Technical Aspects of Bronchoalveolar Lavage: Recommendations for a Standard Procedure

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ABSTRACT

Bronchoalveolar lavage (BAL) has been used widely in the diagnosis of infection. More than 30 years after its description, its use in the clinical management of interstitial lung disease remains controversial. One limitation has been the variations in how the specimen is obtained and handled. Several groups have developed recommendations for obtaining and handling BAL specimens. This article summarizes these recommendations and discusses the variable effects they have on BAL results. The final recommendations provide a standard methodology that can be used by the largest number of pulmonologists.

KEYWORDS: Urea, immunohistochemistry, bronchoalveolar lavage technique

Bronchoalveolar lavage (BAL) via a flexible bronchoscope was introduced as a research tool by Reynolds and Newball in 1974.[1] The technique rapidly gained acceptance and a large number of centers began using the technique to obtain cells and proteins from the lower respiratory tract.[2] The lavage process includes advancing the bronchoscope as far as possible into the airway (wedged). Fluid, usually normal saline, is introduced into the distal airway through the suction channel, and then the fluid is aspirated. Fig. 1 illustrates the lavage process, with the lavage fluid instilled. As shown, there are three populations of cells: (1) cells in the alveoli that are within the BAL fluid and are being sampled, (2) alveolar cells that are not in contact with the BAL fluid and therefore are not sampled, and (3) airway cells that may also be washed into the bronchoscope during the lavage process and therefore are sampled. The process of lavage is not a perfect sampling. One of the difficulties with interpretation of BAL results is dealing with the uncertainties of the BAL process.[3]

OBTAINING BRONCHOALVEOLAR LAVAGE FLUID

Many centers previously used their own technique for performing lavage. A survey done in the mid-1980s emphasized the differences and similarities of the techniques around the world.[4] It became apparent that differences in technique led to differences in results. Therefore, several groups established standardized methods for performing lavage. Two of these originated from the European Respiratory Society (ERS), which provided recommendations about the technical aspects of performing BAL.[5,6] In the United States, four centers performed standardized studies across their centers in both normal controls and patients with interstitial lung disease.[7] They had specific recommendations regarding various aspects of lavage.[8] An American Thoracic Society task force has looked at these recommendations and has developed a consensus recommendation (Keith Meyer and Ganesh Raghu, chairmen, personal communication). The recommendations are summarized in (Table 1).[5,6,8] This section reviews the features of obtaining and processing

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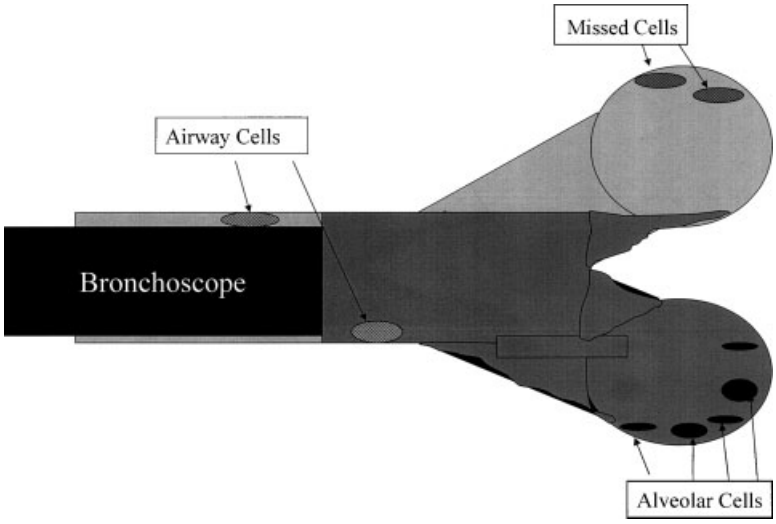

Figure 1 Illustration of the lavage process. Fluid is instilled through the bronchoscope and fills some of the alveolar space. Cells in the alveolar space are sampled. There are cells in the alveolar space that do not come in contact with the instilled fluid (missed cells). There are also airway cells that can be washed into the alveolar lavage fluid.

