The immunological assessment of $\alpha_1$-antitrypsin with reference to its function in bronchial secretions

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Summary
1. The quantification of $\alpha_1$-antitrypsin ($\alpha_1$AT) by standard immunological techniques is altered by interaction of the protein with leucocyte elastase.
2. The results obtained for $\alpha_1$-antitrypsin-leucocyte elastase mixtures in the presence of a functional excess of the inhibitor were relatively accurate for the first 6 h. However, continued incubation for more than 24 h led to a major overestimation of the $\alpha_1$AT as the result of breakdown of the enzyme-inhibitor complex releasing a partially proteolysed form of the inhibitor.
3. In the presence of excess enzyme up to a twofold overestimation of $\alpha_1$AT occurred within 1 h and the degree of overestimation increased with time (up to threefold at 24 h). This was eventually associated with the presence of only a partially proteolysed form of $\alpha_1$AT (mol wt. $\approx$ 50 000).
4. Different results for each sample were obtained when different polyclonal antisera were used to quantify the $\alpha_1$AT.
5. Complete inactivation of $\alpha_1$AT by oxidation resulted in little change in the immunological quantification of the protein. However, further addition of $\text{H}_2\text{O}_2$ led to a progressive underestimation of the $\alpha_1$AT.
6. The effect of physicochemical alteration on the immunological quantification of $\alpha_1$AT by different antisera should be borne in mind for all studies assessing this protein in lung secretions.

Key words: antibody, $\alpha_1$-antitrypsin, leucocyte elastase, lung secretions, proteolysis.

Abbreviations: $\alpha_1$AT, $\alpha_1$-antitrypsin; 2DIEP, two-dimensional immunoelectrophoresis; LE, leucocyte elastase; RIEP, rocket immunoelectrophoresis; SDS, sodium dodecyl sulphate.

Introduction
Proteolytic enzymes have been implicated in the pathogenesis of several acute and chronic lung diseases including emphysema [1], the adult respiratory distress syndrome [2], bronchiectasis [3] and cystic fibrosis [4]. However, the secretions contain several enzyme inhibitors which protect the lung from enzyme induced damage. The exact mechanisms whereby the enzymes can overcome this protective inhibitor 'screen' are poorly understood but it has been suggested that a defect in the 'screen' as a result of the inhibitors may be important.

$\alpha_1$-Antitrypsin ($\alpha_1$-proteinase inhibitor) is a major enzyme inhibitor of lung secretions and has so far received most attention. Evidence has shown that this inhibitor can be present not only in its native (active) form but also as an enzyme-inhibitor complex [5], in a partially proteolysed form [6] and in an oxidized form [7]. All these changes to the native $\alpha_1$-antitrypsin ($\alpha_1$AT) are associated with loss of inhibitory function and would account for a reduction in its inhibitory capacity in the lung secretions, which may partly explain the susceptibility of some individuals to enzyme induced lung damage. However, the degree and therefore importance of any reduction in inhibitory capacity of the $\alpha_1$AT depends largely upon the accuracy of its measurement.

Lung $\alpha_1$AT is measured by conventional immunological techniques such as radial immunodiffusion [8] and rocket immunoelectrophoresis [9], and compared with standard solutions of
known $\alpha_1$AT concentration. The accuracy of these techniques (coefficient of variation $\approx 4\%$) is entirely dependent upon physicochemical and immunological identity of the test protein and the standard solution [10]. Therefore measurement of lung $\alpha_1$AT that has been altered as described above may give inaccurate results when compared with a standard solution of the native (unaltered) protein.

Direct evidence of inaccuracies in the quantification of lung $\alpha_1$AT is lacking but can be implied from our previous data [5]. It is known that both $\alpha_1$AT and albumin (proteins of almost identical size) are present in lung secretions largely as a result of simple diffusion from serum [11]. Thus their relative concentrations in the secretions and serum should be identical. However, our previous studies have shown that sputum $\alpha_1$AT is between 0.5 and 6 times the value expected in comparison with albumin [5]. In view of the accuracy of the immunological techniques employed, this suggests major over- and under-estimation of $\alpha_1$AT in some samples.

There is currently no information concerning the effect of proteolysis, oxidation and complexing with enzyme upon the immunological measurement of $\alpha_1$AT. The present study was designed to investigate this problem in vitro in order to determine what error (if any) might be expected in quantifying $\alpha_1$AT in lung secretions.

