

Comparison of concentrations of two proteinase inhibitors, porcine pancreatic elastase inhibitory capacity, and cell profiles in sequential bronchoalveolar lavage samples

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ABSTRACT Bronchoalveolar lavage is used to obtain cells and proteins from the lower respiratory tract for diagnosis and research. Uncertainty exists about which site in the lung is sampled by the lavage fluid and what effect different lavage volumes have on recovery of the constituents of lavage fluid. Dilution of alveolar lining fluid by lavage fluid is variable and results are usually expressed as protein ratios to surmount this problem. We have compared cell profiles and the concentrations of two proteinase inhibitors—the low molecular weight bronchial protease inhibitor antileucoprotease and α_1 proteinase inhibitor, together with α_1 proteinase inhibitor function and its relationship to the cell profile in sequential bronchoalveolar lavage fluid samples from patients undergoing bronchoscopy. There was no difference in total or differential cell counts or albumin or α_1 proteinase inhibitor concentrations between the first and second halves of the lavage. Both the concentration of antileucoprotease and the ratio of antileucoprotease to albumin were, however, lower in the second half of the lavage ($2p < 0.01$ and $2p < 0.05$ respectively). There was no difference in the function of α_1 proteinase inhibitor (assessed by inhibition of porcine pancreatic elastase—PPE) between aliquots (0.28 mole PPE inhibited/mol α_1 proteinase inhibitor; range 0–1.19 for the first half and 0.37 mol PPE inhibited/mol α_1 proteinase inhibitor; range 0.10–0.80 for the second half). About 60–70% of α_1 proteinase inhibitor in each half of the lavage fluid was inactive as an inhibitor. The function of α_1 proteinase inhibitor did not differ between bronchitic smokers and ex-smokers. Alpha₁ proteinase inhibitor function was not related to the number of total white cells, macrophages, or neutrophils in the lavage fluid. Contamination of lavage by red blood cells was found to alter the concentration of α_1 proteinase inhibitor but not its function when aliquots with and without erythrocytes were compared. These results show that the only difference between the two halves of these lavage samples is in the amount of antileucoprotease present, suggesting that more proximal secretions are being harvested early in the lavage procedure. Much of the α_1 proteinase inhibitor present in the samples is functionally inactive, but this is not clearly related to any particular cell type or to smoking habits, and does not differ between different stages of the lavage procedure. Finally, the presence of erythrocytes probably does affect α_1 proteinase inhibitor concentration and such samples should be excluded from analysis.

Investigation of proteolytic enzymes and anti-proteases in lung secretions has provided valuable insight into the pathogenesis of such diverse diseases

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as emphysema, cryptogenic fibrosing alveolitis, sarcoidosis, and adult respiratory distress syndrome.^{1,2} The use of bronchoalveolar lavage has enabled proteins and cells to be sampled directly from the bronchoalveolar region of the lung and this overcomes the problem of contamination by saliva and nasopharyngeal secretions that may affect results in

sputum. Much has been learned, but unfortunately various problems with both the technique itself and the expression of the results obtained means that caution must be exercised in interpretation of the information generated, comparing results obtained by different research groups, and considering the place of the technique as a diagnostic and research tool.

Several factors may influence the results. Firstly, the method of bronchoscopy and bronchoalveolar lavage differs between laboratories and individual endoscopists, producing results that are not comparable.

Secondly, the amount of lavage fluid returned varies greatly. Local anaesthetic and the lavage fluid itself cause variable dilution of the fluid lining the bronchi and alveoli. Protein measurements are usually expressed as ratios to surmount the uncertainty introduced by dilutional factors, but other methods have been used in an attempt to standardise measurements. A particular protein may be related to the concentration of total protein, albumin, monomeric immunoglobulin A (IgA), secretory IgA, or potassium ion.³ Some workers have also added methylene blue to the fluid used for lavage and measured its dilution in the fluid recovered, to quantify the volume of secretion returned.⁴ Standardising against the albumin concentration in the lavage fluid, however, is the technique used most frequently.

