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 (PiZ), 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 (Bill 315 ZW) and an IL-8 monoclonal antibody, respectively.

Results: Sputum neutrophil chemotactic activity (expressed as % n-formylmethionyl leucylphenylalanine (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.
activity using a specific LTβ 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 chart) 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. 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.

Isolation of blood neutrophils

Polymorphonuclear neutrophils (PMNs) were isolated from the whole blood of healthy volunteers as described previously. 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. 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 LTβ and IL-8

Increasing concentrations of the LTβ 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 LTβ (Sigma Chemicals, Poole, UK), IL-8 (R&D Systems), and a mixture of the two. For each set of experiments the LTβ receptor antagonist was preincubated with normal PMNs and the IL-8 antibody was preincubated with the chemotaxiant(s) for 1 hour before the chemotaxis assay. The effect of the LTβ 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 LTβ receptor antagonist or the IL-8 antibody was taken as the contribution of LTβ, receptor antagonist or the IL-8 antibody respectively, to the total chemotactic activity of individual samples.

Sputum biochemistry

LTβ 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.

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 chemoattractants. 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 height and the ratio of FEV1 to vital capacity (FEV1/FVC). Neither group had significant reversibility (<12% increase in FEV1) to inhaled β2 agonist.

Table 1  Patient and sputum characteristics

<table>
<thead>
<tr>
<th></th>
<th>PiZ (n=11)</th>
<th>PiM (n=11)</th>
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<tbody>
<tr>
<td>Age</td>
<td>47 (8)</td>
<td>64 (6)*</td>
</tr>
<tr>
<td>M/F</td>
<td>8.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Current [ex] smokers</td>
<td>3 (8)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Inhaled corticosteroids</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Stable state FEV1 (% predicted)</td>
<td>28.3 (19.5)</td>
<td>33.7 (14.4)</td>
</tr>
<tr>
<td>Stable state FEV1/VC (%), 30.7 (10.1)</td>
<td>40.1 (17.0)</td>
<td></td>
</tr>
<tr>
<td>Macrophase sputum appearance (M/MF)</td>
<td>2/9</td>
<td>2/9</td>
</tr>
<tr>
<td>Sputum bacterial load 10^10 cfu/ml</td>
<td>4</td>
<td>4</td>
</tr>
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FEV1= forced expiratory volume in one second; VC=vital capacity; M=mucoid; MP=mucopurulent; cfu=colony forming units.

Statistical analysis table. 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 chemoattractants. 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 height 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 the postbronchodilator forced expiratory volume in one second (FEV1) expressed as a percentage of the value predicted for the patient’s age, sex, and height and the ratio of FEV1 to vital capacity (FEV1/FVC). Neither group had significant reversibility (<12% increase in FEV1) to inhaled β2 agonist.
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).

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 preincubated 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 antagonist 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).

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).

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.

**Table 2** Airway inflammation in patients with COPD with (PiZ) and without (PiM) α1-AT deficiency

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PiZ (n=11)</th>
<th>PiM (n=11)</th>
<th>Mean difference (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTB4 (nM)</td>
<td>17.54 (4.14)</td>
<td>11.29 (6.89)</td>
<td>6.25 (-10.53 to 23.04)</td>
<td>0.023</td>
</tr>
<tr>
<td>IL-8 (nM)</td>
<td>8.75 (2.16)</td>
<td>3.50 (0.61)</td>
<td>5.25 (0.56 to 9.94)</td>
<td>0.015</td>
</tr>
<tr>
<td>Elastase (µM)</td>
<td>0.02 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.02 (0.01 to 0.03)</td>
<td>0.010</td>
</tr>
<tr>
<td>MPO (mg/l)</td>
<td>8.58 (2.49)</td>
<td>6.30 (0.82)</td>
<td>0.19 (-0.27 to 0.65)</td>
<td>0.326</td>
</tr>
</tbody>
</table>

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 >10⁷ 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 chemoattractants 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 chemoattractant 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 chemoattractants account for the increase seen in the chemotactic activity of sputum from patients with α₁-AT deficiency.

The source of LTB₄ and IL-8 in airway secretions is uncertain, but a possible explanation for the increased concentrations of these two chemoattractants in the sputum of α₁-AT deficient subjects is the presence of free elastase. Elastase has been shown in vitro to stimulate LTB₄ release from alveolar macrophages, and the addition of purified human α₁-AT to inactivate the elastase removed this effect. In addition, sputum from patients with cystic fibrosis (which also contains high levels of neutrophil elastase) is able to induce IL-8 gene expression in human bronchiol epithelial cells which can also be inhibited by the addition of α₁-AT. The presence of low but detectable elastase activity in the sputum of our patients with α₁-AT deficiency, but not those with normal α₁-AT levels, confirms the findings of our previous study and would be in keeping with this explanation. The lack of detectable elastase activity in all but one sample from patients with normal α₁-AT levels is likely to be due to inhibition of the elastase by protease inhibitors, in particular α₁-AT and secretory leukoprotease inhibitor.

Of further interest was the finding that the mean molar concentration of LTB₄ in the sputum and its contribution to chemotaxis was greater than that of IL-8 in the group as a whole, and this was not significantly influenced by α₁-AT phenotype. In addition, the sputum concentration of LTB₄, but not IL-8, correlated with overall chemotactic activity, although the nature of this relationship suggests that this is likely to reflect the higher levels of LTB₄ present in the samples studied here. We have previously found higher mean molar concentrations of LTB₄ than IL-8 in sputum collected both during exacerbations and in the stable clinical state from patients with COPD, both with and without α₁-AT deficiency. However, to our knowledge this is the first time the contribution of LTB₄ and IL-8 to chemotaxis has been compared in patients with COPD with and without α₁-AT deficiency. The results suggest that LTB₄ is of particular importance to sputum 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 sputum is likely to be of benefit in COPD, particularly when it is associated with α₁-AT deficiency.

### Authors' affiliations

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

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