**Methods**

**Proteins**

Leucocyte elastase (LE) was purified from circulating human polymorphs as described previously [12] and was kindly donated by Dr A. J. Barrett (Strangeways Laboratories, Cambridge). The protein was dissolved in Tris/phosphate buffer (0.05 mol/l, pH 8.0) containing NaCl (0.2 mol/l) and Triton (0.1%). The concentration of LE was determined in a standard way from the absorbance at 280 nm (absorption coefficient = 9.85).

The enzyme activity was determined by active site titration, the method of Nakajima & Powers [13] being used with the chromogenic substrate N-succinyl trialanine $p$-nitroanilide. The elastase was found to retain 68% of the expected activity and this figure was used throughout the studies.

$\alpha_1$-Antitrypsin was purified from human plasma. The (NH$_4$)$_2$SO$_4$ precipitated fraction was subjected to chromatography on DEAE-Sephacel (Pharmacia) and eluted with an increasing salt gradient (0.05–0.5 mol/l Tris/HCl, pH 8.8). The fractions containing $\alpha_1$AT (identified immunologically with monospecific antisera) were then pooled and applied to a Sepharose concanavalin A affinity column (Pharmacia) and eluted with methylmannoside (0.05 mol/l in buffer, pH 6.0: 0.1 mol/l acetate, 1 mol/l NaCl, 1 mmol/l CaCl$_2$ and 1 mmol/l MgCl$_2$).

The remaining minor contaminant was identified immunologically as orosomucoid and this was removed by an immunoadsorbent column prepared from cyanogen bromide-activated Sepharose (Pharmacia) and the immunoglobulin fraction of a monospecific antiserum to orosomucoid (Seward Laboratories).

The remaining protein was kept in 0.05 mol/l Tris/HCl buffer (pH 8.8). The concentration was assessed by rocket immunoelectrophoresis [9] and compared with the British standard reference serum 74/520 (1700 mg/l).

Purity was assessed immunologically and the preparation was shown to produce only one peak on two-dimensional immunoelectrophoresis against anti-(whole human serum) (Seward Laboratories); in particular no cross-reactivity was shown with monospecific antiserum to the other serum proteinase inhibitors ($\alpha_2$-macroglobulin, $\alpha_1$-antichymotrypsin inter-$\alpha$-trypsin inhibitor, $\alpha_1$ esterase inhibitor and antithrombin III).

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis [14] gave two bands, the major one at a mol. wt. of 54 000 and a minor one at 50 000, suggesting the presence of some $\alpha_1$AT that had undergone limited proteolysis. The $\alpha_1$AT activity was determined from its ability to inhibit the characterized LE.

The assays were performed by adding increasing amounts of the $\alpha_1$AT to a fixed amount (4 $\mu$g) of LE in 0.5 ml of Tris/HCl buffer (0.2 mol/l, pH 8.8). The mixtures were incubated for 10 min (37°C), then 0.5 ml of the substrate (N-succinyl trialanine $p$-nitroanilide, 3 mg/ml) was added and incubation continued for a further 10 min. The absorbance was then recorded at 410 nm and compared with that of a blank of substrate alone.

A typical inhibition curve is shown in Fig. 1. The two proteins are known to interact on a 1:1 molar basis and the intercept should occur through the point of functional equivalence. Assuming molecular weights of 30 000 for LE [15] and 54 000 for $\alpha_1$AT [16], as well as only 68% activity of the enzyme, it is possible to determine the activity of the $\alpha_1$AT from the intercept. The $\alpha_1$AT quantity for the intercept obtained was 1.45 times higher than expected for fully active $\alpha_1$AT, suggesting 69.1% remaining inhibitory activity of the preparation.

For subsequent studies molar ratios of LE and $\alpha_1$AT were taken from the functional inhibition curve shown in Fig. 1. Mixtures functionally
Immunological quantification of α1-antitrypsin

Effect of oxidation

Hydrogen peroxide (3%, w/v) was obtained from Thornton and Ross (Huddersfield) and amounts from 5 to 50 μl were incubated with α1AT (17.4 μg of α1AT in 50 μl) for 30 min at 37°C. The total loss of inhibitory activity against LE over 10 min incubation was confirmed with the enzyme assay described above. The α1AT was simultaneously assessed by RIEP as described above and compared with a control sample of α1AT alone.

Antiseras used

Three monospecific antisera to α1AT were assessed. All were the immunoglobulin fraction of sheep antisera raised in the Immunodiagnostic Research Laboratory, University of Birmingham. Two of the antisera have been commercially available (Seward Laboratories).

