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Inhibitory capacity of alpha$_1$ antitrypsin in lung secretions: variability and the effect of drugs

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ABSTRACT The inhibitory function of α_1 antitrypsin (α_1AT) has been studied in the lung secretions of 31 patients with chronic obstructive bronchitis. The inhibitory capacity for a single sample showed a wide range (median 0·13 μg porcine pancreatic elastase (PPE) inhibited per μg α_1 antitrypsin; range 0–0·55 μg), and all but five of 86 samples studied were capable of inhibiting some porcine pancreatic elastase. No sample showed free elastase activity, however. The inhibitory capacity, studied in six patients over five consecutive days, varied daily within the same individual (coefficient of variation 9·0–108·9%). Corticosteroid treatment (40 mg prednisone daily) increased the inhibitory capacity of sputum α_1 antitrypsin in 10 patients ($2p < 0·05$) from a median value of 0·13 μg PPE inhibited per μg α_1AT (range 0·06–0·36) before treatment to 0·22 μg PPE inhibited per μg α_1AT (range 0·09–0·65) after treatment. The inhibitory capacity of sputum was higher than in the corresponding bronchoalveolar lavage sample from the same patient ($2p < 0·05$; $n = 10$). The median value for sputum was 0·22 μg PPE inhibited per μg α_1AT (range 0–0·55) and the value for lavage fluid was 0·07 μg PPE inhibited per μg α_1AT (range 0–0·27).

The demonstration that intratracheal instillation of a proteolytic enzyme (papain) in rats produced emphysema[1] led to the idea that proteinases are important in the pathogenesis of emphysema. Elastases are capable of digesting lung elastin,[2] and human leucocyte elastase has been shown to damage bronchial mucosa and ciliated epithelium,[3] and to cause emphysema in experimental animals.[4] It has been suggested therefore that leucocyte elastase may also play a part in the pathogenesis of cystic fibrosis,[5] bronchiectasis,[6] and the adult respiratory distress syndrome[7] as well as emphysema.

The action of proteolytic enzymes is opposed in vivo by several proteinase inhibitors. Of these, α_1 antitrypsin accounts for 90% of elastase inhibition in the serum and is thought to be a major elastase inhibitor in bronchial secretions and the dominant inhibitor in bronchoalveolar secretions.[8] Its importance is inferred by the association of α_1 antitrypsin deficiency with the development of severe emphysema at an early age, even in non-smokers.[9] Most patients with emphysema, however, have normal serum concentrations of α_1 antitrypsin and the balance between proteinase and antiproteinase activity within the lung is likely to be a major determinant of subsequent damage. This balance might be altered unfavourably by an increase in the elastase burden in the lung, such as may occur during infection[10] or as a result of smoking,[11] or by a reduction of the functional α_1 antitrypsin present in the lung caused by smoking[12] or interaction with other proteinases.[13] Modification of this balance in favour of the inhibitors might provide a means of preventing progression of disease. Alternatively, factors which tip the balance in favour of the enzymes might prove harmful.

Steroid treatment has been shown to decrease the concentration of α_1 antitrypsin in sputum from patients with chronic bronchitis,[14] though it is uncertain whether this also reduces the effective proteinase inhibitor screen. Danazol on the other hand is known to raise serum, and hence alveolar, concentrations in patients with a deficiency of α_1 antitrypsin,[15] and this has been associated with an increase in the inhibitory capacity of the secretions. Little is known about the effect of other commonly prescribed therapeutic agents on the inhibitory capacity of α_1 antitrypsin. There is little information concerning the range of α_1 antitrypsin function in sputum

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and its variation, either from day to day or between subjects. This information is essential for proper interpretation of results obtained after therapeutic intervention. Furthermore, little is known about whether the functional capacity of α_1 antitrypsin at various levels of the bronchial tree is constant or shows significant differences.

