Evaluation of albumin as a reference marker of dilution in bronchoalveolar lavage fluid from asthmatic and control subjects

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Abstract

Background—Standardised expression of results of bronchoalveolar lavage (BAL) is problematical in the absence of a validated "denominator" of epithelial lining fluid dilution. The suitability of albumin in BAL fluid has been investigated in groups of clinically stable asthmatic and control subjects.

Methods—Absolute levels of albumin in BAL fluid were measured in a preliminary study of 21 asthmatic and 10 control subjects. In a more complex study designed to investigate the origin of albumin sampled at BAL in nine asthmatic and seven control subjects, radiolabelled albumin was injected intravenously five minutes before BAL.

Results—In the preliminary study levels of albumin in BAL fluid were very similar, with a geometric mean value of 44 (95% CI 35–54) $\mu\text{g/ml}$ BAL supernatant for the asthmatic subjects and 41 (95% CI 33–52) $\mu\text{g/ml}$ for the controls. The majority of control and asthmatic subjects in the radiolabel study exhibited minimal flux of albumin from the circulation into the BAL aspirate. This finding was not uniform, however, and in a third of the asthmatic subjects an albumin flux equivalent to >20% of the measurable albumin was found in two or more aliquots of a 3×60 ml lavage.

Conclusions—The results of this investigation into the source of albumin sampled at BAL suggest that, in general, albumin would be a reasonable reference solute for normalising the degree of dilution of BAL fluid in the groups studied. The origin of albumin was not always restricted to the bronchopulmonary segment under investigation, however, with significant leakage from the blood compartment in some individuals despite the consistency of absolute levels observed in the preliminary study.

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The complex origin of bronchoalveolar lavage (BAL) aspirate and the variable dilution of the resident lung epithelial lining fluid (ELF) pose fundamental problems for the standardised expression and interpretation of BAL findings.^{1,2}

The ideal way of presenting BAL data, both for cells and solutes, would be with the ELF volume as the denominator—that is, cells or solute concentration expressed per unit ELF. The use of methylene blue³ introduced with BAL fluid as a marker of dilution has almost certainly grossly overestimated the volume of ELF. Large bidirectional movements of water occur between the instilled BAL fluid and the circulation,⁴ with a large net gain to the BAL fluid. The contribution of ELF to the total dilution of an exogenous marker such as methylene blue is therefore likely to be small.

Urea has been advocated as a more physiological marker of dilution.^{5–7} Its low molecular weight, coupled with high rate of membrane diffusion, would suggest that its concentration in ELF and plasma should be the same. It is suggested, therefore, that if plasma and BAL urea concentrations were measured, the volume of ELF could be determined by proportionality:

ELF volume =

$$\frac{\text{BAL [Urea]}}{\text{Plasma [Urea]}} \times \text{volume of BAL aspirate}$$

The finding that amounts of urea sampled at BAL increased with the "dwell" time used between the instillation and aspiration of the BAL fluid⁸ indicated that urea is likely to move from blood to BAL fluid along its concentration gradient. In addition, we have described acute movement of radiolabelled urea from the blood into the BAL fluid which accompanied a large net water influx.⁹ This is consistent with studies in animal models where high hydrostatic pressures and local distortion of the lung (both present at BAL aspiration) have been shown to open up unphysiological "pores" in the epithelium which allow the passage of water and solutes.^{10,11}

As an alternative means of expressing the results of BAL in a standardised form, many workers have quoted the solutes or cells of interest relative to albumin^{12–14} or the total protein measured in BAL fluid.¹⁵ This model has been used on the basis that it has allowed direct comparison of results, taking account of the dilution of ELF, without the need to calculate its actual volume. Inherent assumptions have therefore been made: (1) that albumin concentrations in ELF were similar between the subject groups under study and

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not affected by the disease process under consideration; and (2) that no additional albumin crossed "acutely" from the circulation during the BAL procedure.

The latter may be incorrect in view of the demonstrated movements of water⁴ and small molecular weight solutes⁹ at BAL. The situation may be further compounded by the possible effect of disease processes on the permeability of the alveolar "membrane" which has been suggested as the cause of elevated levels of protein in BAL fluid in patients with some forms of interstitial lung disease,¹ or after acute airway challenge in patients with red cedar asthma¹⁶ or seasonal allergen.¹⁷

A recognition of these potential problems has led to studies that have attempted to validate the use of denominators of ELF dilution. The usual approach to such studies has been to perform absolute determinations of the putative markers being sampled in BAL supernatants. From these assays inferences have then been made regarding the stability and origin of the markers in the subjects studied. Hence, in recent work where urea concentrations were less variable than albumin concentrations in patients from different disease groups, it was concluded that urea might be a preferable denominator for BAL studies.⁶ Similarly, variation of albumin levels sampled in different disease groups has been used to infer the lack of suitability of albumin levels in BAL fluid as a denominator.¹