BAL that appear to affect BAL results and presents the recommendations made by these reports.[5,6,9]

Underlying Condition

The underlying disease has been shown to affect the BAL process in several ways. The disease may be inhomogeneous; therefore, BAL results may differ from various parts of the lung. This has been shown for idiopathic pulmonary fibrosis.[10] For infections, BAL should be performed in the area of most disease, not only for bacterial but also for pneumocystis pneumonia.[11]

Airway obstruction leads to changes in BAL return. This is due to the mechanical problem of aspirating fluid through a tube further narrowed by the disease. In patients with chronic obstructive lung disease, the airway collapse may be variable. The more pressure used to aspirate the fluid, the larger the amount of collapse. It has been found that the ratio of forced expiratory volume in 1 second to forced vital capacity

Table 1 Aspects Affecting Bronchoalveolar Lavage Results

Source of Variability	US BAL Cooperative[8]	European Respiratory Society 1999[6]	American Thoracic Society 2007
Disease process itself	Stated	State underlying disease	State underlying disease
Suction pressure during the procedure	''Gently'' aspirate by handheld syringe	Keep to a minimum (25–100 mm Hg)	Keep below 100 mm Hg; avoid visible airway collapse
The handling of fluid: filtered/ nonfiltered; concentrated	No comment	State technique specifically	No filtering with gauze
Volume instilled	240 mL	Instill at least 100 mL	Instill at least 100 mL
Handling of first aliquot recovered	Pooled all samples	Specify	Pool all samples unless specified
Number of aliquots	Four	Specify and standardize	Specify and standardize
Position of patient	Semirecumbent	Specify	Specify
Area that is lavaged	Right middle lobe/lingula	Specify	Specify
Number of areas lavaged	One	Specify	Specify
Variability of lavage return	Discontinued lavage if difference between instilled and aspirated was > 100 mL	Report volume and percent of fluid returned; establish minimal percent recovered	Report volume and percent of fluid returned; at least 5% of instilled volume must be recovered
Reporting measurements of acellular components	Report per mL of fluid recovered	Report per mL of fluid recovered	Report per mL of fluid recovered
Sample storage	Specified	Specify	Specify

(FEV_1:FVC) is related to the yield of BAL return. The lower the ratio, the lower the proportion of BAL return.[12,13] Reduction in the FEV_1 with a normal FVC does not affect the percent return.[13]

Asthma is another obstructive disease. The obstruction in asthma can be induced by the procedure itself.[14,15] A major concern in asthma is the risk associated with the procedure. Recommendations for performing BAL in asthmatics have been made.[16] Lavage can be done in asthmatics.[17,18]

Cigarette smoking has profound effects on the cellular population found in the BAL fluid.[5] The largest effect is an increase in the number of macrophages, often 10-fold more cells than in nonsmokers.[19] Neutrophils also appear in increased numbers. Many feel that control subjects should be nonsmokers.[6] However, the patient population may be smokers. Therefore, changes attributed to the disease may actually be changes due to the smoking habits of the patient.

BAL is performed in acute respiratory distress syndrome (ARDS) patients.[20,21] However, the procedure can lead to significant hypoxia,[22] and modifications of the procedure may be necessary.[21]

The usefulness of BAL in various diseases is one of its strengths. Performing BAL in various diseases may lead to changes in the return of the fluid. However, this is not cause for avoiding the procedure; one must mention the underlying condition and its effects in any report of the procedure.

One should report the underlying disease of the patients and controls and report on the cigarette smoking history of all groups. Analysis of smokers versus nonsmokers should also be considered.

Suction Pressure during the Procedure

This effect is very apparent to the bronchoscopist during the BAL procedure, where airway collapse can occur if the suction pressure is too high.[23] The majority of physicians in one survey used low-pressure wall suction (< 60 mm Hg).[4] Some used pressure generated by a handheld syringe, adjusting pressure based on visualization of the airways. In either case, visual examination of the airway should allow monitoring for airway collapse during the aspiration process. During the lavage, it is recommended to keep suction pressure below 100 mm Hg and to avoid visible airway collapse.

Handling of Aspirated Fluid

The aspirated fluid can contain mucous material as well as the fluid itself, and the handling of this fluid can affect the results of the BAL.

Some groups have used filtering though gauze to remove the mucous material. However, the cells in the BAL fluid may variably adhere to the gauze, which can affect the cellular return of the BAL sample.[24] Also, gauze may affect the sterility of the sample.

Macrophages retrieved by BAL can be quite adherent to glass and similar surfaces. Macrophage adherence may be altered in various conditions, including cigarette smoking.[25] At the time of the lavage cells should be stored in silicone-coated or similar containers. Centrifugation to concentrate proteins and cells can lead to loss of cells. Washing the cells can change the differential count considerably.[26]

One recommendation is to avoid filtering with gauze. If it is used, it should be specified. Cell counts should probably be made on unfiltered, unwashed, and unconcentrated samples. If concentration is performed, the method should be specified.