The present study was designed to investigate the inhibitory function of α_1 antitrypsin in lung secretions from patients with stable chronic obstructive bronchitis. In particular, we wished to assess the daily variability in the inhibitory capacity of α_1 antitrypsin in sputum and bronchoalveolar lavage samples from the same patient. In addition, we assessed samples from patients before and during treatment with corticosteroids and bromhexine to determine whether these therapeutic agents had any effect on α_1 antitrypsin function.

Methods

PATIENTS

We studied 31 patients with chronic cough and sputum production. These were divided into three groups: (1) Sputum samples were collected from 11 patients both before and during steroid treatment. Eight of these patients were men and the mean FEV_1 was 0·93 (1 SD 0·27) l. All were current smokers. Six of these patients also provided sputum samples on five consecutive days before treatment, which were used to assess the daily variability of the capacity of the sputum for inhibiting porcine pancreatic elastase. (2) A further 10 patients (nine of whom were male) were studied before and during bromhexine treatment. Seven were current smokers and the mean FEV_1 was 1·41 (1·18) l. The average age was 63·2 (SD 5·1) years. (3) A final group of 10 patients (eight male) provided paired sputum and bronchoalveolar lavage samples. The average age was 66 (8·6) years and the mean FEV_1 was 1·65 (0·48) l. Nine were current smokers. All 31 patients were studied at least six weeks after any episode of chest infection, while they were in a stable clinical state.

Sputum samples were collected over a three to four hour period in the morning and then ultracentrifuged at 50 000 *g* for 90 minutes (3°C). The sol phase was removed and stored at −70°C until it was analysed.

EFFECT OF DRUGS

Eleven patients were studied on a single day before and on the sixth day after starting a course of prednisone, 40 mg daily. The procedure has been described in detail previously.[14] Briefly, sputum that had been collected on the morning of one of the control days (chosen by the presence of adequate samples to study the maximum number of patients) was compared with samples from the same 11 patients collected on the sixth day of steroid treatment, when the greatest changes in protein concentrations had been seen.[14]

Ten further patients were studied, from whom sputum samples were collected on a pretreatment control day and on the seventh day of treatment with bromhexine, 32 mg daily.

All samples were processed as described above.

COMPARISON OF SPUTUM WITH BRONCHOALVEOLAR LAVAGE FLUID

Ten patients undergoing diagnostic fibreoptic bronchoscopy for suspected pulmonary neoplasia or unexplained haemoptysis were studied. Sputum was collected before premedication with atropine and processed as described above. After administration of intravenous diazepam and local anaesthesia with intranasal and nasopharyngeal lignocaine, the bronchoscope was passed through the nose and the vocal cords were then anaesthetised with two 2 ml aliquots of 4% lignocaine. The bronchoscope was then wedged in a subsegmental bronchus in either the lingula or the right middle lobe, on the macroscopically or radiologically normal side. Warmed sterile physiological saline, 120 ml in six 20 ml aliquots, was instilled and recovered in a sterile trap by gentle suction. The resultant fluid was initially centrifuged at low speed to remove the cells and then ultracentrifuged at 50 000 *g* for 90 minutes, for consistency with the sputum samples. The supernatant was then concentrated by a known amount (5–10 fold), an Amicon pressure filtration system with a UM2 membrane (molecular weight cut off = 2000 daltons) being used, and the concentrated bronchoalveolar lavage fluid was stored at −70°C until it was analysed.

ASSESSMENT OF SAMPLES

Sputum and bronchoalveolar lavage fluid samples were assessed in the following ways:

1 The concentration of α_1 antitrypsin (α_1AT) was measured immunologically by rocket immunoelectrophoresis into agarose containing sheep antiserum to α_1AT (Immunodiagnostic Research Laboratory, University of Birmingham) and this was compared with a known standard serum (100% = 2·04 g/l α_1AT). The antiserum was selected because it was known to provide accurate quantification of α_1AT that had recently been exposed to enzyme resulting in α_1AT-enzyme complexes or partialy proteolysed α_1AT.[16]