Thirdly, there is uncertainty about the area of the lung being sampled. Since the bronchoscope cannot usually be passed beyond the fourth generation bronchi, clearly many orders of bronchi and peripheral bronchi are lavaged during the procedure, although some of the fluid instilled is assumed to reach the alveoli and to be retrieved. In addition, it has been suggested that peripheral bronchi are sampled during the initial part of a lavage and alveolar cells and proteins are recovered from the subsequent fluid instilled.⁵ Since neutrophils may predominate in the more proximal airways and macrophages are the major cell type in the alveoli,¹ the release of proteolytic enzymes and oxidants from the different cell populations and their interaction with proteinase inhibitors could lead to variation in results of functional inhibitor studies between the early and late fractions of the lavage fluid.

Finally, contamination of secretions obtained by lavage may occur if the bronchial mucosa is traumatised during the procedure. The presence of red blood cells in the lavage fluid is often thought to indicate that serum proteins have entered the bronchial tree as a result of mucosal damage¹ and such samples are frequently discarded, although it is not clear whether this is necessary.

The present study had two objectives. The first was to clarify whether separate sites of the bronchial tree

are sampled by the early and late phases of the lavage procedure. The concentrations of α_1 proteinase inhibitor and low molecular weight bronchial protease inhibitor, and also albumin concentration, were compared in sequential aliquots of lavage fluid from patients undergoing diagnostic fibreoptic bronchoscopy. Secondly, we wished to determine whether the presence of either red blood cells (suggestive of trauma with consequent leakage of serum) or of white blood cells (which produce oxidants and enzymes capable of inactivating α_1 proteinase inhibitor)^{6,7} was related to α_1 proteinase inhibitor function.

Methods

We studied a total of 19 patients (mean age 61.8 (SD 10.8) years); eight were women. All were undergoing fibreoptic bronchoscopy for the investigation of chest symptoms or radiographic abnormalities. Six patients had evidence of interstitial lung disease and showed a restrictive defect on pulmonary function testing with interstitial changes on the chest radiograph. Four of them had cryptogenic fibrosing alveolitis and two had sarcoidosis. Two patients presented with haemoptysis for which no cause was found, and the remaining 11 patients had carcinoma of the bronchus. The latter two groups had similar lung function (mean FEV₁/FVC ratio 69.6% (17.3%)) and they are considered together.

Fibreoptic bronchoscopy and bronchoalveolar lavage were performed by the same person (RAS). The patients received intramuscular atropine (600 μ g) and intravenous diazepam (5–10 mg) as premedication and the bronchoscope was passed into the lower respiratory tract with the aid of lignocaine as a local anaesthetic. It was wedged in the middle lobe or lingula (and, in the case of the patients with tumours, in the radiologically or macroscopically normal lung) and three 20 ml boluses of warmed sterile physiological saline were instilled and gently aspirated in turn. The volume returned formed the first aliquot of lavage fluid. The procedure was repeated to obtain the second aliquot.

Each aliquot was then processed in a similar way. The lavage fluid was filtered through a double layer of gauze to remove mucus and the volume of fluid was noted. One millilitre was removed for a total cell count, a Coulter counter being used. The remaining fluid was centrifuged at 3000 rev/min for five minutes and the cell pellet was resuspended in a drop of the supernatant. The cell suspension was placed on an albuminised slide, fixed in 95% alcohol and stained with Papanicolaou's stain. One slide was air dried and stained with Giemsa. At least 200 cells were then counted to obtain a differential cell count. The lavage

fluid remaining after removal of the cells was concentrated in an Amicon pressure filtration system, with a YCO5 membrane (molecular weight cut off 500 daltons). The concentration factor was recorded.

