMN chemotaxis to the mucoid and mucopurulent sputum pools was investigated in a similar way. The suppression of chemotaxis by optimal concentrations of the LTB4 receptor antagonist or the IL-8 antibody was taken as the contribution of LTB4 and IL-8, respectively, to the total chemotactic activity of individual samples.

**Sputum biochemistry**

LTB4 and IL-8 were measured by ELISA using commercially available kits (Amersham International plc, Buckinghamshire, UK and R&D Systems, respectively). Neutrophil elastase and myeloperoxidase (MPO) activity were both measured by chromogenic substrate assay, as described and validated previously.22 23

**Statistical analysis**

Categorical data between patients with and without α1-AT deficiency were compared using the Fisher’s exact test. The age of the subjects in each group was compared using an independent t test. Lung function, chemotaxis, and sputum biochemistry data were compared using the Wilcoxon test for paired and unpaired data (where appropriate). The Spearman’s rank correlation test was used to examine the relationship between chemotactic activity and sputum chemotacticants. A p value of less than 0.05 was considered to be statistically significant. Sputum and blood sample collection was approved by the South Birmingham Health Authority ethics committee and all subjects provided written informed consent.

**RESULTS**

Demographic data for the α1-AT deficient and non-deficient patients are shown in table 1. The α1-AT deficient group were younger but otherwise both groups were closely matched. No patients were on oral corticosteroid therapy and neither group had evidence of bronchiectasis on high resolution computed tomographic scanning of the chest. The results shown are for the postbronchodilator forced expiratory volume in one second (FEV1) expressed as a percentage of the value predicted for the patient’s age, sex, and height24 and the ratio of FEV1 to vital capacity (FEV1/FVC). Neither group had significant reversibility (<12% increase in FEV1) to inhaled β2 agonist.

**Chemotactic response to sputum**

Preliminary dose-response experiments with pooled sputum revealed that mean (SE) PMN chemotaxis was maximal to neat sputum from both the α1-AT deficient patients (mucopurulent 0.96 (0.10) mm, mucoid 0.64 (0.10) mm; mean difference 0.32 mm (95% CI 0.15 to 0.48), p=0.05) and the control patients (mucopurulent 0.70 (0.14) mm, mucoid 0.54 (0.10) mm; mean difference 0.16 mm (95% CI –0.03 to 0.36), p=0.08). At 1:2 sol phase dilution the difference between mucoid and mucopurulent samples from both groups reached conventional levels of significance. The mean (SE) PMN

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**Table 1**: Patient and sputum characteristics

<table>
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<tr>
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<th>PiZ (n=11)</th>
<th>PiM (n=11)</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>47 (8)</td>
<td>64 (6)*</td>
</tr>
<tr>
<td>M/F</td>
<td>8:3</td>
<td>5:6</td>
</tr>
<tr>
<td>Current [ex] smokers</td>
<td>3 (8)</td>
<td>7</td>
</tr>
<tr>
<td>Inhaled corticosteroids</td>
<td>28.3 (19.5)</td>
<td>33.7 (14.4)</td>
</tr>
<tr>
<td>Stable state FEV1 (%) predicted</td>
<td>30.7 (10.1)</td>
<td>40.1 (17.0)</td>
</tr>
<tr>
<td>Macroscopic sputum appearance (M/MP)</td>
<td>2/9</td>
<td>2/9</td>
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<tr>
<td>Sputum bacterial load &gt;10^7 [cfu/ml]</td>
<td>4</td>
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FEV1= forced expiratory volume in one second; VC=vital capacity; M=mucoid; MP=mucopurulent; cfu=colony forming units.

Data for age, FEV1 (% predicted) and FEV1/VC (%) are mean (SD).

*p<0.05.
chemotaxis to pooled sputum from α1-AT deficient patients was 0.80 (0.10) mm for mucopurulent samples and 0.47 (0.10) mm for mucoid samples (mean difference 0.33 mm (95% CI 0.27 to 0.40, p<0.05), and for samples from control patients the mean (SE) PMN chemotactic activity was 0.53 (0.12) mm and 0.37 (0.11) mm, respectively (mean difference 0.16 mm (95% CI 0.07 to 0.24), p<0.05). Since such sputum samples can differ widely in their neutrophil content, a 1:2 dilution was used for all further experiments.

PMN chemotaxis to diluted sputum (expressed as % fMLP control) from matched COPD patients with and without α1-AT deficiency is shown in fig 1. A wide range in response was seen but the mean (SE) chemotactic activity was significantly higher in samples from the α1-AT deficient patients than in those from control non-deficient patients (63.4 (8.9)% v 36.7 (5.5)%; mean difference 26.7% (95% CI 4.9 to 48.4), p<0.05). Since such sputum samples can differ widely in their neutrophil content, a 1:2 dilution was used for all further experiments.