2 The function of α_1AT in the samples was assessed by its ability to inhibit porcine pancreatic

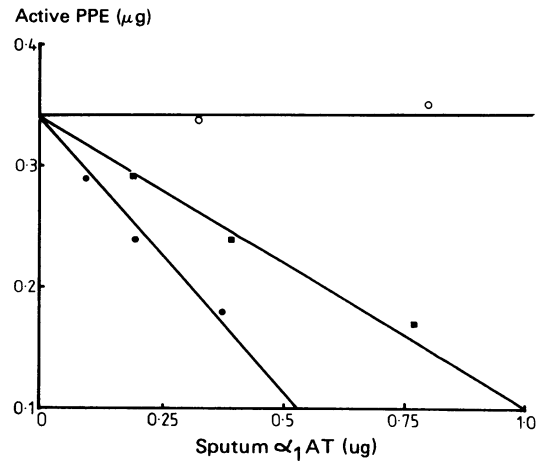

Fig 1 *Ability of sputum α_1 antitrypsin (α_1AT) to inhibit porcine pancreatic elastase (PPE): results for three sputum samples, one of which did not inhibit the enzyme. Increasing amounts of sputum (or bronchoalveolar lavage fluid) were incubated with 0·34 μg active PPE, Succ(Ala)$_3$pNA being used as substrate to assess residual elastase activity; and the intercept volume and hence the amount of α_1 antitrypsin totally inhibiting the enzyme were calculated.*

elastase (PPE), which had been assessed for enzyme activity by comparison with leucocyte elastase of known activity determined previously by active site titration.[16] A fixed amount (0·34 μg) of active PPE was preincubated for 10 minutes alone or with increasing volumes of sputum or bronchoalveolar lavage fluid made up to 0·5 ml with 0·2 mol/l tris hydrochloride buffer (pH 8·6) with 1% triton, at 37°C. The mixture was then incubated for a further 30 minutes at 37°C with 0·5 ml of succinyl-L-alanyl-L-alanyl-L-alanine-*para*-nitroanilide (Succ(Ala)$_3$pNA) obtained from the Peptide Institute, Osaka (initial concentration 1 mg/ml). The substrate was chosen to assess residual elastase activity because its hydrolysis had been shown to correlate in a linear manner with elastase activity.[17] Optical density (absorbance) was then measured at 410 nm and compared with a blank of substrate and buffer alone. The fall in absorbance against the volume of secretion added was determined for each sample by linear regression analysis (method of least squares) and the intercept volume, where enzyme activity (absorbance) would be zero, was calculated. This point was taken as the volume of secretion required to inhibit 0·34 μg PPE completely (between batch coefficient of variation 9·9%; n = 13). From this volume the inhibitory capacity was calculated as the amount of PPE (μg) inhibited per μg of α_1AT. The method is summarised for three samples in figure 1.

The effects of drugs on inhibitory capacity, expressed as amount of enzyme inhibited per μg α_1AT and per 100 μl sputum, as well as any difference between sputum and bronchoalveolar lavage fluid, were assessed with the two tailed Wilcoxon rank sum test.

Results

In a single sputum sample obtained from each of the 31 patients, the inhibitory capacity of α_1AT ranged from zero to 0·55 μg PPE inhibited/μg α_1AT (median 0·13). Two of the samples failed to inhibit any PPE, despite the presence of clearly measurable α_1AT.

The daily within patient variability of inhibitory capacity is shown for the six patients in the table. In most patients this was relatively stable, though unexplained large differences did occur (patient 4, day 5). In the six patients studied the coefficient of variation of α_1AT inhibition in sputum ranged from 9% to 108·9%.

EFFECT OF DRUGS

Steroids

Sputum samples from these 11 patients showed greater inhibition of PPE per μg α_1AT during than before treatment ($2p < 0·05$). The median value on the control day was 0·13 (range 0·06–0·36) and on the sixth day of steroid treatment 0·22 (range 0·09–0·65) μg PPE inhibited per μg α_1AT. The inhibitory capacity of a given volume of the secretions was, however, unaltered by treatment (median before steroid treatment 0·14 (range 0·10–0·50) and after treatment 0·19 (range 0·11–0·45) μg PPE per 100 μl sputum).

