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Elastase inhibitors of sputum sol phase: variability, relationship to neutrophil elastase inhibition, and effect of corticosteroid treatment

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ABSTRACT The concentrations of three known elastase inhibitors (α_1 proteinase inhibitor, antileucoprotease, and α_2 macroglobulin) have been determined in the sputum of six patients with obstructive bronchitis over five consecutive days. Antileucoprotease was the major inhibitor measured and potentially could provide more than 80% of the elastase inhibition, whereas the contribution of α_2 macroglobulin was less than 0.2%. Comparison with the inhibitory capacity of the secretions active against human neutrophil elastase showed that the inhibitors could account for only about half of the inhibition measured. This suggests the presence of a substantial amount of unrecognised inhibitor. Corticosteroid treatment in 10 patients reduced the mean α_1 proteinase inhibitor concentration ($p < 0.025$) from 18.6 μg/ml (SD 22.5) to 9.8 (6.6). Antileucoprotease, however, increased ($p < 0.05$) from 20.5 μg/ml (24.3) to 39.3 (23.4). These changes were associated with an increase in elastase inhibition ($p < 0.025$) from 180 (160) μg elastase/ml secretion to 310 (130), suggesting a beneficial effect of steroid treatment on the antielastases in lung secretions.

Although the cause of emphysema remains uncertain, the currently favoured hypothesis is that proteolytic enzymes with elastolytic properties digest lung elastin, leading to structural changes and hence dilatation of air spaces.[1] Before the enzymes can attack lung elastin, however, they must overcome the local inhibitors. The enzyme that has been implicated in human emphysema is neutrophil elastase.[1] Hence the balance between this enzyme and elastase inhibitors may play a crucial part in the development of emphysema.

There has been controversy about the nature of antielastases in lung secretion. Gadek *et al*[2] initially presented data suggesting that the only antielastase of the alveolar structure was α_1 antitrypsin (also called α_1 proteinase inhibitor). Although several low molecular weight inhibitors of elastase have been identified in lung secretions,[1] it was thought that these were inhibitors protecting bronchial rather than alveolar structures.[3]

Recent immunohistochemical evidence, however, has shown that these inhibitors are present in peripheral airways.[4] Furthermore, they can be recovered by bronchoalveolar lavage[5] and contribute substantially to the elastase inhibition in lavage samples.[5 6] In addition, we have shown that the relative concentrations of the low molecular weight inhibitors and α_1 proteinase inhibitor, as well as their function, are broadly similar in bronchial secretions (sputum) and bronchoalveolar lavage fluid.[5]

The purpose of the present study was to assess the known antielastases in sputum sol phase from patients with chronic bronchitis and emphysema. In particular, we were interested in their contribution to the antielastase activity of the secretion and whether or not this varied from day to day in a group of subjects. Furthermore, we have obtained preliminary evidence that corticosteroid treatment increases the elastase inhibition of lung secretions.[7] Alpha$_1$ proteinase inhibitor concentrations, however, are lower during steroid treatment,[8] and we wished to determine whether the increase in elastase inhibition could be explained by changes in the other known inhibitors.

Methods

The study was divided into two parts. Firstly, we studied six patients with smoke related chronic

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obstructive bronchitis (mean FEV_1 1.23 (SD 0.39) aged 54–67 years. All were current smokers and collected their sputum (as free from saliva as possible) during the first four hours from rising on five consecutive days. Secondly, we studied 10 similar patients (four of them female) aged 45–64 years (mean FEV_1 0.981 (0.27)) while they were in hospital for a trial of steroid treatment. Sputum was collected during their first week of placebo treatment and after five days of steroid treatment (40 mg prednisone a day). This time interval had previously been shown to be sufficient to show changes in secretion proteins.[8] All other treatment remained unaltered.

The sputum was ultracentrifuged to obtain the sputum sol phase, which was divided into two aliquots and stored at −70°C until it was analysed.