In this study we have attempted to evaluate the use of albumin as a denominator of ELF dilution at BAL in stable asthmatic subjects. We chose to do this because over recent years increasing interest has been shown in trying to measure inflammatory mediators and other solutes in this group, despite a lack of consensus on how to express the results obtained.²

Initially the amounts of albumin in the BAL aspirate from an asthmatic and control group were measured, with the asthmatic patients having a second measurement made after treatment with inhaled steroids. In a further study radiolabelled albumin was injected into the circulation before bronchoscopy in asthmatic and non-asthmatic control subjects, in order to investigate the source of this putative denominator being sampled at BAL.

Methods

Ethical permission for all studies was obtained from the Joint Ethics Committee in Newcastle.

SUBJECT GROUPS

All subjects studied were either volunteer patients with asthma with a well documented disease history, or control subjects undergoing diagnostic bronchoscopy who had normal lung function, no evidence of general airway or pulmonary disease, and no local abnormality at the site lavaged. The most common indication for bronchoscopy in these subjects was an episode of minor haemoptysis.

BAL METHODOLOGY

A 3 × 60 ml BAL was performed with sterile, pyrogen free, phosphate buffered saline, prewarmed to 37°C, in a segment of the right middle lobe, with an Olympus BF P10 bronchoscope (5 mm diameter, Keymed Ltd, UK). Aspiration was performed at a pressure of -50 to -100 mm Hg into an iced siliconised glass vessel.

Study 1

In this study in which the albumin levels in BAL fluid in asthmatic patients and controls were compared, BAL was performed in 21 asthmatic and 10 control subjects (table 1). Cells were separated from BAL supernatant within one hour by centrifugation (15 minutes at 100 g). The pooled BAL supernatant was subdivided into 4 ml aliquots and stored at -20°C until subsequent assays. BAL was performed before and after treatment with high dose inhaled beclomethasone dipropionate (1000 µg twice daily) for a median of 2.5 (range 2-10) months in the asthmatic patients.

Study 2

In this study a more extensive experiment to investigate the dynamics of albumin sampling at BAL was performed. 1.48 MBq of iodine-125 labelled human serum albumin in 0.5 ml normal saline was injected intravenously five minutes before a 3 × 60 ml sequential BAL in a further nine asthmatic subjects (seven men, median FEV₁ 76% (range 39-82%) predicted, one current smoker), and seven non-asthmatic subjects (four men, median FEV₁ 96% (range 68-126%) predicted, one current smoker). BAL aspirate was processed as above.

ISOTOPE COUNTING

In study 2, gamma counts were performed on aliquots taken from the three separate BAL supernatants and on venous blood plasma samples taken simultaneously from the arm contralateral to that used for the isotope injection. In order to optimise the accuracy of quantifying the low levels of activity in the BAL supernatants, a count time of 60 minutes was used.

For this study it was planned to exclude any subject with macroscopic blood staining of the BAL aspirate, which in the event was never necessary. Subclinical, microscopic bleeding was evaluated by enumerating erythrocytes from unprocessed BAL aliquots with a Neubaur counting chamber. A red

Table 1 Demographic details of 21 asthmatic and 10 control subjects included in the preliminary study

	Asthmatics	Controls
n	21	10
Age range (years)	20-52	19-67
Sex (M:F)	10:11	7:3
No of smokers	3	4
Inhaled medication	17 salbutamol only, 4 on ≤200 µg BDP	Nil
Mean FEV ₁ (% predicted) (95% CI)	83 (73-93)	92 (82-102)

BDP—beclomethasone dipropionate.

Table 2 The above background iodine-125 activity (counts per minute) detected from the bronchoalveolar lavage samples of each patient studied with the corresponding coefficients of variation (CV) for the counts observed expressed as a percentage

Subjects*	Aliquot 1		Aliquot 2		Aliquot 3	
	Counts	%CV	Counts	%CV	Counts	%CV
A1	9	13	12	10	67	2
A2	2	52	3	35	2	52
A3	3	36	4	27	3	36
A4	7	16	9	12	12	9
A5	32	4	8	14	23	5
A6	0	—	0	—	0	—
A7	0	—	0	—	0	—
A8	0	—	0	—	0	—
A9	0	—	2	43	2	44
C1	1	105	2	53	1	105
C2	7	16	9	12	12	9
C3	1	105	0	—	1	105
C4	8	14	4	27	1	104
C5	5	22	1	10	22	6
C6	0	—	0	—	0	—
C7	0	—	2	48	2	48

*Asthmatic subjects A1–A9, control subjects C1–C7.

blood cell count was performed on the corresponding venous blood samples with an automated analyser (Coulter STKR). These counts were used to estimate, by proportionality, how much labelled albumin might have crossed into the BAL aspirate as a result of bleeding and consequent plasma movement.