Bromhexine

Treatment with oral bromhexine for seven days was not associated with change in relative or total inhibitory capacity in the 10 patients studied ($2p > 0·1$). The median values before treatment were 0·08 (range 0·04–0·25) μg PPE inhibited μg α_1AT and 0·20 (range 0·09–0·78) μg PPE inhibited per 100 μl sputum respectively, whereas during treatment the median values for relative and total inhibitory capacity were 0·11 (range 0·02–0·19) PPE inhibited per μg α_1AT and 0·28 (range 0·06–0·92) μg PPE inhibited per 100 μl sputum respectively.

COMPARISON OF SPUTUM WITH BRONCHOALVEOLAR LAVAGE FLUID

The sputum samples showed greater inhibitory capacity than the paired bronchoalveolar lavage samples ($2p < 0·05 > 0·01$) in the 10 patients studied although the ranges were wide. The median

Inhibitory capacity of sputum samples from six patients on five consecutive days expressed in μg porcine pancreatic elastase inhibited per μg α_1 antitrypsin for individual samples

Patient No	Day					Mean (1 SD)	Coefficient of variation
	1	2	3	4	5		
1	0·43	0·23	0·42	0·41	0·37	0·37 (0·08)	22·2
2	0·11	0·13	0·09	0·09	0·08	0·10 (0·02)	20·2
3	0·11	0·07	0·05	0·09	0·25	0·12 (0·08)	68·7
4	0·08	0·06	0·11	0·07	0·46	0·16 (0·17)	108·9
5	0·45	0·36	0·30	0·40	0·22	0·34 (0·09)	25·3
6	0·07	0·06	0·06	0·07	0·07	0·07 (0·01)	9·0

value for sputum was 0·21 (range 0–0·55) μg PPE inhibited per μg α_1AT and 0·07 (range 0–0·27) for bronchoalveolar lavage fluid.

Three samples (two sputum samples collected during steroid treatment and one sputum sample from the paired sputum and bronchoalveolar lavage fluid samples) inhibited slightly more PPE than could be accounted for by the total amount of α_1AT present measured immunologically (0·65, 0·65, and 0·55 μg PPE/μg α_1AT. On the basis of molecular weights of 26 400 and 54 000 respectively for PPE[18] and α_1AT,[19] 1 μg α_1AT should be capable of inhibiting 0·49 μg of PPE, on the assumption of a 1:1 molar interaction.[20] To investigate this discrepancy further, we adsorbed α_1AT from one of the three samles using a sepharose bound anti-α_1AT antibody immunoadsorption column. After the main protein peak (peak 1) had passed through the column the adsorbed protein (peak 2) was eluted with 0·2 mol/l glycine hydrochloride, pH 2·5, and then dialysed against 0·2 mol/l tris hydrochloride, pH 8·6. The protein containing samples were tested immunologically for the presence of α_1AT to ensure that α_1AT was excluded from the main peak (peak 1) and the α_1AT was present in the eluted fractions (peak 2). The fractions containing the main protein peak were pooled, as were those from peak 2 which contained the previously adsorbed protein. The pooled fractions were concentrated 20–30 fold as described for the bronchoalveolar lavage fluid samples and tested for inhibitory activity towards PPE. Only the fractions containing α_1AT (peak 2) showed any inhibition (fig 2).