PROTEIN CONCENTRATIONS

The sputum albumin and α_1 proteinase inhibitor were measured by rocket immunoelectrophoresis with well characterised antiserum known to give accurate results, even for α_1 proteinase inhibitor that had recently interacted with enzyme.[9] An aliquot of the samples was flown in dry ice to Leiden for measurement of the low molecular weight bronchial inhibitor antileucoprotease by enzyme linked immunosorbent assay (ELISA[10]).

$Alpha_2$ macroglobulin was measured by ELISA in our laboratory with a known standard serum and antiserum obtained from the Immunodiagnostic Research Laboratory (University of Birmingham). The lower limit of detection was 2 ng/ml and the between batch coefficient of variation at 1800 ng/ml (n = 5) was 2.4%.

Comparison of molar amounts of the elastase inhibitors was taken from their concentrations and molecular weights. The values used were 54 000 daltons for α_1 proteinase inhibitor[11]; 14 500 for antileucoprotease[10] and 725 000 for α_2 macroglobulin.[12]

ENZYME INHIBITION

The ability of each secretion to inhibit neutrophil elastase was determined by adding increasing volumes of the sample to a known amount of enzyme,[5] and the inhibitory capacity (volume capable of totally inhibiting the enzyme) was determined by interpolation of the inhibition curve. The amount of enzyme inhibited per ml of secretion was thus obtained (the neutrophil elastase inhibitory capacity).

On the assumption that the molecular weight of neutrophil elastase is 30 000 daltons[13] we could determine the number of moles in neutrophil elastase inhibited by a given volume of secretion. This result was compared with the number of moles of inhibitor present, as described above. Thus we could determine whether the inhibitors present could account for the inhibition measured. For these calculations we took into account the known observations that α_1 proteinase inhibitor and antileucoprotease inhibit neutrophil elastase in a 1:1 molar ratio and α_2 macroglobulin in a 2:1 molar ratio.

Comparisons between results were tested statistically using the Wilcoxon test for paired data (single tailed).

Results

All samples contained measurable quantities of the three inhibitors. The range between individuals, however, was wide for each of the days, as indicated by the SD values shown in table 1. The coefficients of variation between subjects (SD/mean × 100) for antileucoprotease on the five days were 55%, 48.3%, 33.8%, 54.9%, and 51.3%. The variability for α_1 proteinase inhibitor showed a wider range—from 109.8% (day 1) to 30.5% (day 3)—and that for α_2 macroglobulin ranged from 80.8% (day 1) to 57.9% (day 3).

Antileucoprotease was the major inhibitor in terms of moles/100 moles of total measured inhibitor. This inhibitor accounted for more than 60% of the measured inhibitors in all secretions. $Alpha_1$ proteinase inhibitor was the second major inhibitor, representing about 10–20% of the inhibitors measured. The contribution of α_2 macroglobulin was usually less than

Table 1 *Mean (SD) concentrations and percentage molar concentrations (molar ratios) of known protease inhibitors, neutrophil elastase inhibitory capacity, and calculated percentage of unknown inhibitor in sputum from six patients on five consecutive days*

Day	Concentration (μg/ml)			Molar ratio (%)			NEIC (μg/ml)	Unknown inhibitor (%)
	ALP	α_1PI	α_2M	ALP	α_1PI	α_2M		
1	37 (20.5)	23.5 (25.8)	1.98 (1.6)	84.7 (12.3)	15.2 (12.2)	0.11 (0.08)	167.9 (87.8)	50.0 (16.0)
2	46.2 (22.3)	21.3 (11.4)	1.47 (0.94)	87.8 (5.3)	12.1 (5.3)	0.08 (0.08)	193.9 (80.7)	51.0 (18.0)
3	48.3 (16.3)	16.4 (5.0)	0.88 (0.51)	90.6 (5.3)	9.4 (5.2)	0.04 (0.04)	216.4 (55.9)	51.5 (12.5)
4	42.8 (23.5)	19.7 (9.1)	1.16 (0.88)	87.2 (5.8)	12.7 (5.8)	0.08 (0.08)	207.6 (66.9)	59.2 (26.5)
5	34.7 (17.8)	16.9 (9.9)	0.97 (0.77)	82.4 (11.1)	17.5 (11.0)	0.08 (0.14)	182.2 (57.0)	68.5 (36.7)

ALP—antileucoprotease; α_1PI—α_1 proteinase inhibitor; α_2M—α_2 macroglobulin; NEIC—neutrophil elastase inhibitory capacity.

