

THORAX

Review

Bronchoalveolar lavage as a research tool

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Bronchoalveolar lavage as a technique is relatively easy to describe. A fiberoptic bronchoscope is passed into the airways and gently impacted, or "wedged," into a subsegmental bronchus. Sterile saline, buffered and warmed to body temperature, is then injected into the subsegment through the bronchoscope biopsy channel, and then aspirated. Details, such as the amount of fluid injected, the number of aliquots used, the "dwell time," and the aspiration pressure, vary greatly between laboratories, and are frequently poorly described in research reports, though there have been attempts in both North America and Europe to obtain some standardisation.^{1,2} As an attempt at compromise between the methods on offer, we have adopted a standardised 3 × 60 ml aliquot regimen, each aliquot being injected under minimal hand pressure over about 10 seconds, with immediate aspiration—that is, a minimal dwell time—and a "low" aspiration suction pressure of about 80 cm H₂O. By choice we use the middle lobe, because it is relatively easy to establish a wedge and fluid return is said to be best.³ Siliconised glassware is used to reduce cellular adhesion.

It is more difficult to be sure about what exactly we are doing with bronchoalveolar lavage, how to handle the material obtained, and what the data generated actually mean. The basic assumption is that the injected fluid reaches the area of pathological interest and that the aspirate will then be a representative sample of the "epithelial lining fluid" containing solutes and a population of cells relevant to the pathophysiology of the disease process. Sampling the site of pulmonary disease directly in this way, with a relatively atraumatic and simple procedure, is obviously attractive. Not surprisingly therefore bronchoalveolar lavage has become an extremely popular research technique generating a very large body of published reports—initially mainly concerned with interstitial lung disease but increasingly also on airway disease and asthma. There seems to be an acceptance that bronchoalveolar lavage can be used as a tool for quantitative analysis of pulmonary disease processes, and a tendency to ignore some awkward but fundamental questions relating to potential procedural artefacts. Many of the

assumptions underlying this body of work have been relatively little tested. In this article we attempt to address some of these issues.

Distribution and anatomical origin of the lavage fluid

In the study of interstitial lung disease it is assumed that the intubated bronchopulmonary segment can be regarded as a large sump which the bronchoalveolar lavage fluid fills, so that samples can be removed from the peripheral alveolar compartment. The picture is somewhat less complex for those studying the airways as there is little doubt that the fluid introduced and aspirated has moved through the bronchi. A central question in both areas of research is what is the optimum volume of fluid for obtaining the best data for a proximal ("bronchial") wash as opposed to a distal ("alveolar") wash.

A small but influential study in a rather non-specific group of patients with interstitial lung disease (eight patients and nine controls) showed slightly more lymphocytes and polymorphs in the second half of a 240 ml lavage than in the first half in the patients with disease, whereas in the controls numbers of both lymphocytes and neutrophils had fallen in the second half of the lavage.⁴ The difference between groups was therefore greater with the large volume, supporting the need for a large volume of lavage fluid to provide an optimum sample from lung with parenchymal disease. In asthma⁵ another influential study showed that with a small volume (5–20 ml) lavage considerably more epithelial cells and acute inflammatory cells were obtained than in a 100 ml lavage, and it has been concluded that such small volumes are appropriate for studying airway disease. The actual number of cells obtained with the small volume lavage, however, was inevitably low, viability of the cells was poor, and, interestingly, the number of lymphocytes increased dramatically with the large volume.⁵ Others, however, have been unable to show such clearcut differences in acute inflammatory cell numbers between small and large volume lavage in patients with asthma.^{6,7}

In a radiographic study using a computerised digital subtraction imaging technique we

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showed that bronchoalveolar lavage fluid containing niopam did indeed reach the periphery of the lung segment.⁸ Aspiration of the first 60 ml aliquot caused fluid movement towards the bronchoscope only in the proximal part of the lung segment, whereas in the periphery fluid at this stage continued to move away from the bronchoscope. When larger volumes were introduced there was more movement of fluid at aspiration back towards the bronchoscope throughout the segment. This study has been quoted as evidence that the basic assumption that a small lavage volume will provide an optimum sample of airway epithelial lining fluid whereas a large volume will preferentially sample lung parenchyma is correct. Further, a study we performed in patients with sarcoidosis seemed to give support to this general hypothesis, with cellular metabolic activity in macrophages and polymorphs, as assessed by induced chemiluminescence, increasing dramatically between the first and the second and third 60 ml aliquots.⁹ A subsequent study, however, has shown a very similar pattern in asthma—that is, a dramatic increase in cell metabolic activity in macrophages and polymorphs between the first and subsequent aliquot, which differed significantly from that seen in control material only in the latter samples.¹⁰