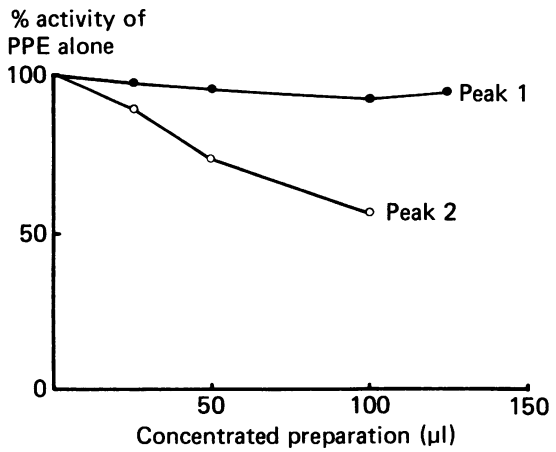

Fig 2 *Inhibition curves obtained from one of three sputum samples in which the observed functional capacity of α_1 antitrypsin (α_1AT) exceeded the expected value. The sample was passed through an α_1 antitrypsin immunoadsorption column (see text for details) to remove the α_1 antitrypsin and the porcine pancreatic elastase (PPE) inhibitory capacity of the unadsorbed protein was assessed (peak 1). The adsorbed α_1 antitrypsin was then eluted and its inhibitory capacity determined (peak 2).*

Discussion

Despite the presumed importance of α_1 antitrypsin in protecting the lung from damage by proteolytic enzymes, there have been very few studies of the variability in the functional capacity of α_1 antitrypsin and these have been performed on bronchoalveolar lavage fluid obtained from normal subjects at irregular intervals over several months.[21 22] In the present study we have assessed the short term variability in secretions from the upper part of the lower respiratory tract from several patients over five consecutive days.

The within subject variation of inhibitory capacity differed for each patient and was occasionally large. The reason for this wide variation is not clear. All the patients were current smokers, but the variations in the cigarettes they smoked in a day during the period of study may have led to similar fluctuations in α_1 antitrypsin function, perhaps as a result of vari-

able degrees of oxidation of the α_1 antitrypsin by cigarette smoke.

The effect of drugs is of interest. In our previous studies the concentration of α_1 antitrypsin in sputum tended to fall in patients having steroids,[14] perhaps owing to a decrease in protein transudation from serum as part of the general anti-inflammatory effect of corticosteroids. Despite this, the inhibitory function of α_1 antitrypsin improved in that these secretions were able to inhibit more porcine pancreatic elastase per μg α_1AT. This would suggest that although the lung α_1 antitrypsin has fallen there has been a greater fall in the proteinase burden, perhaps owing to a reduction in proteinase bearing cells (such as neutrophils and macrophages) or a decrease in release of proteinase due to stabilisation of lysosomal membranes.[23] Whether such changes could be advantageous either in the short term or over periods of years by having a beneficial effect on the lung proteinase-antiproteinase balance remains to be determined.

Bromhexine treatment in the dosage used seemed to have no effect on the inhibitory capacity of α_1 antitrypsin in the lung. No attempt was made to determine whether a true mucolytic action occurred.

Three sputum samples inhibited more porcine pancreatic elastase than could be accounted for by the α_1 antitrypsin present as measured immunologically. A similar slight discrepancy has been noted by Carp *et al*[21] in their studies of bronchoalveolar lavage fluid from 24 non-smokers. They obtained an inhibition of 0·59 ± 0·08 μg PPE per μg α_1AT (the theoretical value should be 0·5 μg PPE inhibited per μg α_1AT) and thought that this discrepancy was due to impurities in the commercially prepared porcine pancreatic elastase. The interpretation of these slight discrepancies is also dependent on several other factors. Firstly, accurate quantification of lung α_1 antitrypsin is imperative and we have previously indicated that such inaccuracies are likely to occur even with the best polyclonal antisera.[16] Immunological errors in estimation, however, are likely to occur only if the α_1 antitrypsin has been inactivated as an inhibitor and would therefore be unlikely to account for the apparent overestimation of α_1 antitrypsin inhibitory function. Secondly, the observation could also be explained by the presence of another inhibitor of porcine pancreatic elastase in the sputum or bronchoalveolar lavage fluid that was contributing to the total elastase inhibition screen; but, despite a decade of research on this subject, α_1 antitrypsin and α_2 macroglobulin are the only inhibitors present in sputum which have been shown to be active against porcine pancreatic elastase in stable chronic obstructive bronchitis. Although Hochstrasser *et al* have recently demonstrated the presence of another inhibitor of porcine pancreatic elastase in sputum, it has to be activated by acidification and is inactive in the stable clinical state.[20]

Alpha$_2$ macroglobulin is capable of causing partial inhibition of porcine pancreatic elastase activity against small peptide substrates such as Succ(Ala)$_3$pNA,[24] but it is present only in low concentrations in sputum (usually less than 5% of the α_1 antitrypsin molar concentration[10]) and it is thus likely to contribute little if anything to the inhibition of the enzyme in the present studies. In view of these studies most research groups, including Boudier *et al* in their recent study,[25] have used methods similar to those described here to determine α_1 antitrypsin function in secretions.