Validation of methodology to assess the contribution of LTB4 and IL-8

Figure 2A summarises the suppression of chemotaxis to 500 nM LTB4 by the LTB4 receptor antagonist from a control mean (SE) of 0.56 (0.06) mm to 0.00 (0.01) mm when PMNs were incubated with 1 µM of the antagonist. Figure 2B shows the suppression of chemotaxis to 500 nM IL-8 by the IL-8 antibody from a control mean (SE) of 0.67 (0.12) mm to 0.00 (0.01) mm when the chemoattractant was incubated with 0.5 mg/ml antibody. At the above concentrations of LTB4, receptor antagonist and IL-8 antibody there was no detectable effect on cell viability, as assessed by trypan blue exclusion.

When optimal concentrations of LTB4, and IL-8 were mixed the chemotactic response increased, but this was not completely additive (mean (SE) chemotaxis 0.51 (0.09) mm for 500 nM LTB4 alone, 0.76 (0.04) mm for 500 nM IL-8 alone, and 1.03 (0.10) mm for the mixture). When the LTB4 receptor antagonist (1 µM) was preincubated with the PMNs or the IL-8 antibody (0.5 mg/ml) was added to the mixture, chemotaxis towards the mixture was suppressed appropriately to the level expected for the remaining chemoattractant (0.83 (0.06) mm with the LTB4 antagonist and 0.58 (0.08) mm with the IL-8 antibody). In combination, the LTB4 receptor antagonist and the IL-8 antibody reduced PMN chemotaxis to a mixture of both chemoattractants to 0.04 (0.01) mm (n=6 for all experiments).

Chemotaxis to diluted mucoid and mucopurulent sputum pools was suppressed in a dose dependent manner after preincubation with the LTB4 antagonist. At 10 µM of antagonist chemotaxis was suppressed to 57.9 (10.2)% of the control (no antagonist) for mucoid samples (mean difference 42.1% (95% CI 15.8 to 68.4), p<0.05) and to 53.5 (7.7)% for mucopurulent samples (mean difference 46.5% (95% CI 26.7 to 66.3), p<0.05). Similar results were seen for the IL-8 antibody which also suppressed chemotactic activity in a dose dependent manner to 67.6 (14.0)% of control for mucoid samples (mean difference 32.4% (95% CI 4.2 to 60.6), p<0.05) and 62.3 (9.9)% (mean difference 37.7% (95% CI 17.6 to 57.8), p<0.05) for mucopurulent samples at 1 mg/ml antibody. At these concentrations, which were used for the subsequent experiments described below, there was no detectable effect on cell viability as assessed by trypan blue exclusion. In addition, the PMNs retained their ability to migrate towards IL-8 in the presence of the LTB4 receptor antagonist and to LTB4 with the IL-8 antibody, as well as to fMLP with either (data not shown).

Chemotactic contribution of LTB4 and IL-8 in sputum samples

The results of the contribution of LTB4 and IL-8 to chemotactic activity of diluted sputum from matched COPD patients with and without α1-AT deficiency are shown in fig 3. The mean contribution of LTB4 (expressed as % fMLP control) was significantly higher in the samples from α1-AT deficient subjects than in non-deficient patients (mean (SE) 31.9 (6.3)% v 18.0 (3.7)%; mean difference 13.9% (95% CI –1.4 to 29.1), p<0.05). In addition, the mean (SE) contribution of IL-8 (expressed as % fMLP control) was significantly higher in the samples from α1-AT deficient subjects than in subjects with normal levels of α1-AT (24.1 (5.2)% v 8.1 (1.2)%; mean difference 15.9% (95% CI 4.7 to 27.2), p<0.05). The remaining chemotactic activity—that is, the difference between overall chemotactic activity and the combined contribution of LTB4 and IL-8 (expressed as % fMLP control)—did not differ significantly between the two groups (mean (SE) 7.5 (5.7)% v 10.6 (2.0)%; mean difference –3.1% (95% CI –15.8 to 9.5), p=NS).
When all the subjects were considered together, the mean (SE) contribution of LTB₄ (expressed as % total chemotactic activity) was significantly higher than IL-8 (46.8 (3.5)% vs 30.8 (4.6)%; mean difference 16.0% (95% CI 2.9 to 29.2), p<0.05) and this difference was not influenced by α₁-AT phenotype (p=0.606).

**Sputum biochemistry**

Sputum elastase activity, LTB₄, and IL-8 levels were significantly higher in the α₁-AT deficient group whereas MPO activity was similar in the two groups (table 2). When all the subjects were considered together, the mean (SE) concentration of LTB₄ in the 22 samples was higher than that of IL-8 (14.4 (4.0) nM vs 6.1 (1.2) nM; mean difference 8.3 nM (95% CI 4.9 to 16.5), p<0.05). Again this difference was not significantly influenced by α₁-AT phenotype (p=0.140). The LTB₄ levels correlated strongly with overall chemotactic activity (r=0.823, p<0.001) and the results are summarised in fig 4A using a semi-log plot for convenience. On the other hand, sputum IL-8 levels did not correlate with overall chemotactic activity (r=0.174, p=NS; fig 4B).

