ORIGINAL ARTICLE

Sputum chemotactic activity in chronic obstructive pulmonary disease: effect of α_1-antitrypsin deficiency and the role of leukotriene B_4 and interleukin 8

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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 α_1-antitrypsin (α_1-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) α_1-AT deficiency (PiZ), was assessed using the under-agarose assay. The contribution of leukotriene B_4 (LTB_4) and interleukin 8 (IL-8) to the chemotactic activity was examined using an LTB_4 receptor antagonist (BIIL 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 α_1-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 LTB_4 and IL-8 (expressed as % fMLP control) was also significantly higher in α_1-AT deficient patients than in patients with COPD with normal levels of α_1-AT (LTB_4: 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 LTB_4 (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 α_1-AT phenotype (p=0.606).

Conclusions: These results suggest that the bronchial secretions of COPD patients with α_1-AT deficiency have increased neutrophil chemotactic activity. This relates to the increased levels of IL-8 and, in particular LTB_4, 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 α_1-AT deficiency via increased neutrophil recruitment and release of neutrophil elastase.

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Increased numbers of neutrophils are found in bronchial lavage samples[1–3] and bronchial biopsy specimens from subjects with chronic obstructive pulmonary disease (COPD),[4] even when they are clinically stable, and there is evidence that the decline in forced expiratory volume in 1 second (FEV_1) is related to airway neutrophilia.[5] For these reasons, and the fact that neutrophil enzymes can cause all the pathological features of COPD,[6] it has long been thought that neutrophils play a central role in the pathogenesis and progression of COPD. In the lower airways α_1-antitrypsin (α_1-AT) is thought to be the major inhibitor of neutrophil elastase,[7] and subjects with severe deficiency of α_1-AT (PiZ phenotype; serum α_1-AT concentration <11 μM) develop early onset and rapidly progressive pulmonary emphysema.[8–10] Although the major function of α_1-AT is considered to be as 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 airways[11] and the larger airways of deficient subjects with chronic bronchitis compared with non-deficient subjects.[12 13] This increased neutrophil recruitment is thought to contribute further to the development of the rapidly progressive lung destruction seen in α_1-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 B_4 (LTB_4) and interleukin 8 (IL-8) are both potent chemotactic agents capable of promoting neutrophil transendothelial migration,[14 15] and increased levels of these two chemoattractants have been found in secretions from patients with COPD, particularly those with severe α_1-AT deficiency.[11 13] Hubbard *et al* also reported higher levels of LTB_4 and chemotactic activity in alveolar macrophage supernatant from α_1-AT deficient patients compared with non-deficient subjects.[16] These workers concluded that LTB_4 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 α_1-AT deficiency has not previously been assessed in detail, so it is not known whether the higher levels of LTB_4 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 α_1-AT levels, LTB_4 accounted for approximately 30% of the total chemotactic activity at presentation of an exacerbation,[17] 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 α_1-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 α_1-AT deficiency and (2) to assess the contribution of both LTB_4 and IL-8 to this chemotactic

Table 1 Patient and sputum characteristics

	PiZ (n=11)	PiM (n=11)
Age	47 (8)	64 (6)*
M/F	8:3	6:5
Current (ex) smokers	3 (8)	5 (6)
Inhaled corticosteroids	7	7
Stable state FEV_1 (% predicted)	28.3 (19.5)	33.7 (14.4)
Stable state FEV_1/VC (%)	30.7 (10.1)	40.1 (17.0)
Macroscopic sputum appearance (M/MP)	2/9	2/9
Sputum bacterial load $>10^7$ (cfu/ml)	4	4

FEV_1= forced expiratory volume in one second; VC=vital capacity; M=mucoid; MP=mucopurulent; cfu=colony forming units.
Data for age, FEV_1 (% predicted) and FEV_1/VC (%) are mean (SD).
*$p<0.05$.