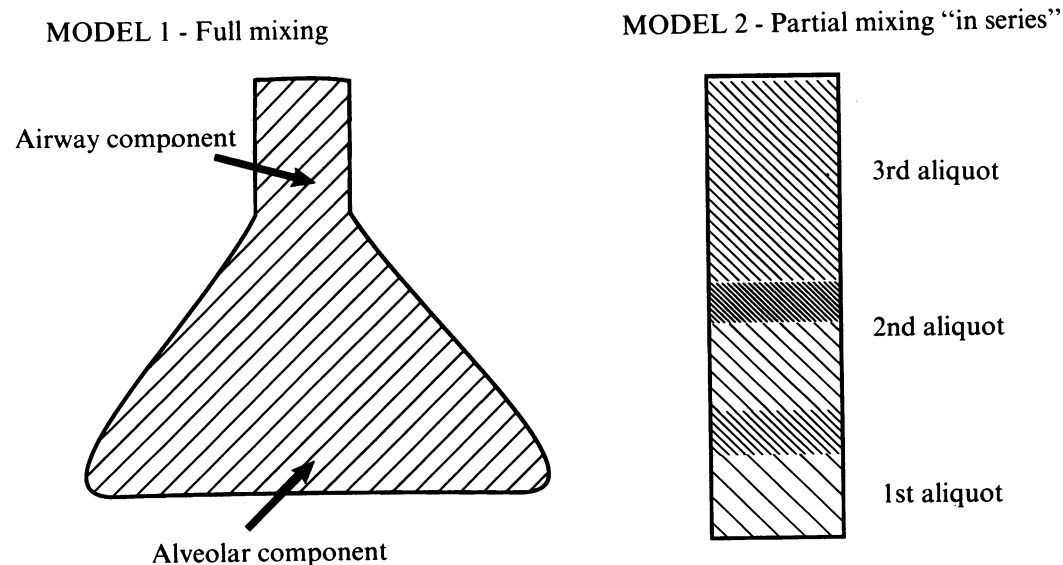
In a further study¹¹ we attempted to define the “mixing model” for the lavaged bronchopulmonary segment (fig 1), asking whether the system behaves in the way initially assumed, particularly by those investigating interstitial disease—that is, as predominantly a large “alveolar sump”—or whether it behaves more as an essentially tubular structure with relatively little mixing between subsequent aliquots in series. In subjects undergoing a standard 3×60 ml aliquot bronchoalveolar lavage the first aliquot was labelled with 0.005% methylene blue, and the second with 1 MBq of technetium-99m colloid. When the degree of mixing between the second and first aliquots was calculated from the amount of methylene

blue recovered in the second aspirate, 24% (median) of the second aspirate came from the residuum of the first aliquot. From the technetium colloid and methylene blue in the third aspirate we calculated that 16% of the third aspirate came from the residuum of the second aliquot, 8% from that of the first aliquot, and 76% from the fluid injected as the third aliquot. Thus our data do not support a simple sump model of alveolar filling and aspiration but are more in keeping with a tubular model of aliquots in series, in which there is relatively little admixing at the interfaces between subsequent aliquot injectates, each aliquot pushing the one before ahead of it and then moving back at aspiration largely unmixed.

This does not mean that bronchoalveolar lavage is inappropriate for studying interstitial lung processes—indeed, it has proved very informative, and studies have shown some correspondence between bronchoalveolar lavage cell profiles and biopsy findings or at least cells extractable from lung tissue,^{12,13} though interlobar variations in disease activity need to be taken into account.¹⁴ We would, however, emphasise the complexity of the process of sampling, and the possibility that some of the cells being sampled originate from within the airways rather than at alveolar or interstitial level. In asthma, paradoxically, a small volume may not be optimal, and some relevant information may be available only from large volume lavages. Indeed, a very small volume wash may be predominantly contaminated with senescent cells and superficial cellular debris, no longer representative of the active airway inflammatory process.

