A comparison of albumin and urea as reference markers in bronchoalveolar lavage fluid from patients with interstitial lung disease


ABSTRACT: Lavage fluids were investigated for 67 subjects in 6 groups: 12 with active sarcoidosis, 8 with inactive sarcoidosis, 17 with pigeon breeder’s disease, 10 asymptomatic pigeon breeders, 12 with idiopathic pulmonary fibrosis (IPF) and 8 normal subjects. Albumin and urea per ml of bronchoalveolar lavage fluid (BALF) were determined for each subject together with percentage return of fluid (BAL%). Novel assay systems were employed to measure urea and albumin and these were compared with existing analytical techniques. When compared with the control group, we found that urea per ml of BALF was not statistically different for all other groups, except those with pigeon breeder’s disease who had significantly raised levels. For albumin, however, three groups had significantly higher levels than the controls, namely those with active sarcoidosis, pigeon breeder’s disease and IPF. BAL% return showed no significant differences for any group when compared with the controls. We conclude that since albumin is significantly raised in most patients with interstitial lung disease it does not represent a suitable marker for the quantitation of reactive proteins in BALF. Urea shows much less variability between groups than does albumin, and hence in the absence of a proven alternative represents the most reliable estimate available of epithelial lining fluid dilution during the lavage procedure, providing dwell time is kept to a minimum.

There is an urgent need to establish a suitable marker in bronchoalveolar lavage fluid (BALF) which will allow accurate comparisons of recovered epithelial lining fluid (ELF) in various pulmonary disorders. This need is magnified by the differing techniques of lavage between centres which makes inter-centre comparisons difficult.

One widely used method is to present data in relation to the concentration of albumin or total protein [1, 2]. The rationale is that such a representation negates the problem of ELF dilution in BALF and therefore allows direct comparison of results. However, since BAL involves dilution of ELF to a variable degree, the initial concentration of albumin and total protein in ELF is not known. Conclusions therefore cannot be made regarding the original concentration of cells or secretary products in vivo in the ELF. In addition, by virtue of their size, these compounds may not readily diffuse across the capillary membrane into the alveolar space; moreover since certain disease states can alter the permeability of these membranes, such components may enter the ELF more readily in these circumstances and so increase protein concentration in situ [3].

The use of methylene blue added to the lavage fluid to determine ELF volume by its dilution in the aspirated BALF has also been proposed [4]. More recently Rennard et al. [5] have proposed the use of urea for the estimation of ELF dilution in BALF. It is considered that because of its low molecular weight and high rate of membrane diffusion, urea concentrations in ELF and plasma would be in equilibrium. The amount of ELF in BAL could therefore be determined by comparing serum urea with levels in BALF. However, it has been shown that urea can diffuse from the plasma into the BALF during the BAL procedure thereby leading to an overestimation of ELF volume [6–9]. Moreover, Weinberg et al. [10] have suggested that diffuse lung disease decreases the percentage of BAL% return, an occurrence which some believe would increase accumulation of urea in BALF from such patients [8].

The aim of this study was to investigate the assumption that both albumin and urea would be increased in the BALF of patients with diffuse lung disease compared with normal controls and other disease groups.
Materials and methods

Subject groups

Lavage fluids from 67 subjects were investigated. None of these were taking inhaled or systemic steroids or were suffering from an acute respiratory disease at the time of investigation. These were:

a) 8 male patients referred with non-specific respiratory symptoms. All investigations including chest radiograph and bronchoscopy were normal. Three were current smokers.

b) 12 patients with biopsy proven sarcoidosis, all considered to have active disease on the basis of raised serum angiotensin converting enzyme (SACE) and/or a high intensity lymphocytic alveolitis (greater than 28% T-lymphocytes) [11]. Two were current smokers.

c) 8 patients with biopsy proven sarcoidosis without elevated SACE or a lymphocytic alveolitis considered to have inactive disease. None were smokers.

d) 17 pigeon breeders who gave a history of at least three episodes of extrinsic allergic alveolitis in the six months prior to lavage. All gave a positive response to inhalation challenge with nebulized pigeon serum at 1/10 to 1/100 dilution, and all had circulating precipitating antibodies to pigeon antigens. Three were current smokers and five were ex-smokers.

e) 10 pigeon breeders with no history of extrinsic allergic alveolitis and with a normal chest radiograph, no functional lung impairment and who gave a negative reaction to inhalation challenge with pigeon serum. Four were current smokers.

f) 12 patients with biopsy proven idiopathic pulmonary fibrosis (IPF). Five were current smokers.