activity using a specific LTB_4 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[18]) sol phase was obtained from six patients with COPD with α_1-AT deficiency (PiZ) and six patients with normal α_1-AT (PiM), as described previously.[19] For subsequent studies a sample was collected over 4 hours (from rising) from 11 patients with α_1-AT deficiency (PiZ) and 11 matched patients with normal α_1-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 LTB_4 and IL-8

Increasing concentrations of the LTB_4 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 LTB_4 (Sigma Chemicals, Poole, UK), IL-8 (R&D Systems), and a mixture of the two. For each set of experiments the LTB_4 receptor antagonist was preincubated with normal PMNs and the IL-8 antibody was preincubated with the chemoattractant(s) for 1 hour before the chemotaxis assay. The effect of the LTB_4 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 LTB_4 receptor antagonist or the IL-8 antibody was taken as the contribution of LTB_4 and IL-8, respectively, to the total chemotactic activity of individual samples.

Sputum biochemistry

LTB_4 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 α_1-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 α_1-AT deficient and non-deficient patients are shown in table 1. The α_1-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 (FEV_1) expressed as a percentage of the value predicted for the patient's age, sex, and height[24] and the ratio of FEV_1 to vital capacity (FEV_1/FVC). Neither group had significant reversibility (<12% increase in FEV_1) to inhaled β_2 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 α_1-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

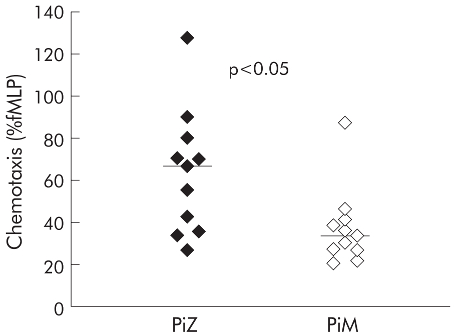

Figure 1 Chemotactic response to diluted sputum (1:2) from matched COPD patients with (PiZ, n=11) and without (PiM, n=11) α_1-antitrypsin deficiency. Individual results are represented by the symbols. The horizontal bars represent the median values for each group.

chemotaxis to pooled sputum from α_1-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,[18] 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 α_1-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 α_1-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 LTB_4 and IL-8

Figure 2A summarises the suppression of chemotaxis to 500 nM LTB_4 by the LTB_4 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 LTB_4 receptor antagonist and IL-8 antibody there was no detectable effect on cell viability, as assessed by trypan blue exclusion.

When optimal concentrations of LTB_4 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 LTB_4 alone, 0.76 (0.04) mm for 500 nM IL-8 alone, and 1.03 (0.10) mm for the mixture). When the LTB_4 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 LTB_4 antagonist and 0.58 (0.08) mm with the IL-8 antibody). In combination, the LTB_4 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 LTB_4 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 LTB_4 receptor antagonist and to LTB_4 with the IL-8 antibody, as well as to fMLP with either (data not shown).