Results

Effect of complexing and proteolysis

The interaction of α1AT and LE in the presence of excess inhibitor (LE/α1AT molar ratio <1:1) produced increasing enzyme-inhibitor complex (expressed as a proportion of total α1AT) seen on 2DIEP with increasing amounts of LE added (Fig. 2). The presence of these complexes was confirmed by SDS-polyacrylamide gel electrophoresis (data not shown).

The effect of this complexing upon the immunological assessment of α1AT is shown as part of Figs. 3 and 4. After 10 min incubation of enzyme with inhibitor there was a progressive diminution in the quantification of α1AT immunologically as the functional molar ratio of LE/α1AT increased and the proportion of enzyme-inhibitor complex increased. The average value at molar ratio 0.75 : 1 (LE: α1AT), when about 60% of the α1AT was present as enzyme-inhibitor complex, was 74.5% (SD ± 2.61; n = 6) of that obtained for α1AT alone. The value obtained at functional molar equivalence, 1 : 1 (LE/α1AT), when 69% of the α1AT was present as complex, was 89.7% (SD ± 5.7) of the control value.

However, this reduction in α1AT quantification was short-lived and by 6 h incubation the results obtained in inhibitor excess had returned to the control values (Fig. 4), although the α1AT value for functional equivalence (1 : 1 molar ratio) was now found to exceed the control value (= 130%). Over the subsequent 24-48 h the α1AT values obtained for these mixtures showed a continued

FIG. 1. Inhibition of leucocyte elastase by α1AT. The vertical axis indicates the remaining enzyme activity. Each point is the mean of five studies. The point of complete inhibition indicates functional molar equivalence. The molar interaction studies were determined by ratios of the quantities of α1AT and LE giving the point of functional equivalence.

Protein assessment

The immunological changes to α1AT were assessed by standard rocket immunoelectrophoresis (RIEP) as described by Laurell [9]. In brief, aliquots of the LE/α1AT reaction mixtures were placed in wells cut into a 1 mm thick agarose gel containing monospecific antisera to α1AT. The plates were electrophoresed overnight (3 V/cm), then dried and pressed. After this the plates were stained with 2% kenacid blue and the heights of the precipitation arcs were measured and compared with fresh aliquots of the α1AT used as a standard on each plate. A further control of α1AT incubated alone at 37°C for the same length of time as the LE/α1AT mixtures, and at the same concentrations as in the mixtures, was also included on each plate.

In some experiments the α1AT in the reaction mixtures was compared with α1AT alone by both RIEP and radial immunodiffusion as described by Mancini et al. [8].

The presence of α1AT/LE complexes was confirmed by standard SDS-polyacrylamide gel electrophoresis [13] and the proportion of complexed α1AT was assessed by two-dimensional immunoelectrophoresis (2DIEP) as described previously [5]. The presence of α1AT that had undergone limited proteolysis was assessed by SDS-polyacrylamide gel electrophoresis alone.

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FIG. 2. Two-dimensional immunoelectrophoretic plates of α1AT/LE mixtures showing increasing proportions of complex. Samples were run from right to left in plain agarose and then at right angles into agarose containing antibody to α1AT. Plates A–D are for increasing quantities of LE added to a fixed amount of α1AT. The free α1AT (1) and α1AT/LE complex (2) are indicated in plate A.

FIG. 3. Quantification of α1AT by RIEP 10 min after addition of LE. The vertical axis is the quantity expressed as a percentage of α1AT alone and the horizontal axis the functional LE/α1AT molar ratio. Each point is the mean of six experiments and the bar lines are ± 1 SD.

The results obtained for α1AT in the presence of excess enzyme (LE/α1AT molar ratio > 1:1) showed that although enzyme-inhibitor complexes were formed initially the proportion of α1AT present as complex was slightly less at 10 min [61.8% ± 4.4 for molar ratio 2:1 (LE/α1AT) and 55.9% ± 3.6 for 3:1; n = 3] than seen at functional molar equivalence, suggesting some early breakdown of the complex. This was associated with α1AT measurements nearer to the control value (Fig. 3).

However, with longer periods of incubation the enzyme-inhibitor complexes formed in the presence of excess enzyme showed more rapid
disappearance as seen on both 2DIEP and SDS-polyacrylamide gel electrophoresis than samples in inhibitor excess. These results are being reported in detail elsewhere but after 6 h incubation the concentration value obtained for α₁AT in the presence of excess enzyme had risen to about 150% of the control value. A continued rise in quantification was again seen over the next 24–48 h, reaching more than 300% of the control value (Fig. 4), and this was associated with no enzyme-inhibitor complexes seen on 2DIEP or SDS-polyacrylamide gel electrophoresis and only the presence of partially proteolysed α₁AT (mol. wt. ≈ 50,000) demonstrated by the latter technique.