The concentrations of α_1 proteinase inhibitor and albumin in the lavage fluid were determined by rocket immunoelectrophoresis. The antibody used to quantitate α_1 proteinase inhibitor was known to give accurate results even for protein that had recently interacted with enzyme.⁸ The ratio of α_1 proteinase inhibitor to albumin was calculated for each sample. A portion of each aliquot of lavage fluid was coded, frozen in dry ice, and flown to Leiden for the measurement of the low molecular weight inhibitor (antileucoprotease) by an enzyme-linked immunosorbent assay.⁹ The ratios of antileucoprotease to albumin were also calculated for each pair of aliquots.

The method of determining α_1 proteinase inhibitor function has been described.¹⁰ In summary, increasing amounts of lavage fluid were preincubated with a fixed amount of porcine pancreatic elastase of known activity¹⁰ obtained from PL Biochemicals, Milwaukee, Wisconsin. After further incubation with the substrate succinyl-trialanyl-paranitroanilide the residual enzyme activity was determined spectrophotometrically and expressed as a percentage of the activity of porcine pancreatic elastase and substrate alone. The amount of lavage fluid necessary to inhibit the enzyme totally (that is, to give zero absorbance) was determined by interpolation by the method of least squares and expressed as micrograms of porcine pancreatic elastase inhibited per millilitre of lavage fluid, after correction for concentration (between batch coefficient of variation = 5.6%).

The amount of active α_1 proteinase inhibitor in 1 ml of unconcentrated lavage fluid was determined by calculating the number of moles of porcine pancreatic elastase inhibited by 1 ml concentrated lavage fluid, on the assumption of a 1:1 molar interaction between porcine pancreatic elastase and α_1 proteinase inhibitor,¹¹ and corrected with the concentration factor. This was compared to the total amount of α_1 proteinase inhibitor in the unconcentrated lavage fluid measured immunologically, and the amount of inactive α_1 proteinase inhibitor in each sample equalled the difference between the two values.

Wilcoxon's rank sum test for paired samples (two tailed) was used to assess any difference between the two aliquots, and least squares analysis (two tailed) was used to determine the relationship between α_1 proteinase inhibitor and the cell counts.

Results

The average volume of returned lavage fluid was 27.7 (SD 8.8) ml for the first aliquot but was greater

($2p < 0.02$) for the second aliquot (32.8 (12.5) ml). There was no difference in recovery of fluid for either aliquot when patients with chronic bronchitis were compared with those with interstitial lung disease.

The total white cell yield did not differ between the first and the second aliquots (medians 13.0 and 12.0×10^6 cells; ranges 2.0–34.2 and 3.0–37.5 $\times 10^6$). The differential cell count was also similar for the two aliquots of lavage fluid (fig 1).

The protein concentrations and the α_1 proteinase inhibitor (α_1 PI):albumin and antileucoprotease (ALP):albumin ratios are shown in the table. There was no difference in α_1 PI or albumin concentrations or in α_1 PI:albumin ratio between the first and the second aliquots of lavage fluid. In contrast, however, the ALP concentration in the second aliquot was significantly lower in 15 of the patients ($2p < 0.01$) and the ALP:albumin ratio was significantly lower in 12 ($2p < 0.05$).

Thirteen of the 19 patients had red blood cells in the first aliquot, the average number of cells being $0.02 (0.03) \times 10^9$ /ml. The concentrations of α_1 PI, ALP, and albumin, as well as the α_1 PI and ALP concentrations related to albumin for these samples, are shown in the table and compared with the results in the remaining six patients. The α_1 PI concentration, α_1 PI:albumin ratio and ALP:albumin ratio were lower in the aliquots without red blood cells ($2p < 0.05$).

The ability of lavage fluid to inhibit porcine pancreatic elastase (PPE) was the same for the two aliquots, but showed wide variation. The median value