Given the current uncertainty we prefer to pool all our aspirates and analyse all the material obtained, rather than make presumptive value judgments on the relative merits of different samples. It seems paradoxical that in some centres only the first small volume aliquot is analysed, whereas in others this sample is thrown away even when the same disease is being studied. Moreover, if multiple investiga-

Figure 1 Hypothetical models for mixing between aliquots of introduced fluid in bronchoalveolar lavage. In the partial mixing model (model 2) the residual fluid from aliquots 1 and 2 would be smaller in volume than the newly introduced aliquot 3.



tions are to be performed on the bronchoalveolar lavage cell population—for example, several cytopsin preparations, cell subset analysis by flow cytometry, and cell function studies—even the 15–20 million cells usually available from our standard procedure may not be adequate, a point to be borne in mind in the planning of studies.

Processing of bronchoalveolar lavage cells

The subsequent processing of aspirated bronchoalveolar lavage fluid is even more variable, if anything, than the initial procedure. The first step is to obtain an accurate total cell count, which is vital for adequate interpretation of the subsequent differential cell count. Most laboratories have used a haemocytometer chamber as automated cell counters have proved unreliable in these circumstances (personal observations). This total cell count, however, has been performed by different workers at various stages within the sequence of lavage fluid processing steps, though at each stage there will be a variable degree of cell loss. Presumably as a result largely of these technical variations, the total number of cells recovered, or at least eventually counted, varies between studies to an extraordinary degree. To take an almost random selection of studies of lavage fluid in asthma, for example, approximately the following average cell returns ($\times 10^3/\text{ml}$ of lavage aspirate) from large volume protocols are found: Flint *et al*¹⁵ 12; Gerblich *et al*¹⁶ 40; Diaz *et al*¹⁷ 59; Kirby *et al*⁷ 63; Lam *et al*⁵ 144; Kelly *et al*¹⁸ 190; and Godard *et al*¹⁹ 207. In other and sometimes much quoted publications such data are just not available, and indeed quite frequently the description of methods is quite inadequate. A recent large multicentre American study¹ may provide a useful yardstick for investigators, though even here it is difficult to know whether fluid was filtered before counting and a hand held syringe under poorly quantified negative pressure was used for aspiration. The final number of countable cells expected should be in the region of 120–200 $\times 10^3/\text{ml}$ for patients with asthma and normal control subjects, with higher values in smokers and in patients with active interstitial disease. Lower total cell yields should prompt reflection on why the apparent cell loss (or loss of recruitment) is occurring.

In the protocol most frequently used for processing of bronchoalveolar lavage aspirates the raw fluid is filtered through variable layers of cotton gauze (sometimes presoaked) and centrifuged for a variable time and at a variable speed, and then the cell pellet is resuspended in a physiological solution; sometimes these steps are repeated. Concern has been expressed by several workers that some of these processing steps may themselves cause cell loss or distort the final cell differential.^{20–23}

Over the past few years we have attempted a systematic analysis of these potentially major problems. We have adopted for general use a stainless steel mesh of pore size 200 μm for filtration as we regarded cotton gauze of varying

thickness as inherently too imprecise, and as there was also the possibility that, as a biological substance, it may activate cells we wished to study functionally.