Photographs of RIEP plates from one such experiment are shown in Fig. 5. The precipitation rockets for all samples are well formed and similar to those for the standard and control samples of α₁AT. Furthermore the results obtained by this technique were similar to those obtained by conventional radial immunodiffusion [8], suggesting that the immunological changes observed are not confined to immunoelectrophoresis (data not shown).

Effect of different antisera

Fig. 6 summarizes the results of four experiments measuring α₁AT after incubation with various amounts of LE for 1 h. The antisera all produced results similar to the control value in the presence of excess inhibitor. However, at molar equivalence, and particularly in enzyme excess, clear differences were seen. Further details are given in Table 1. One antiserum (Z820) gave results very close to the control value for all samples, whereas the others gave results up to 200% of the control value (Z567).

Photographs of the rocket plates are shown in Fig. 7 for the two antisera giving the results closest to and furthest from the control value. The rockets showing most change from the control value (antiserum Z567 functional equivalence and enzyme excess) also appear less distinct.

These differences between antisera were more pronounced after 24 h and are summarized in Fig. 8.

Effect of oxidation

The addition of H₂O₂ solution for 30 min resulted in complete loss of inhibitory function against LE, as shown by the method described previously, even when only 5 μL was added to 17.4 μg of α₁AT in 50 μL of buffer. Despite this, immunological changes were minimal until larger quantities of H₂O₂ were added (Fig. 9).

Discussion

α₁-Antitrypsin is frequently quantified in the lung secretions in order to understand its function and protective role in the pathogenesis of several acute and chronic lung diseases. This involves the use of
TABLE 1. Summary of some of the results for each antiserum

The average results for α1AT quantification are shown for various functional molar ratios of LE/α1AT. The results are expressed as the percentage of a control sample of the α1AT alone. Each column is for a different α1AT antiserum (laboratory codes Z820, Z567 and Z456). Figures in parentheses are ± 1 SD (n = 4).

<table>
<thead>
<tr>
<th>Antiserum .</th>
<th>Z820</th>
<th>Z567</th>
<th>Z456</th>
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</thead>
<tbody>
<tr>
<td>LE/α1AT molar ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 : 1</td>
<td>89.3</td>
<td>104.7</td>
<td>96.9</td>
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<tr>
<td>1.0</td>
<td>83.6</td>
<td>138.1</td>
<td>102.8</td>
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<tr>
<td>1.5 : 1</td>
<td>95.8</td>
<td>204.6</td>
<td>129.3</td>
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<tr>
<td>2.0 : 1</td>
<td>99.3</td>
<td>209.3</td>
<td>139.5</td>
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standard immunological techniques comparing the secretion with a known reference sample.

However, in the secretion α1AT is often exposed to many factors including enzymes and oxidants within the lung for various times before sampling. Furthermore the standard α1AT with which these samples are quantified is usually a reference serum or pure native protein. Unless the antibody used is directed against antigenic determinants on the α1AT molecule which are unaltered by such factors in the lung the result is likely to be inaccurate.

The present studies confirm that interaction of α1AT with enzymes can lead to major errors in estimating the protein immunologically. The interaction of α1AT and LE in the presence of excess α1AT leads to the formation of enzyme–inhibitor complexes demonstrable by both 2DIEP and SDS–polyacrylamide electrophoresis. This is associated with a rapid but small (up to 25%) underestimation of the α1AT immunologically. However, over the next 4–6 h the result obtained returned to the expected value even for the antiserum, which was thought to be least satisfactory (Fig. 4). Over the subsequent 24–48 h further assessment of the α1AT/LE mixtures resulted in more significant overestimation of the α1AT, with results up to twofold greater than expected (Fig. 4), and this was associated with a breakdown of the complex seen on 2DIEP.

The results obtained at molar equivalence and in the presence of enzyme excess when none of the α1AT is functionally active were different from those in inhibitor excess. The early results (up to 1 h incubation of enzyme and inhibitor) are associated with very little alteration in the immunological
Immunological quantification of α1-antitrypsin

FIG. 7. RIEP plates for 'best' (Z820) and 'worst' (Z567) antiserum. Standard control samples are shown (std). Two samples in inhibitor excess (left of arrows) and enzyme excess (right of arrows) are shown either side of the functional equivalent samples (indicated by the arrows). Samples of enzyme and inhibitor were incubated for 1 h before analysis.