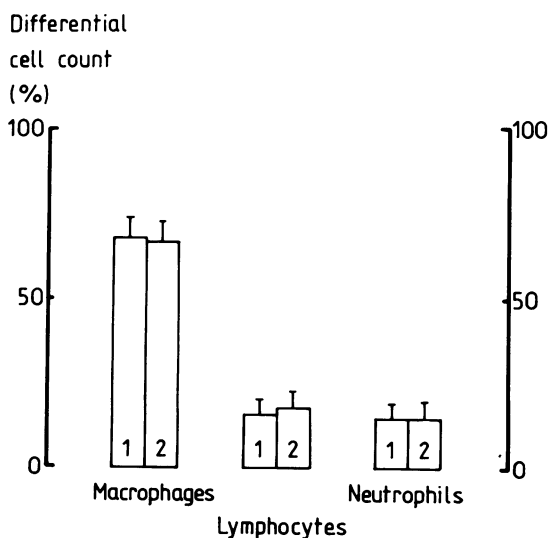


Fig 1 Differential cell counts (means and standard errors) for the first (1) and second (2) aliquots.

Protein concentrations and ratios for the first and second aliquots and for those patients with and without red blood cells in the first aliquot (medians with ranges in parentheses)

	α_1 PI ($\mu\text{g/ml}$)	ALP (ng/ml)	Albumin ($\mu\text{g/ml}$)	α_1 PI/Alb ($\mu\text{g/mg}$)	ALP/Alb (ng/ μg)
1st aliquot n = 19	2.4 (0.1–17.9)	142.8 (9.1–4625.8)	31 (12–205)	87.3 (7.1–358)	5.6 (0.41–121.7)
2nd aliquot n = 19	3.3 (0.7–18.3)	78.6* (6.4–1806.7)	38 (14–153)	100.0 (5.0–264)	3.4** (0.14–41.1)
RBC present n = 13	2.8 (0.6–17.9)	142.8 (41.9–4625.8)	27 (12–205)	125.9 (21.6–358.3)	6.0 (0.7–121.7)
RBC absent n = 6	1.5** (0.1–7.3)	120.4 (9.1–437.0)	42 (14–191)	30.6** (7.1–102.8)	2.37** (0.4–5.8)

*2p < 0.01; **2p < 0.05.

RBC—red blood cells; α_1 PI— α_1 proteinase inhibitor; ALP—antileucoprotease; Alb—albumin.

for inhibition in the first aliquot was 0.67 (range 0–1.55) μg PPE/ml lavage fluid and 0.63 (range 0.06–2.80) μg PPE/ml in the second aliquot. In molar terms (median values and ranges), the first aliquot inhibited 0.28 (0–1.19) mol PPE/mol α_1 PI and the second inhibited 0.37 (0.10–0.80) mole PPE/mol α_1 PI. Much of the α_1 PI present was inactive, but the proportion of inactive inhibitor was similar in the two halves of the lavage fluid (median 72.0% (range 0–100%) in the first aliquot and 63% (18.9–89.5%) in the second aliquot). Moreover, α_1 PI function did not differ (2p > 0.1) between the seven bronchitic smokers and the four ex-smokers (median 0.28 (range 0.06–1.19) mol PPE inhibited/mol α_1 PI; and 0.40 (0.16–0.51) mol PPE inhibited/mol α_1 PI respectively).

The PPE inhibitory capacity, the total amount of α_1 PI, and the amount of inactive α_1 PI were not related to the number of neutrophils or macrophages

present in the lavage fluid, but there was a significant relationship between PPE inhibitory capacity (2p < 0.01), total α_1 PI (2p < 0.001), and inactive α_1 PI (2p < 0.001) and the lymphocyte count. Figure 2 summarises the data for the total amount of α_1 PI and the lymphocyte counts in all 38 aliquots.

There was no difference in PPE inhibitory capacity (median values and ranges) in those samples containing red blood cells (0.52 (0.02–1.55) μg PPE/ml lavage fluid and in those without red blood cells (0.11 (0–2.06) μg PPE/ml). In addition, the proportions of α_1 PI that was inactive were similar, being 68.3% (35.1–89.5%) and 85.0% (0–100%) in samples with and without red blood cells.