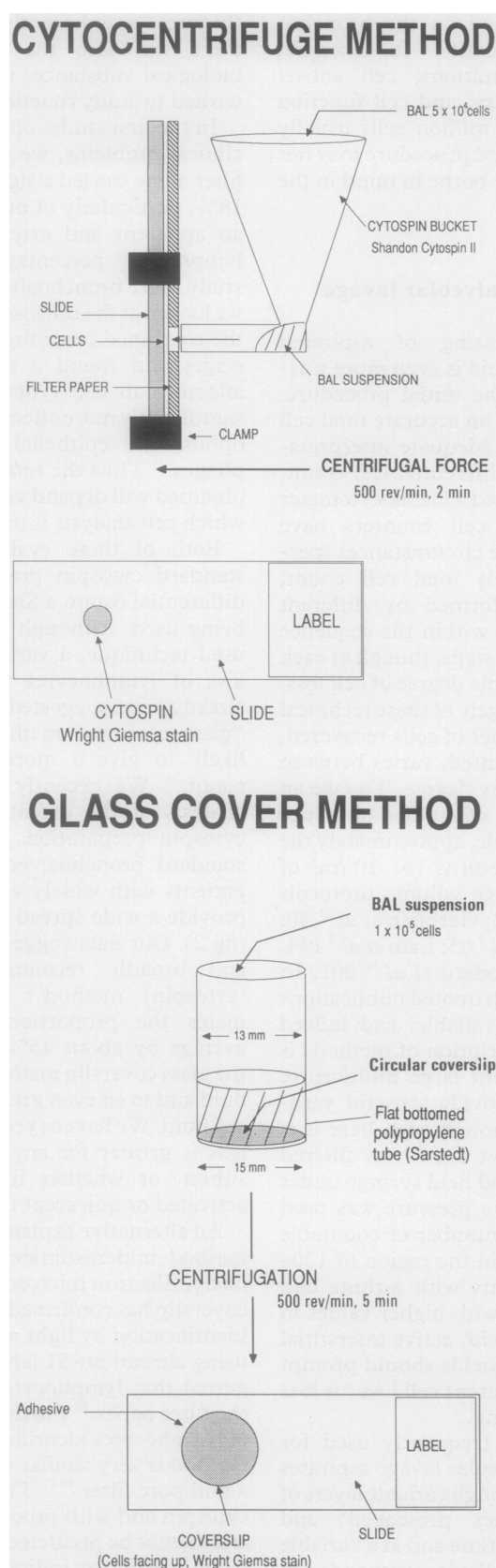
In the first study, of 18 patients with various clinical problems, we showed that use of this filter alone caused a significant total cell loss of 18%, particularly of macrophages, which gave an apparent and artefactual increase in the lymphocyte percentage.²⁴ In a subsequent study of 51 bronchoalveolar lavage procedures we looked at the cell loss after both filtration and the combined centrifugation and resuspension stages and found a total cell loss of 30%, affecting all cell types but this time having significantly more effect on lymphocytes, eosinophils, and epithelial cells than on macrophages.²⁵ Thus the total and differential count obtained will depend very much on the point at which cell analysis is made.

Both of these evaluation studies used a standard cytopsin preparation for a manual differential count, a Shandon II cytocentrifuge being used. Although this is the most widely used technique, a variable and unpredictable loss of lymphocytes with the cytopsin has already been suggested.²⁰ More recently a new “glass coverslip method” was suggested as likely to give a more accurate lymphocyte count.²⁶ We recently attempted a detailed analysis of the extent of the problem with cytopsin preparations, using 27 consecutive standard bronchoalveolar lavage aspirates in patients with widely varying disease states to provide a wide spread of lymphocyte counts²⁷ (fig 2). Our data suggest that the conventional and broadly recommended cytocentrifuge (cytopsin) method^{1,2} alarmingly underestimates the proportion of lymphocytes—on average by about 45%—by comparison with the glass coverslip method used with raw lavage fluid and to an even greater extent with processed fluid. We have as yet no data on whether this loss is greater for any particular lymphocyte subset, or whether it affects cells that are activated or quiescent to a greater extent.

An alternative explanation, that the coverslip method underestimates macrophages, is unlikely. Electron microscopy of cells on the glass coverslip has confirmed the accuracy of cellular identification by light microscopy, and a study using chromium-51 labelled lymphocytes suggested that lymphocytes were being lost into the filter paper.²⁶ Furthermore, the proportion of lymphocytes identified by the glass coverslip method is very similar to that determined with a millipore filter.^{26,27} These cell losses with the cytopsin and with processing are very variable and cannot be predicted on any one occasion or in any particular individual. Processing of the lavage fluid also caused a large loss of eosinophils by both methods in our studies. These large potential artefacts need to be taken into account when bronchoalveolar lavage data are being assessed. We recommend the coverslip method for manual analysis, with unfiltered raw lavage fluid whenever possible.

Computerised FACScan flow cytometry is becoming popular in research centres for counting bronchoalveolar lavage cells and their

Figure 2 Schematic representation of (a) the cytocentrifuge method and (b) the glass cover slide method as used by us for a comparative study of apparent lymphocyte loss. BAL—bronchoalveolar lavage fluid.



phenotypic subtypes. This uses a combination of size and scatter properties and monoclonal antibodies to cellular antigens. It has the major advantage of a much better coefficient of reproducibility than conventional manual immunofluorescence methods²⁸ because it counts thousands of cells rather than a few hundred. We have also recently compared FACS as a method for obtaining a three part

differential (using Becton Dickinson Leucogate reagent and Paint-a-Gate software) versus both the cytospin and the glass coverslip method. Only processed lavage fluid can be used as a sample containing mucus is unsuitable for FACS. FACS and the coverslip method proved very similar results, whereas again the cytospin method apparently "lost" about half the lymphocytes.