All patients gave informed written consent and the study was approved by the local Ethical Committee.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed using 180 ml of bicarbonate buffered saline pH 7.4 at 37°C. Percentage return of lavage fluid (BAL%) was determined for each subject. The mean dwell time was 3.2±0.4 min and lavages exceeding 4 min were excluded in order to reduce passive diffusion of urea into the BALF [8]. The right middle lobe was the lavage site chosen for each subject. The mean dwell time was 3.2±0.4 min and lavages exceeding 4 min were excluded in order to reduce passive diffusion of urea into the BALF [8]. The right middle lobe was the lavage site chosen for each subject. The mean dwell time was 3.2±0.4 min and lavages exceeding 4 min were excluded in order to reduce passive diffusion of urea into the BALF [8]. The right middle lobe was the lavage site chosen for each subject. The mean dwell time was 3.2±0.4 min and lavages exceeding 4 min were excluded in order to reduce passive diffusion of urea into the BALF [8]. The right middle lobe was the lavage site chosen for each subject. The mean dwell time was 3.2±0.4 min and lavages exceeding 4 min were excluded in order to reduce passive diffusion of urea into the BALF [8].

Urea assay

Urea estimation was performed using a modification of a commercially available assay system (Sigma Diagnostics, Urea Nitrogen, Sigma Chemical Company). The normal procedure was modified to enable the assay to be performed in microtitre plates and the resulting colour measured using a Titertek Multiskan MC plate reader at 620 nm.

100 μl of test or a 1/100 dilution of standard were incubated with 200 μl urease for 20 min at room temperature (RT) in a microtite plate. 50 μl of the contents of each well were then transferred to another plate and 100 μl of phenol nitroprusside and 100 μl of alkaline hypochlorite were added. This plate was incubated for a further 30 min at RT before reading.

Eighteen known standards in the range 0–150 μg·ml⁻¹ were assayed using both the modified microplate method and the method described in the assay kit. Results from the two methods were compared using the techniques described by Bland and Altman [13].

Albumin assay

A sandwich double antibody assay was employed using microtitre plates. Each well on the plate (Nunc Immuno-plate F) was sensitized with “capture” antibody (50 μl of 1/500 sheep anti human serum albumin, HSA, Sigma, Poole U.K.) plus 150 μl of 0.067 M carbonate/bicarbonate buffer (pH 9.5) 4°C/16 hr.

Serial fourfold dilutions of lavage fluid from 1/25 to 1/25,600 were made up in phosphate buffered saline (pH 7.2) with sodium azide (0.2 g·l⁻¹) Tween 20 (0.5%) and bovine serum albumin (1 g·l⁻¹) - PBSNTB. 200 μl of each dilution was incubated per sensitized well (35 min at RT). Bound albumin was detected using rabbit anti HSA (200 μl of 1/1000 in PBSNTB, 35 min at RT) followed by alkaline phosphatase conjugated sheep anti rabbit IgG (200 μl of 1/100 in PBSNTB, 35 mins at RT).

Enzyme function was determined using pnitro phenyl phosphatase (Sigma 104, 200 μl at 1 g·l⁻¹ in 10% diethanolamine pH 9.8) and the reaction stopped with 50 μl of 5N sodium hydroxide. Released p-nitrophenol was determined at 405 nm. All inter-reagent washings were PBSNTB x 3.

Each plate had a series of standard BALF dilutions incorporated and the results of each individual BALF (in quadruplicate) determined from the standard plot.

Albumin in the standard BALF was determined similarly in comparison with human serum albumin (Sigma).

Statistical Methods

The six groups of subjects were compared by separate analyses of variance for each of the three measures: urea concentration, albumin concentration and BAL%. Since there was clear evidence of positive skewness in the distributions of these measures, with a corresponding increase in variance for the groups with higher mean values, the analyses of variance were carried out on the log scale to satisfy more closely the assumptions of this technique.

Comparisons between the control group and each of the other five groups were made by computing 95% confidence intervals for the differences in the relevant means, using Dunnett's procedure as described by Zar.
As a result of working on the log scale, when transformed back to the original unit of measurement these confidence intervals represent proportional changes in the average values across the groups (strictly, they represent proportional changes in the geometric mean values).

The F-test from analysis of variance indicates the significance of the overall differences between the groups; the confidence intervals reflect both the statistical significance and the likely magnitude of the selected contrasts.

Results

Comparison of the “micro” method with the standard method

The two measurements are very strongly associated (correlation coefficient = 0.998) but to address the issue of agreement an analysis of paired differences is required [13].

The mean difference, as an estimate of the bias (micro-standard), is -1.1 μg·ml⁻¹ (95% confidence interval [-2.25 to +0.05 μg·ml⁻¹]); the bias is thus both small and not statistically significant at the 5% level. Assuming a normal distribution for the differences, we would expect 95% of differences to lie between -5.6 and +3.4 μg·ml⁻¹, the so-called “limits of agreement” [13]. Compared with the magnitudes of the measurements themselves such discrepancies could be considered small. The precision of these estimated limits of agreement is reasonable; 95% confidence intervals are given here by adding and subtracting 2.0 from each limit, for example -7.6 to -3.6 μg·ml⁻¹ for the lower limit of -5.6 μg·ml⁻¹.