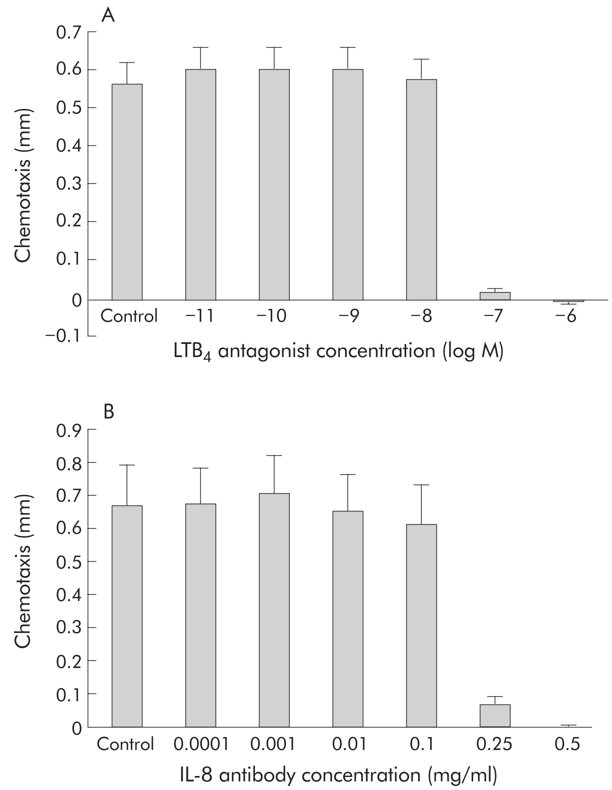

Figure 2 Inhibition of chemotactic response to (A) 500 nmol LTB_4 showing the effect of preincubation of neutrophils with increasing concentrations of LTB_4 antagonist and (B) 500 nmol IL-8 showing the effect of increasing concentrations of IL-8 antibody. The results shown are the mean (SE) values of six experiments.

Chemotactic contribution of LTB_4 and IL-8 in sputum samples

The results of the contribution of LTB_4 and IL-8 to chemotactic activity of diluted sputum from matched COPD patients with and without α_1-AT deficiency are shown in fig 3. The mean contribution of LTB_4 (expressed as % fMLP control) was significantly higher in the samples from α_1-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 α_1-AT deficient subjects than in subjects with normal levels of α_1-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 LTB_4 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).

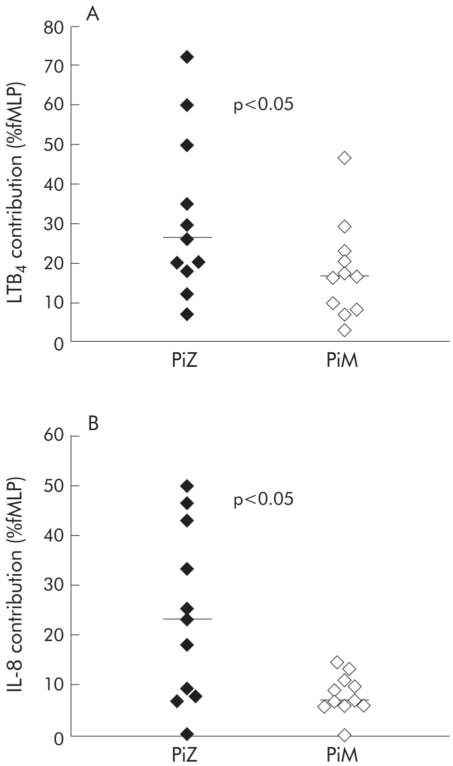

Figure 3 Contribution of (A) LTB_4 and (B) IL-8 to the chemotactic activity of sputum from matched COPD patients with (PiZ, n=11) and without (PiM, n=11) α_1-antitrypsin deficiency. Individual values are represented by the symbols. The horizontal bars represent the median values for each group.

When all the subjects were considered together, the mean (SE) contribution of LTB_4 (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), $p<0.05$) and this difference was not influenced by α_1-AT phenotype ($p=0.606$).

Sputum biochemistry

Sputum elastase activity, LTB_4, and IL-8 levels were significantly higher in the α_1-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 LTB_4 in the 22 samples was higher than that of IL-8 (14.4 (4.0) nM *v* 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 α_1-AT phenotype ($p=0.140$). The LTB_4 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).

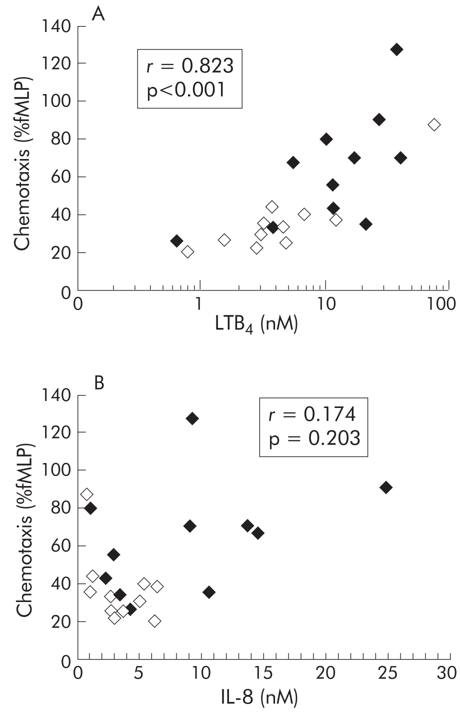

Figure 4 Relationship between total chemotactic activity and sputum LTB_4 (A) and IL-8 (B) levels. Closed symbols indicate patients with COPD with α_1-antitrypsin deficiency (PiZ); open symbols indicate patients with COPD with normal α_1-antitrypsin (PiM). Correlation coefficients (*r*) and significance (p) for all data are shown.