Assessment of lavage fluid solutes

Many different solutes have been measured in bronchoalveolar lavage fluid, including immunoglobulins, proteins, enzymes, a whole range of inflammatory mediators and surfactant like lipids and phospholipids, and drugs. The main problem that workers face, whether acknowledged or not, is how to present the data rationally, taking into account the complex and almost certainly very variable dilution of the solute of interest. The same problem of "dilution" perhaps ought to apply to cell counts, but is certainly particularly acute with the handling of data on solutes.

Using radioisotope markers we have shown that the fluid dynamics during bronchoalveolar lavage are complex (fig 3).²⁹ During injection of fluid water passes from the bronchopulmonary segment, presumably into the blood compartment or possibly the interstitium, and at aspiration water is drawn from plasma. Thus from an average of 85 ml aspirate from five 3×60 ml injectates a median of 33.3 ml (39%) apparently came directly from the blood compartment. Within the aspirate there will be a small amount of the fluid of real interest, the epithelial lining fluid, which will be diluted not only by the injectate (some of which will be aspirated, some pushed out of the lung segment, and some retained in the bronchopulmonary segment) but also by this extra source of water. Our data are very consistent with the results of studies on animal models, where both high hydrostatic pressures and local distention of the lung segment will open up large pores in the epithelium to allow passage of both water, as bulk flow, and solutes of lower molecular size by the process of "solvent drag."³⁰⁻³² Such movements are much greater than would occur with diffusion alone.

Various methods have been used to present data on the solutes in bronchoalveolar lavage. Some authors present the total amount of solute aspirated per procedure or give a concentration—that is, solute per ml of lavage fluid aspirated—and ignore probable differences in dilution between subjects and between the groups studied. This problem is greatly exacerbated if the injected volumes or the aspirate volumes (or both) are very different. In some studies the apparent difference between disease groups seems to depend very largely on such technical differences.³³

Other authors have tried to calculate the volume of epithelial lining fluid in the lavage fluid aspirate by using an endogenous marker of epithelial lining fluid dilution. Urea and albumin have been used most often.^{34 35} The assumptions underlying this correction are that

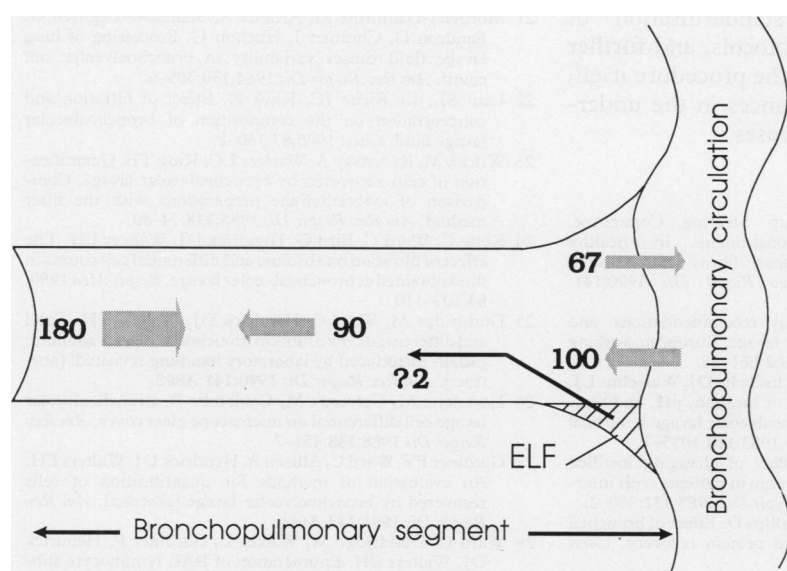


Figure 3 Schematic representation of the fluid fluxes that occur during bronchoalveolar lavage (all numbers in millilitres). Out of the 180 ml introduced, about 67 ml of the water moves out of the bronchopulmonary segment, while about 100 ml of water is drawn from the circulation. Of the 90 ml that might be aspirated, about 33 ml will come directly from the circulation, 55 ml will be from the injected fluid, and at most 2 ml will be from the epithelial lining fluid (ELF), though this last figure is likely to be a substantial overestimate because of "solvent drag" of urea with the water influx.