ALBUMIN ASSAYS

Albumin concentrations were measured in the BAL supernatants by a double antibody, liquid phase radioimmunoassay (Diagnostic Products Ltd, Abingdon, UK) performed in duplicate. The between batch variation for the assay was 6% and the limit of detection 0.9 µg/ml. Plasma albumin levels were measured directly with a Hitachi 717 continuous flow autoanalyser.

Calculations

Calculation of the dilution volumes for the injected radiolabelled albumin was made by comparing the specific activities of the injected isotope against activity in the plasma samples following the five minute equilibration period.

The amount of albumin in the BAL aspirate derived acutely by influx from the circulation into the BAL fluid was calculated by proportionality.

$$A = \frac{\text{cpm/ml BAL}}{\text{cpm/ml plasma}} \times [\text{plasma albumin}]$$

where A = amount of albumin "leaked", and cpm/ml represents the gamma counts in

counts per minute per ml of BAL supernatant or plasma.

STATISTICAL ANALYSIS

Statistical analysis was performed with the statistical package Minitab (CLE COM Ltd, Birmingham). Comparisons between the control subjects and the asthma patients were made with analysis of variance (ANOVA) or, where appropriate, the fitted general linear model which allows analysis of variance to be performed on unbalanced designs. *p* values of <0.05 were taken as significant.

Results

The total return volumes in study 1 were similar: median 86 (interquartile range 63–110) ml for the baseline in the asthmatic patients, 94 (79–116) ml after treatment, and 94 (53–113) ml in the controls. This was also the case for study 2: median 105 (interquartile range 72–114) ml in the asthmatic patients, 84 (62–110) ml in the controls. There was no statistical difference between the various subject groups (*p* = 0.91). Results of albumin assays were subsequently analysed in terms of µg albumin/ml BAL supernatant, although the outcome of the study was very similar if analysed in terms of total returns.

STUDY 1

BAL albumin levels were very similar in the 21 asthmatic and 10 non-asthmatic subjects, with a geometric mean value of 44 (95% CI 35–54) µg/ml BAL supernatant for the asthmatic subjects and 41 (95% CI 33–52) µg/ml for the controls. There was no significant effect of treatment on albumin concentrations in the asthmatic patients with a geometric mean level after treatment of 37 (95% CI 29–36) µg/ml.

STUDY 2

The dilution volumes for the injected iodine-125 labelled albumin were within predicted values of plasma volumes in all subjects. This indicated that there was no significant movement of albumin from the circulation into the extracellular space by the time BAL was performed.

The above background iodine-125 counts per minute detected from the BAL samples of each patient are presented in table 2, together with the corresponding coefficients of variation for the counts. Isotope counts follow a Poisson distribution so that the coefficients of variation are high where counts are low and

Table 3 Albumin levels measured in the nine asthmatic and seven control subjects in study 2, with the amounts of albumin calculated to have been derived from the circulation by acute flux by radiolabelling (µg/ml, geometric mean values and 95% confidence intervals (CI)).

	Aliquot 1		Aliquot 2		Aliquot 3	
	Total	Fluxed	Total	Fluxed	Total	Fluxed
Asthma patients						
Geometric mean (95% CI)	55 (30–102)	7 (2–28)	47 (25–91)	6 (3–12)	44 (25–76)	10 (3–34)
Controls						
Geometric mean (95% CI)	62 (35–110)	3 (1–12)	68 (48–98)	3 (1–9)	56 (35–93)	2 (1–6)

vice versa, hence the apparent fluxes observed were not due to inadequacy of measurement.

The amounts of albumin calculated by radioisotopes to have crossed acutely from the circulation for each aliquot are summarised in table 3 together with the corresponding total albumin levels. Total albumin levels were not significantly different between the subject groups in study 1 and 2 ($p = 0.122$). The regression between total levels of albumin assayed and acute movement of albumin indicated that there was no significant relationship between the two in these subjects (regression equation: total = $71.2 - 0.579$ fluxed albumin: $p = 0.541$). There was a trend towards greater albumin flux in the asthma patients, but this did not reach conventional levels of statistical significance ($p = 0.08$). There was no significant difference in albumin flux between the BAL aliquots ($p = 0.748$).

Albumin flux from the circulation, expressed as a percentage of the total assayed, is shown in the figure for the two groups. Correction of these data for any albumin flux attributable to microscopic bleeding made no difference to the results.