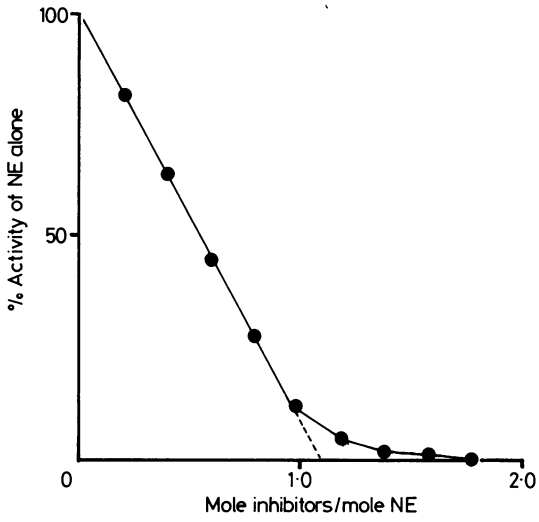

Fig 1 *Inhibition of neutrophil elastase (NE) by a mixture of antileucoprotease, α_1 proteinase inhibitor, and α_2 macroglobulin. The vertical axis indicates the remaining enzyme activity and the horizontal axis the total moles of inhibitor added per mole of enzyme. The regression line passes through 1:08 moles per mole neutrophil elastase.*

1% in all the samples and was on average under 0.11%. These results are also summarised in table 1.

The average between subject results for each protein were similar though not identical on each of the five days. There were no significant differences for any of the results between any two days.

INHIBITION STUDIES

Before we undertook inhibition studies on samples of sputum the validity of the technique was assessed with a mixture of all three proteins in quantities similar to the average results obtained for the six patients. The inhibition of neutrophil elastase by this mixture is shown in figure 1. Extrapolation of the inhibition line shows that the x intercept occurs close to the point of molar equivalence of enzyme and the total amount of inhibitor.

The results of inhibition studies using samples of sputum are shown in table 1. The average amount of neutrophil elastase inhibited per ml of secretion was relatively constant on each of the days, although the range was again wide. The between subject variability ranged from 52.3% (day 1) to 25.8% (day 3).

Despite the variability the amount of enzyme inhibited clearly was more than twice as great on average as the total capacity of the inhibitors measured. Thus more than half of the inhibition could not be accounted for by the total amount of antileucoprotease, α_1 proteinase inhibitor, or α_2 macroglobulin. This proportion was relatively constant

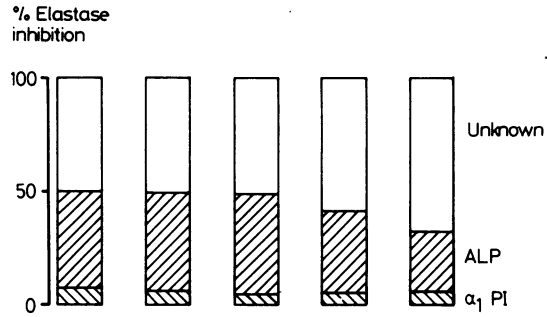

Fig 2 *Average potential contribution of antileucoprotease (ALP) and α_1 proteinase inhibitor (α_1PI) to total elastase inhibition for the six patients on each of the five days.*

(table 1); the between subject variability (cofficient of variance for the five days was 32%, 35.3%, 24.3%, 44.8%, and 53.6%.

Figure 2 summarises the relative contributions of antileucoprotease and α_1 proteinase inhibitor to total inhibition and to the proportion unaccounted for. The potential contribution of α_2 macroglobulin was too small to be illustrated graphically.