the concentration of urea or albumin in epithelial lining fluid is the same as that in plasma (which is measured), that it does not change with the disease process, and that no urea or albumin enters the lavage fluid from elsewhere during the lavage itself. The volume of epithelial lining fluid would then be calculated from the plasma:lavage fluid ratio. Urea, unfortunately, almost certainly does move into lavage fluid during the procedure,^{36,37} both by solute flux and by diffusion down a concentration gradient, and would seem to be inherently inappropriate as a marker of epithelial lining fluid volumes, though it still has its advocates.³⁸ Data from studies that have used the urea method need to be interpreted with caution, particularly where urea concentrations in lavage fluid seem to vary considerably between disease groups.³⁹

Albumin as a marker of epithelial lining fluid dilution would seem to be even more flawed. It will almost certainly not be present in epithelial lining fluid at a concentration near to that in plasma. The oncotic pressure of most interstitial fluids and therefore the albumin concentration, which is its main determinant, is about one third that of plasma. Furthermore, the albumin concentration in lavage fluid aspirate increases in some disease states,³⁸ and can be affected by treatment.⁴⁰ Current studies are addressing the question of whether this implies increased background permeability of the epithelium or local production or concentration of albumin, or whether albumin just moves across more freely from the plasma during bronchoalveolar lavage in these circumstances. At the moment there is no obviously suitable marker of epithelial lining fluid volume, and this is likely to be variable and will depend, among other things, on its albumin content. A better approach may be to present data on

solute in terms of the total that would be present in the total dilution volume of which the aspirate is a representative sample. As it is, the only reasonably convincing data are where ratios of one solute to another differ in different diseases,¹ or where differences between solute measurements are extremely large, however presented (as with major basic protein in asthma⁴¹), or where a given substance is found in a particular condition with none in the control lavage fluid (as with tissue kallikrein and platelet activating factor in asthma).^{42,43}

A new method for attempting to calculate epithelial lining fluid volume is described in this issue by Baldwin *et al.*⁴⁴ They have used a 20 ml "microlavage" through a thin plastic tube placed distally as a preliminary procedure before a formal large volume bronchoalveolar lavage. They use the urea concentration in the microlavage aspirate to calculate its epithelial lining fluid volume, and use the protein concentration to back calculate epithelial lining fluid protein concentration. By proportionality, protein concentration in the subsequent formal lavage aspirate is then used to calculate its epithelial lining fluid volumes. This is an interesting paper and a novel and promising approach, but several aspects will need fuller evaluation—in particular whether water and urea flux into the microlavage aspirate occurs as a result of the negative hydrostatic pressures applied, which would once more lead to an overestimate of the urea concentration in the epithelial lining fluid and so of its volume. The difficulty of aspirating fluid from the microlavage and the frequency of apparent trauma would suggest that substantial distortion of the epithelium is likely to occur distally, which could possibly facilitate much more bulk water and solvent movement than would be expected from permeability coefficients calculated from experiments based on osmotically induced fluid fluxes. Furthermore, if protein fluxes occur acutely at aspiration of the lavage fluid, which may be the case when the epithelium is inflamed,⁴⁵ then the ability to use protein concentrations as suggested to calculate epithelial lining fluid volume would also be lost.

Conclusions

This review has concentrated particularly on the quantitative aspects of bronchoalveolar lavage and lavage fluid processing as a guide to those who may be tempted to use the technique as a research tool. Close attention to detail is necessary at all stages as even the most routine and apparently innocent procedure can affect the ultimate results. We also hope that the article will help in the reading and critical appreciation of research reports, where inadequately described or inappropriate methods, or naive acceptance of some conventional but unlikely assumptions, may distort interpretation of the data.

Despite its major methodological and interpretational difficulties, bronchoalveolar lavage used as a research tool continues to provide exciting insights into the pathophysiology of an extremely wide range of diseases.

Improvements in the standardisation of bronchoalveolar lavage protocols, and further insights into the nature of the procedure itself, can only hasten these advances in the understanding of pulmonary diseases.

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