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 LTB_4 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,[19] the chemotactic response to optimal concentrations of LTB_4 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 LTB_4 antagonist and the IL-8 antibody.

Table 2 Airway inflammation in patients with COPD with (PiZ) and without (PiM) α_1-AT deficiency

	PiZ (n=11)	PiM (n=11)	Mean difference (95% CI)	p value
LTB_4 (nM)	17.54 (4.14)	11.29 (6.89)	6.25 (–10.53 to 23.04)	0.023
IL-8 (nM)	8.75 (2.16)	3.50 (0.61)	5.25 (0.56 to 9.94)	0.015
Elastase (μM)	0.02 (0.00)	0.00 (0.00)	0.02 (0.01 to 0.03)	0.010
MPO (mg/l)	8.58 (2.49)	6.30 (0.82)	0.19 (–0.27 to0.65)	0.326

LTB_4=leukotriene B_4; 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 LTB_4 antagonist, and the IL-8 antibody would provide a valid assessment of the overall sputum chemotactic activity and the contributions of LTB_4 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 α_1-AT deficiency. Sputum inflammation and hence chemotactic activity could be influenced by variations in patient characteristics, such as the degree of lung function impairment,[5] the presence of acute exacerbations,[17] cigarette smoking,[25] corticosteroid treatment,[26] sputum macroscopic appearance,[19] and a bacterial load of $>10^7$ colony forming units per ml.[27] 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 LTB_4 and IL-8 in patients with α_1-AT deficiency, which is in keeping with previous studies of sputum[13] and bronchoalveolar lavage fluid[11] from α_1-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 LTB_4 and IL-8 was significantly higher in the sputum from patients with α_1-AT deficiency, although the remaining sputum chemotactic activity (not accounted for by LTB_4 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 α_1-AT deficiency.

The source of LTB_4 and IL-8 in airway secretions is uncertain, but a possible explanation for the increased concentrations of these two chemoattractants in the sputum of α_1-AT deficient subjects is the presence of free elastase. Elastase has been shown in vitro to stimulate LTB_4 release from alveolar macrophages, and the addition of purified human α_1-AT to inactivate the elastase removed this effect.[16] 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 bronchial epithelial cells which can also be inhibited by the addition of α_1-AT.[28] The presence of low but detectable elastase activity in the sputum of our patients with α_1-AT deficiency, but not those with normal α_1-AT levels, confirms the findings of our previous study[13] and would be in keeping with this explanation. The lack of detectable elastase activity in all but one sample from patients with normal α_1-AT levels is likely to be due to inhibition of the elastase by proteinase inhibitors, in particular α_1-AT and secretory leukoprotease inhibitor.[7]

Of further interest was the finding that the mean molar concentration of LTB_4 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 α_1-AT phenotype. In addition, the sputum concentration of LTB_4, 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 LTB_4 present in the samples studied here. We have previously found higher mean molar concentrations of LTB_4 than IL-8 in sputum collected both during exacerbations and in the stable clinical state from patients with COPD, both with[13] and without α_1-AT deficiency.[17] However, to our knowledge this is the first time the contribution of LTB_4 and IL-8 to chemotaxis has been compared in patients with COPD with and without α_1-AT deficiency. The results suggest that LTB_4 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 α_1-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 α_1-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 α_1-AT deficiency compared with those with normal levels, and this relates to increased contributions from IL-8 and, in particular, LTB_4. 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 α_1-AT deficiency.

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