FIG. 8. Results of 'best' (●, Z820) and 'worst' antiserum (○, Z567) are shown for the same mixtures measured after 1 and 24 h incubation.

measurement of α1AT even with the least satisfactory antiserum (Fig. 4). However, with the more rapid breakdown of enzyme–inhibitor complexes seen on 2DIEP and the appearance of more inactive α1AT that had undergone limited proteolysis (confirmed by SDS-polyacrylamide gel electrophoresis), major overestimation of the α1AT occurred and this increased from 1.5 times the control value after 6 h to almost threefold after 48 h.

Three different antisera were tested and the result obtained for each α1AT/LE interaction mixture was dependent upon which one was used (Table 1 and Fig. 6). One of the antisera (Z820) was much better than the other two for assessing the altered α1AT (i.e. the results obtained were closer to the control value). This antiserum gave the results close to the control value for all α1AT/LE mixtures over the first 6 h of incubation, though it still overestimated the α1AT in the
mixtures of 24 h by 1.3-1.8 times the control value, depending on the molar ratio of the α1AT/LE mixtures (Fig. 8).

The results suggest that the native, complexed and partially proteolysed forms of α1AT all react differently in immunological assays. When native α1AT is used as a standard the value for a secretion α1AT will be slightly low for recently formed complex and high for the partially proteolysed form. The actual result obtained will vary depending on the relative proportions of these components, which in itself will depend upon how recently the complexes have been formed (i.e. how much degradation of the complex to the partially proteolysed form of α1AT has occurred).

In general the results obtained in the presence of excess α1AT (where some functional inhibitory activity would remain) tend to be relatively accurate for up to 6 h after interaction with enzyme. On the other hand, samples in excess enzyme (where no functional inhibitory activity would remain) tend to give up to a 1.5-fold over-estimation of the α1AT concentration within 6 h.

The results obtained for different antisera are of major importance. All gave a different result for the same sample and often the immunoprecipitate was indistinguishable from the native protein. This may not be of importance when assessing α1AT function in lung secretions where excess enzyme is present since no inhibitory activity would remain. However, it is of major importance when comparing α1AT concentrations in lung secretions from different patients where the proportions present as native, complexed and proteolysed α1AT vary, and when assessing the α1AT function in secretions where inhibitory activity persists. The results from these latter studies will not only be dependent upon the varying proportions of α1AT inactive due to enzyme-inhibitor complexes or proteolysis, but also the source and batch of antisera used. Furthermore, even when relatively accurate and well-characterized antisera are used, such as the one studied here (Z820), the results are likely to be accurate only if the α1AT has been present in the lung and exposed to enzyme for less than 6 h. This in itself may depend upon whether bronchial or alveolar secretions are studied or whether they are collected during the day or after an overnight sleep.

On the other hand total inactivation of α1AT by an oxidant (H2O2) produced very little immunological change until it was present in quantities well exceeding the damaging threshold. This is in contradiction to recent studies with cigarette smoke solution, which results in significant immunological changes to α1AT even when it is only partially inactivated [17]. The current results would suggest that the immunological changes due to cigarette smoke are independent of the oxidant effect which inactivates the α1AT [18].

The relevance of such immunological changes in attempts to understand the pathogenesis of diseases such as emphysema may seem uncertain. However, although it is generally accepted that emphysema develops in subjects who have functional α1AT in the alveoli decreased by about 60% [19], factors which produce such a change are uncertain and thus possible preventive measures cannot be considered (unless subjects who smoke can be persuaded otherwise). The only study assessing lung α1AT function in smokers is that of Gadek et al. [20], who found only a 40% reduction in lung α1AT function. This was thought to be the result of oxidation of the α1AT, and the present study with the antisera used here suggests that if the reduction in inhibitory activity is due to oxidation the α1AT measurement and hence the degree of inactivation is probably correct.

Overcoming such problems is likely to be necessary if the major determinants of proteolytic lung diseases are to be identified. There remain several alternative approaches. Firstly, knowing the serum quantities of α1AT and albumin and the secretion value for albumin it may be possible to assume the expected value of α1AT in the secretion, although this is dependent upon similar catabolism of both proteins in the lung. Secondly, a more satisfactory solution will be to measure the α1AT directly with antiserum that detects only antigenic determinants that remain unaltered in the secretions. This latter approach may require the development of monoclonal antiserum and until then all results from different centres may not be comparable.

In conclusion antigenic alteration of α1AT in the lung secretions may lead to major errors in its estimation. This indicates that without well-characterized antiserum the results of quantitative
and functional assessment of lung $\alpha_1$AT and comparison between laboratories should be viewed with caution.

Acknowledgments

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References