Discussion

Many investigators have advocated the use of denominators of ELF dilution in BAL studies and "endogenous" albumin,¹²⁻¹⁴ total protein¹⁵ and urea⁵⁻⁷ have been proposed for this purpose. There are two key assumptions made in such studies: (1) that the disease process studied is not affecting the ELF marker itself, and (2) that no acute leakage of the marker occurs during the BAL procedure from outside the bronchopulmonary segment.

In this study we found no difference between albumin levels in BAL aspirate in a group of stable asthmatic patients compared with non-asthmatic subjects. Further, there was no change in albumin concentrations in the asthmatic subjects after a lengthy period

of treatment with inhaled corticosteroids which had a significant effect upon airway responsiveness to methacholine. From this it might be thought that albumin was likely to be a robust and suitable marker of ELF dilution in such subjects. Indeed, in the second part of this study we did show that, in the majority of the controls and about half the asthmatic subjects studied, the amount of acute flux of albumin from the circulation was minimal, and in these subjects albumin would be a very reasonable denominator for ELF dilution.

This finding was not, however, uniform. The movement of radiolabelled albumin from plasma to BAL fluid that we observed indicated that an apparently normal total albumin level in BAL fluid did not necessarily imply that its source was from ELF alone. An unpredictable and sometimes substantial amount of albumin could be accounted for by acute movement from the circulation, particularly in the nine asthmatic subjects studied. In a third of these individuals an albumin flux equivalent to >20% of the measurable albumin was found in two or more aliquots. Individual variation in the amount of flux that occurred was from none detected to an amount equivalent to the total albumin measured in one individual aspirate. Similarly, in the non-asthmatic control subjects, although the phenomenon was less common and of a lesser magnitude, the albumin flux in an aspirate from one individual accounted for 32% of the albumin sampled. In any one individual the general tendency for albumin flux, if not the absolute amounts, was similar throughout all three aliquots (fig), but even this was erratic in some cases. The asthmatic subjects in this study were clinically stable and with no obvious clinical differences between those with high albumin flux and those in which it was absent.

For the groups as a whole one might perhaps regard the albumin leakage observed as small and its use as a denominator for BAL results appropriate. In a situation where a solute was only moderately elevated in asthmatic subjects, however, the use of the albumin method could potentially lead to an artefactual underestimate of the differences, and a possible false negative result.

Plasma leakage associated with microscopic bleeding in these BAL samples did not account for the levels of albumin movement detected. The occurrence of significant clinical bleeding during BAL would cause doubt about the true origin of the albumin measured in the BAL fluid. It should also be noted that the degree of albumin flux is likely to be greater in any situation of acute inflammation of the airway wall such as after inhalation challenge.^{16,17} Our data only refer to the clinically stable asthmatic and control subjects in this study who had no overt blood staining of the BAL aspirates.

Albumin is a solute with a relatively high molecular mass which is relatively impermeant to the alveolar epithelial membrane.¹¹ Diffusion along the concentration gradient

Albumin fluxes calculated to have occurred into aspirates 1-3 by radiolabelling, expressed as a percentage of the total albumin measured. □, control subjects; ●, asthma patients.

created by the instillation of albumin free saline into the lung was therefore unlikely to explain the albumin movements we observed, especially over the time course of our BAL procedure. Normal "steady state" characteristics of epithelial permeability are unlikely to pertain during the dynamic process of BAL. Given the thousand fold difference in albumin concentration between plasma and BAL fluid, the variability of albumin flux observed is consistent with small acute changes in epithelial permeability to albumin, presumably related to the BAL procedure itself, and possibly exacerbated in the asthmatic subjects by airway inflammation. It is likely that the relatively high aspiration pressures used at BAL tend to open up pores in the airway and pulmonary epithelium.^{10,11} The creation of a variable number of such pores, some large enough to allow passage of significant amounts of albumin despite its high molecular radius (3.5 nm), could therefore account for the variable nature of the acute movement of albumin we observed. Previous work on the permeability of alveolar epithelia is inconsistent. Data from a number of species would suggest that stable pulmonary epithelial membranes have "physiological" pore sizes of up to 1.5 nm, while deliberate perturbation of a lung segment may open up pores as large as 4 nm.¹¹

We conclude that overall albumin would be a reasonable candidate for use as a reference solute in allowing for differential dilution of BAL fluid in the groups studied. This use should, however, be tempered with caution. Our data would suggest that its use could underestimate the difference between an asthmatic and control group where another solute of interest was only moderately elevated in asthma due to leakage of albumin from plasma in a significant and unpredictable minority of the asthmatic subjects.

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