Discussion

Bronchoalveolar lavage is a procedure that is relatively easy to perform, and because it is well tolerated by the patient it may be used to assess disease progression and the effect of treatment by repeating lavages over a period of time. It can also be used to investigate the protein and cell content of the lower respiratory tract in normal individuals. The method of collection may, however, influence the results obtained, and these must therefore be interpreted with caution. The purpose of this study was to compare the first and second halves of the fluid collected during bronchoalveolar lavage—firstly, to determine whether different aliquots of the lavage fluid come from different sites within the lung and, secondly to investigate whether the presence of erythrocytes (suggesting that blood may have leaked into the airway) alters protein concentration and function.

Total and differential cell counts

The total white cell yield was similar to that reported by Davis *et al* for healthy smokers and non-smokers.¹² We were unable to show a difference in total cell count between smokers and ex-smokers with bronchitis, but this may merely reflect the presence of

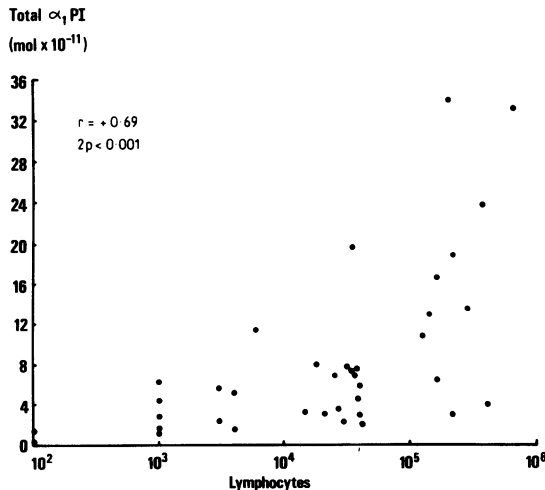


Fig 2 Relationship between total α_1 proteinase inhibitor (α_1 PI) and lymphocyte count per millilitre of bronchoalveolar lavage fluid for all 38 aliquots.

established airways disease in both groups. The differential cell counts remained the same throughout the lavage, confirming the results of Weinberger *et al.*¹³

α_1 proteinase inhibitor and albumin concentrations

We were unable to find a difference in the concentration of either α_1 proteinase inhibitor or albumin between the two halves of the lavage fluid, a result that is at variance with the findings of Davis *et al.*¹² and Merrill *et al.*⁵ The latter group showed that in normal subjects the concentrations of both total protein and albumin fell with successive aliquots but that the albumin:total protein ratio remained constant, suggesting that dilution was responsible for the observed reduction in concentration. In the present study no difference in α_1 proteinase inhibitor or albumin concentration or in α_1 proteinase inhibitor:albumin ratio has been shown between the aliquots, suggesting that the dilution that has occurred has been equal in the two halves of the lavage and is perhaps due to greater quantities of protein in the secretions of patients than in those of healthy subjects. Although the present study includes a mixture of patient groups, each patient acted as his or her own control. No difference was seen in the 13 patients with obstructive lung disease. However, it remains possible that differences could be found in larger groups of normal subjects or those with interstitial lung disease.

Antileucoprotease concentration

In contrast, the concentration of antileucoprotease decreased during sequential lavages in our patients. This was not due to dilution of secretion as the other protein concentrations did not alter and the antileucoprotease:albumin ratio also fell. Although it has been detected in peripheral airways by immunohistochemical techniques¹⁴ this inhibitor is thought to be located predominantly in the upper part of the respiratory tract rather than in the alveoli.¹⁵ Our results show that it is present in greater quantities relative to albumin in the first half of the lavage fluid. This might be predicted as it is thought to be associated with mucus.¹⁶ Most of our patients had cough with excess sputum production and hence greater quantities of bronchial mucus. The mucus would theoretically be harvested early in the lavage procedure and subsequent lavage would then contain relatively less mucus and more alveolar secretions, resulting in a reduction of antileucoprotease. This study provides the first evidence that secretions obtained from different regions of the lung by bronchoalveolar lavage may be biochemically different. Nevertheless, antileucoprotease could still

be measured in samples recovered later from more peripheral regions of the lung, confirming the findings of previous workers in normal subjects.¹⁷

The function of α_1 proteinase inhibitor and the lack of relationship to cell profile in these samples is of interest. Both macrophages and neutrophils produce oxygen radicals⁶ and contain enzymes capable of inactivating α_1 proteinase inhibitor.⁷ It has been proposed that these cells may be important mediators of damage to α_1 proteinase inhibitor *in vivo*.^{6,7} Despite this possibility, there was no relationship between these cells and the amount of inactive or total α_1 proteinase inhibitor present or the PPE inhibitory capacity.