EFFECT OF STEROIDS

Because of the large between subject variability, 10 subjects were included in the study of the effect of steroid treatment. Measurements of α_2 macroglobulin were not made because of its minimal contribution to elastase inhibition. The results are summarised in table 2. There was a significant increase ($p < 0.05$) in antileucoprotease concentration during steroid and a significant decrease ($p < 0.025$) in α_1 proteinase inhibitor concentration. The neutrophil elastase inhibitory capacity rose with steroid treatment ($p < 0.025$), although the proportion of inhibition that could not be accounted for by antileucoprotease and α_1 proteinase inhibitor together remained unaltered.

Discussion

The results of this study show that wide inter-individual variability exists for both the quantity and

Table 2 *Concentration of protease inhibitors, neutrophil elastase inhibitory capacity, and calculated percentage of unknown inhibitor in 10 patients before and during treatment with corticosteroids*

	Concentration (μg/ml)			*Unknown inhibitor (%)*
	ALP	*α_1PI*	*NEIC*	
Control	20.5 (24.3)	18.6 (22.5)	180 (160)	64.9 (30.3)
Steroid	39.3 (23.4)	9.8 (6.6)	310 (130)	69.2 (19.5)
Significance (p)	<0.05	<0.025	<0.025	NS

Abbreviations as in table 1.

the function of proteinase inhibitors in sputum. The variability is similar to that found previously for other proteins in sputum from similar patients.[14] The reasons for such variability have been discussed in detail[15] and reflect the problems of sample collection, local production, and lung inflammation that may vary even in an apparently homogenous group of patients. This emphasises the importance of the requirement that clinical studies in which minor biochemical changes are expected must include sufficient subjects for them to be detected.

The results show that antileucoprotease is the major elastase inhibitor of those measured in bronchial secretions, accounting on average for over 80% of the total molar amount of known inhibitors. On the basis of the results presented here α_2 macroglobulin is unlikely to have a significant antielastase role in these secretions. The low levels found confirm our previous findings[16] and reflect the restriction of α_2 macroglobulin diffusion from serum because of its large molecular size.

Of greater interest are the results of neutrophil elastase inhibition. Again, we found wide variability between subjects (table 1). Furthermore, the degree of enzyme inactivation could not be explained by the measured inhibitors. On average about half of the enzyme inhibition appeared to be due to an unknown inhibitor or inhibitors. Although this supports the recent work of Tournier and colleagues,[17] its validity clearly depends on the accuracy of the methods. If the inhibitors are underestimated or the inhibition is overestimated by a sufficient degree, the discrepancy observed will clearly reflect technical problems alone.

The measurement of lung inhibitors, particularly α_1 proteinase inhibitor, may present problems when the protein in the secretion is physicochemically different from the protein standards.[15] The assay for α_2 macroglobulin is clearly reproducible and because of its low concentration even major inaccuracies in its measurement would be unlikely to effect the overall results. The α_1 proteinase inhibitor assay is also reproducible, but α_1 proteinase inhibitor can be overestimated in immune assays if a major proportion has undergone proteolytic cleavage.[9] Proteolytic cleavage, however, also inactivates α_1 proteinase inhibitor as an inhibitor. Thus if such a change had occurred it would lead to *overestimation* of its possible contribution to elastase inhibition as studied here.

The assay for antileucoprotease is the least precise, although even the 13% between batch coefficient of variation would not be sufficient to produce a major *underestimation* of its concentration. Such a change would have to occur regularly to explain the discrepancy between measured inhibitors and the inhibitory capacity. Inaccuracies in the estimation of inhibitor are therefore unlikely to account for the results, which have led to the suggestion that unknown inhibitors exist.

Several errors may arise in assessing enzyme inhibition with low molecular weight substrate such as that used here.[15] In particular, it is vital that the activity of the enzyme is determined by active site titration as a degree of inactivation occurs with purification. Failure to take this into account will result in an *overestimation* of enzyme inhibition. This is not the case in the studies reported here since it is our routine practice to report results in terms of active enzyme only.