In conclusion, over the range covered by these standards as a whole (0-150 μg·ml⁻¹) there does appear to be reasonable agreement between the methods.

Urea

Using analysis of variance for urea concentration on the log scale, the overall comparison of the six groups of subjects yielded an F-value of 3.6 (on 5 and 61 degrees of freedom), which is significant at the 1% level. Along with basic descriptive statistics on the original scale of measurement (mg·ml⁻¹), the back-transformed 95% confidence intervals described above are given in table 1. Since only that for group 6 is significant and all the others overlap considerably, these intervals indicate that the overall significance for (log) urea concentration is a result of relatively high values among symptomatic pigeon breeders (see fig. 1).

<table>
<thead>
<tr>
<th>Group (i)</th>
<th>n</th>
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<th>sd</th>
<th>95% CI for proportional change relative to controls</th>
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Mean values and sd for albumin and urea (in μg·ml⁻¹) BAL and % BAL return in control groups (group 1), IPF (group 2), Sarcoidosis (Inactive group 3, Active group 4), Pigeon Breeders (asymptomatic group 5, symptomatic group 6). On the right hand column the 95% confidence intervals (CI) for proportional change relative to the control. *: significant differences (p<0.05) for urea group 6, for albumin groups 2, 4 and 6.
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Albumin

The F-statistic for the overall comparison of log albumin concentrations was 13.1 (on 5 and 61 degrees of freedom), which is highly significant (p<0.001). For albumin it is the symptomatic pigeon breeders and active sarcoidosis groups that are chiefly responsible, with also the IPF group having a marginal effect (see table 1 and fig. 1) even though there is little difference between the latter group and groups 3 and 5 (inactive sarcoidosis and asymptomatic pigeon breeders respectively).

BAL%

For log values of this measure, the F-statistic was 1.16 (on 5 and 61 degrees of freedom), which is not significant at the 5% level. In accordance with this, the five confidence intervals in table 1 are very similar.

It should be noted from table 1 that except for those indicated all the confidence intervals for the three measures include 1 (meaning that the group is not significantly different from the controls). However, the width of some of the confidence intervals, both the significant and particularly a number of the non-significant ones, makes them difficult to interpret since neither 1 (that is, no change) nor some quite large changes can be ruled out. This reflects the large variability and the small sample sizes.

Discussion

With regard to the two most commonly used markers for the quantitation of cells and reactive proteins in BALF, this study shows urea giving the most consistent average results across subject groups. Albumin concentration in BALF is significantly higher in both active sarcoidosis and the symptomatic pigeon breeders when compared with controls. Of the remaining three groups, the IPF patients only just reach significance and the asymptomatic pigeon breeders and inactive sarcoidosis group just fail to reach a statistically significant difference when compared with the control group, though these are all small groups with wider confidence intervals.

Increases in proteins (especially immunoglobulins) have been previously reported in the BALF of patients with sarcoidosis [15], and also in smokers [16]. Albumin has also been shown to be raised in active sarcoidosis [15].

A number of theories for these increased levels of BALF proteins have been proposed. These include transudation from the peripheral circulation [17], active transport into the lung [18] and production in situ [19].

The data from the present study indicate that albumin concentration in BALF is increased in patients with interstitial lung disease in comparison with controls, suggesting that it would be unwise to consider albumin when comparing such groups.

The concentration of urea in BALF showed little variation between patient groups and controls on average, with the exception of the symptomatic pigeon breeders. Since the percentage return of BALF was similar in all groups and all lavages were carried out within 4 min this seems difficult to explain on the basis of studies by previous authors. It has been suggested by Marcy et al. [8] that there is reduced BALF return in interstitial disease and that this may lead to increased urea concentrations in the BALF of such patients. Dwell times of above 4–5 min would also allow passive diffusion of urea into the BALF thus giving falsely increased values for that derived from ELF.

Both albumin and urea were significantly raised in this patient group; indeed, mean albumin concentration in the symptomatic pigeon breeders is some 30% greater than in the active sarcoidosis group. Taken together, these results may reflect a true increase in ELF volume recovered from these patients. This may in part be due to the continued antigenic exposure of patients within this group giving continued stimulation of effector cells and immune complex production, which may in turn cause increased secretion of ELF. Baughman et al. [20] have demonstrated a change in the phospholipid content of
B(ALF in certain disease states leading to changes in the microviscosity of the ELF. Such changes in the proportions of phospholipids have also been demonstrated in this patient group by Molina et al. [21]. This requires further investigation to determine the role such changes play in ELF recovery during BAL.

References