The use of the immunoadsorption column to fractionate one of the sputum samples with greater inhibitory capacity for porcine pancreatic elastase than could be accounted for by the α_1 antitrypsin present confirmed that all inhibition of the commercially prepared enzyme resided in the protein peak containing α_1 antitrypsin. This suggests that the discrepancy between the expected and observed inhibition of porcine pancreatic elastase is likely to be a result of minor impurities in the commercially prepared enzyme, as suggested previously by Carp *et al*,[21] and not due to the presence of another inhibitor of the elastase.

In contrast to these three sputum samples, one paired sputum and bronchoalveolar lavage sample and one additional sputum and two additional bronchoalveolar lavage samples failed to inhibit any porcine pancreatic elastase, despite the presence of easily measurable quantities of α_1 antitrypsin in the secretions. The inactivation may be related to cigarette smoking, although other samples from this group of smoking patients, with similar amounts of α_1 antitrypsin (as estimated by rocket immunoelectrophoresis), were able to inhibit the enzyme. The amount of leucocyte elastase present in these samples and the degree of enzyme-inhibitor complexing and partial proteolysis of α_1 antitrypsin were not estimated, so it is uncertain whether one of these other factors or oxidation or a combination of them was responsible for the observed result.

Variation in the inhibitory capacity of α_1 antitrypsin between patients was also large. Strict comparison of our results with those of other workers is difficult, since results are usually expressed as means and standard deviations and this is probably inappropriate for most of our results, which do not show a normal distribution. In samples from our paired sputum and bronchoalveolar lavage fluid study the average inhibitory capacity was 0·24 (0·18) μg PPE/μg α_1AT for sputum and 0·10 (0·10) for lav-

age fluid. This result would give coefficients of variation of 75% and 100% respectively. Although these within patient variabilities are similar they are clearly greater than the coefficient of variation for lavage fluid from smokers obtained by Carp and his coworkers[21]—namely 13·6%. The difference may be due to the selection of patients since the current results are from patients with established lung disease and those of Carp *et al* are from normal subjects. Similar reasons may account for the relatively constant results obtained from normal subjects studied by Carp *et al* on different occasions, which contrast with the appreciable variability found in some of our patients.

The inhibitory capacity of α_1 antitrypsin in bronchoalveolar lavage fluid was less than that found in sputum from the same subject. The reasons for this finding are not clear. Possibly drugs given at the time of bronchoscopy could play a part, although only atropine and diazepam were used systemically, 30 and five minutes respectively, before the procedure. Although we have shown that lignocaine instilled into the area to be lavaged does not affect α_1 antitrypsin function in vitro (unpublished observations), it could cause cellular disruption and release of some proteinase during the lavage procedure, which does not occur during sputum collection. Alternatively, the difference in the inhibitory capacity of α_1 antitrypsin might be due to differences in the local environment and cells found at bronchoalveolar and at bronchial level. In this context the preponderance of macrophages at bronchoalveolar level may be important since it is known that macrophage elastase can inactivate α_1 antitrypsin.[26] Further studies will be necessary before clear conclusions can be drawn.

In conclusion, we have shown that the variability of α_1 antitrypsin inhibitory capacity in sputum, both between and within patients, is large. Steroids alter the inhibitory capacity of α_1 antitrypsin in sputum and this may have long term implications affecting the management of patients with proteinase related lung disease. Bromhexine had no effect on the inhibitory capacity. Finally, the ability of α_1 antitrypsin to inhibit proteinases is lower in bronchoalveolar lavage fluid than in sputum, although the reason for this is not clear.

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