**DISCUSSION**

Using the under-agarose chemotaxis assay we have confirmed that pooled sputum from patients with COPD is able to induce neutrophil chemotaxis in a dose dependent manner, with higher activity in mucopurulent samples than in mucoid samples. The LTB₄ antagonist BIIL 315 ZW and an IL-8 monoclonal antibody were able to remove the appropriate contribution of each agent from the combined chemotactic response towards a mixture of the two chemoattractants. It is worthy of note that, unlike the Boyden chamber method, the chemotactic response to optimal concentrations of LTB₄ and IL-8 in a mixture was not completely additive. The exact reasons for the difference between methodologies remain unknown, but it may relate to the way chemotaxis is quantified: in the under-agarose assay chemotaxis is expressed as the difference between directed movement and chemokinesis whereas the Boyden chamber method simply measures directed movement. Nevertheless, abrogation of each chemoattractant effect produced the expected result for the remaining agent, suggesting that their contribution to the global activity could be determined by this methodology. The chemotactic response to pooled sputum from patients with COPD could also be suppressed by both the LTB₄ antagonist and the IL-8 antibody.

**Figure 3** Contribution of (A) LTB₄ and (B) IL-8 to the chemotactic activity of sputum from matched COPD patients with (PiZ, n=11) and without (PiM, n=11) α₁-antitrypsin deficiency. Individual values are represented by the symbols. The horizontal bars represent the median values for each group.

**Figure 4** Relationship between total chemotactic activity and sputum LTB₄ (A) and IL-8 (B) levels. Closed symbols indicate patients with COPD with α₁-antitrypsin deficiency (PiZ); open symbols indicate patients with COPD with normal α₁-antitrypsin (PiM). Correlation coefficients (r) and significance (p) for all data are shown.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Airway inflammation in patients with COPD with (PiZ) and without (PiM) α₁-AT deficiency</th>
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<tr>
<td></td>
<td>PiZ (n=11)</td>
</tr>
<tr>
<td>LTB₄ (nM)</td>
<td>17.54 (4.14)</td>
</tr>
<tr>
<td>IL-8 (nM)</td>
<td>8.75 (2.16)</td>
</tr>
<tr>
<td>Elastase (µM)</td>
<td>0.02 (0.00)</td>
</tr>
<tr>
<td>MPO (mg/l)</td>
<td>8.58 (2.49)</td>
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LTB₄=leukotriene B₄, IL-8=interleukin8, MPO=myeloperoxidase. Data are presented as mean (SE).
Thus, on the basis of these preliminary studies it was felt that the use of the under-agarose chemotaxis assay, the LTB₄ antagonist, and the IL-8 antibody would provide a valid assessment of the overall sputum chemotactic activity and the contributions of LTB₄ and IL-8, respectively, in individual samples from patients with COPD.

We found that the overall sputum chemotactic activity was significantly higher in COPD patients with α₁-AT deficiency. Sputum inflammation and hence chemotactic activity could be influenced by variations in patient characteristics, such as the degree of lung function impairment, the presence of acute exacerbations, cigarette smoking, corticosteroid treatment, sputum macroscopic appearance, and a bacterial load of >10⁹ colony forming units per ml. We were careful to ensure that the two groups of patients were well matched in terms of these characteristics and that sputum was collected from patients when they were in a stable clinical state (at least 2 months after the last exacerbation). This suggests that the difference we detected in the study was independent of these factors. Assessment of sputum biochemistry, however, revealed significantly higher levels of the potent neutrophil chemotactic agents LTB₄ and IL-8 in patients with α₁-AT deficiency, which is in keeping with previous studies of sputum and bronchoalveolar lavage fluid from α₁-AT deficient patients. In the second part of the study we therefore assessed the contribution of each chemotactic agent to the sputum chemotactic activity. The absolute contribution of both LTB₄ and IL-8 was significantly higher in the sputum from patients with α₁-AT deficiency, although the remaining chemotactic activity (not accounted for by LTB₄ or IL-8) did not differ significantly between the two groups. Taking these data together suggests that the increased levels of these two chemotactic activity in the stable clinical state, and may be central to the increased neutrophil recruitment which is thought to be a key event in α₁-AT deficient and non-deficient patients with COPD.

It is worthy of further comment that, despite the increased sputum chemotactic activity seen in patients with α₁-AT deficiency, the mean sputum levels of MPO (a marker of neutrophil influx and activation) were not statistically different between the two groups. This may reflect the fact that, in the relatively small number of patients studied here, MPO is not a sensitive enough marker to detect small, yet clinically significant, differences in sputum neutrophil numbers. Further studies, including the assessment of absolute neutrophil counts, will be required to clarify this possibility.

In summary, our data show that the chemotactic activity of sputum in COPD is increased in patients with α₁-AT deficiency compared with those with normal levels, and this relates to increased contributions from IL-8 and, in particular, LTB₄. This, together with the deficiency, may explain the more rapid disease progression seen in this condition (via increased neutrophil recruitment). Targeting new treatments at reducing the chemotactic activity of sputum is likely to be of benefit in COPD, particularly when it is associated with α₁-AT deficiency.

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