Lymphocytes and α_1 proteinase inhibitor

The lymphocyte count was, however, positively correlated with the amount of inactive and total α_1 PI present, and also with the PPE inhibitory capacity, expressed as μ g PPE inhibited per ml of lavage fluid. This association was loose, although statistically significant, and its relevance therefore remains uncertain. Lymphocytes have a protease present on their cell membrane and α_1 proteinase inhibitor has been shown to bind to this enzyme, modulating cell function.¹⁸ This could explain the relationship observed between total and inactive α_1 PI and the number of lymphocytes present in lavage fluid. Clearly further studies will be necessary to establish the validity of the lymphocyte- α_1 proteinase inhibitor relationship and, if it is confirmed, whether it represents cause and effect.

α_1 proteinase inhibitor function

A large proportion of α_1 proteinase inhibitor appears to be non-functional in bronchoalveolar lavage fluid from these patients (confirming the study of Boudier *et al.*¹⁹) and the proportion of inactive inhibitor did not differ between aliquots. Carp *et al.* reported that lavage fluid from smokers was 40% less active in its ability to inhibit enzyme than that recovered from non-smokers,²⁰ but failed to indicate the degree of activity of α_1 PI in non-smokers' lavage fluid. Those results are, however, still at variance with those of the present study, which shows that α_1 PI function was no different in lavage fluid from smokers and from non-smokers, although levels were reduced in both. Previous studies in normal subjects^{19,21} and bronchitic patients²² have also failed to show a difference between smokers and non-smokers. Perhaps the discrepancies are technical, relating both to the use of well characterised enzyme (as with the porcine pancreatic elastase used in the current study), and to inaccuracies in quantitation of lung α_1 proteinase inhibitor.⁸ Without collaboration between laboratories, however, this problem may not be resolved.

Presence of erythrocytes

The concentration of α_1 proteinase inhibitor was greater in lavage samples with red blood cells present, and the α_1 proteinase inhibitor:albumin and antileucoprotease:albumin ratios were also greater where red blood cells were present ($2p < 0.05$). The antileucoprotease and albumin concentrations alone were similar for the two groups. Possibly these findings relate to changes in concentration as a result of leakage of blood due to bronchoscopic trauma. Both α_1 proteinase inhibitor and albumin are major components of serum but antileucoprotease is present only in small amounts in blood.⁹ Contamination of lavage fluid by serum should therefore theoretically increase the concentration of α_1 proteinase inhibitor and albumin, reduce the antileucoprotease:albumin ratio, and have no effect on the α_1 proteinase inhibitor:albumin ratio. Clearly this is not the case. The result may, however, be an artefact due to the patients studied. Most of the patients with interstitial lung disease had red blood cells in their first aliquot of lavage fluid and higher protein concentrations. When these six patients were excluded from analysis, there were eight bronchitic subjects with red blood cells in the first aliquot and five with none. Although the numbers were reduced, the only remaining difference between the groups was an increase in the antileucoprotease:albumin ratio in those aliquots with red blood cells, despite the fact that the concentrations of antileucoprotease and albumin were not significantly different. The result may be due to the cumulative effect of small changes or may be statistical artefact. Nevertheless, whether this is a real change or not, the results suggest that it would be safer to discard any samples contaminated with red blood cells.

A further technical problem is the conventional use of ultrafiltration to concentrate lavage proteins. The albumin concentrations in the samples containing red blood cells were lower than could be expected for the potential leakage of serum. This suggests a degree of protein loss and perhaps it would be preferable to study unconcentrated lavage fluid in future.

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