Alpha$_2$ macroglobulin does not inactivate neutrophil elastase when small peptide substrates are used.[18] This could lead to an apparent discrepancy between the expected and observed inhibition of neutrophil elastase when the current methods are used. The contribution of α_2 macroglobulin is potentially very small, however, and would if anything lead to an *overestimation* of its contribution to the neutrophil elastase inhibition seen here.

On balance the results seem to indicate that elastase inhibitors other than those measured are present in considerable quantities in these samples. As a further confirmation, we prepared mixtures of active antileucoprotease, α_1 proteinase inhibitor, and α_2 macroglobulin in ratios similar to those in our bronchitic sputum. The inhibition of neutrophil elastase determined by the same assay is shown in figure 1 and confirms that the regression line passes close to molar equivalence (see under "Results").

The nature of the unidentified inhibitor or inhibitors is uncertain. Several low molecular weight inhibitors of elastase have been purified by different research groups.[1] The relationship between these inhibitors is not clear and may partly represent methodological problems of purification. Indeed, Keuppers and Bromke[19] were able to purify several different molecular weight inhibitors with immunological similarity from the same samples. It is thus possible that all of the inhibitors isolated by different groups may be fragments of the same protein. If this is the case, the purification that takes place before an antiserum is developed may result in immunological assays that do not quantify all forms of the parent protein equally. The net result could be an *underestimation* of antileucoprotease or its precursors and hence an *underestimation* of its contribution to the observed enzyme inhibition, and this could explain our results. Clearly further studies are required to clarify these possibilities. Indeed, whether or not antileucoprotease, as measured here, and the bronchial mucus proteinase inhibitor measured in a previous collaborative study[5] are the same protein can be determined only by careful comparison of samples from each research group.

In addition, recent studies have identified an

inhibitor that is functionally distinct from the mucus inhibitors.[20] This protein can inhibit both neutrophil and porcine pancreatic elastase but not trypsin or cathepsin G.[20] This inhibitor is, however, present only in low concentrations in sputum, providing about 10% of the potential inhibition produced by antileucoprotease.[20] This inhibitor alone is therefore unlikely to account for the discrepancy between inhibition and measured inhibitors observed here.

Corticosteroid treatment was shown to have three major effects. Firstly, the concentration of α_1 proteinase inhibitor in the secretions fell. This is consistent with a decrease in diffusion of this protein from serum into the secretions as a result of the anti-inflammatory effect of the drug. Secondly, there was a rise in the concentration of the locally produced protein antileucoprotease. The reasons are at present uncertain. It is, however, in keeping with the previously noticed rise in another "locally produced" proteinase inhibitor, α_1 antichymotrypsin.[8] The result could be due to increased production of antileucoprotease in the lung or a reduction in secretion volume due to the anti-inflammatory properties of corticosteroids. Further studies will be required to determine the exact mechanism. Thirdly, the neutrophil elastase inhibitory capacity rose during steroid treatment. This was not just the result of an increase in antileucoprotease concentration since the proportion of inhibition not accounted for by antileucoprotease and α_1 proteinase inhibitor remained unaltered. Whatever the mechanisms, the results suggest a beneficial change with an overall increase in the elastase inhibitory capacity of secretions.

Similar effects have also been seen on other enzyme-inhibitor systems in lung secretions. Corticosteroid treatment reduces cysteine proteinase activity[21] and leads to greater concentrations of a metalloproteinase inhibitor and collagenase inhibition in sputum.[22] Further studies of the true effect of corticosteroids on proteinase release and inhibitor function in lung secretions may provide valuable information on their role in protection and damage of lung tissues.

Long term corticosteroid treatment is unlikely to become a routine measure for the prevention of emphysema. Nevertheless, the results presented here suggest that therapeutic intervention can modify the antielastases in lung secretions and this is worthy of further study.

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