Volume Instilled

The volume instilled during the BAL affects the BAL results. This has been shown for both the cellular and the protein contents of the BAL sample.[27–29] The major changes seem to occur during the first 100 mL of the process. The fluid aspirated after 60 mL instilled is significantly different from that after 120 mL instilled. From then on, the cells and proteins seem to level off. In a study of interstitial lung disease and control subjects, differences were not appreciated until at least 120 mL of fluid were instilled (Fig. 2).[27] This effect of volume on interstitial lung diseases has been noted by others.[30]

Rennard et al have further analyzed the first portion of the BAL fluid. They compared the results of the first 20 mL aliquot versus further BAL samples.[31] They found the first 20 mL to contain more epithelial cells. They also found proteins such as lactoferrin, and suggested that this 20 mL sample was a "bronchial" lavage.[31,32]

For a bronchoscopic BAL, most physicians choose to use at least 100 mL of instilled fluid. This is in line with the recommendations from all the groups summarized in Table 1.

Handling the First Aliquot Separately

The aspirated fluid after the first 20 mL is distinctly different from further aliquots.[31] Some investigators have proposed that the first aliquot be handled in a different manner,[31] or even discarded if one is studying diffuse interstitial lung diseases. However, the later aliquots contain a much larger number of cells than those retrieved after the earlier volumes of lavage.[30,33] In one study, it was determined that adding the results of fluid after the first 60 mL aliquot did not significantly change the overall lavage results.[33]

One can handle the initial fluid separately, especially if one is studying diseases that originate in the larger airways. For most diseases, it is acceptable to pool

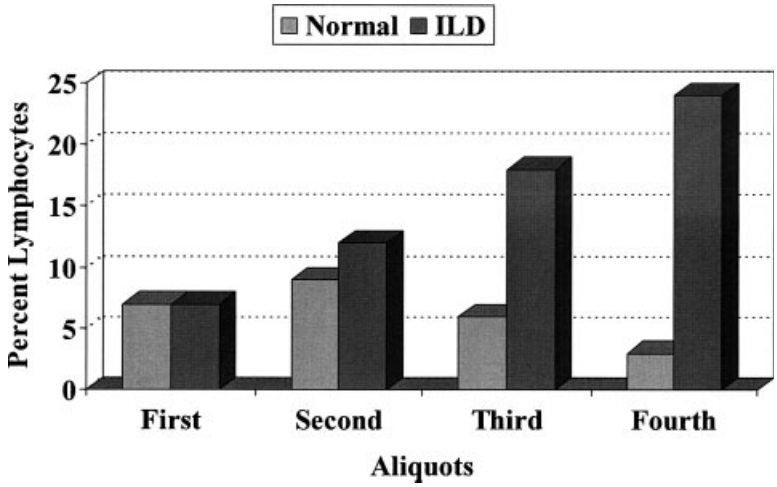

Figure 2 Percentage of lymphocytes in the aspirated fluid after each 60 mL aliquot of a bronchoalveolar lavage in either healthy volunteers or patients with interstitial lung disease, mostly sarcoidosis. The percentage of lymphocytes is significantly higher after the second aliquot (a total of 120 mL instilled). The differences become larger with larger volumes instilled, but the percent lymphocytes was not significantly different between aliquots three and four.[27]

all the aspirated fluid. One needs to specify what technique was used.

Number of Aliquots

Some have recommended a specific number of aliquots be performed during the lavage.[5] However, the number of aliquots is often based on the size of the syringes one has available at the procedure. As noted earlier, the volume of the lavage is crucial for BAL sampling. One can use six 20 mL syringes or two 60 mL aliquots to instill 120 mL fluid. There is little evidence to support one versus another aliquot number for the procedure.

Position of the Patient

The lavage process depends on gravity. Because suction pressure affects airways, one also relies on the fluid falling back toward the bronchoscope. The patient's position can affect the lavage. There are no studies comparing BAL performed with the patient standing versus supine.

Area That Is Lavaged

More important than the position is the area one wishes to lavage. Early on, it was appreciated that lavage in the lower lobes led to a smaller proportion of return than lavage in other areas. Therefore, larger volumes were instilled into patients who underwent lower lobe lavages.[34] Procedures were usually performed in the right middle lobe or lingua.[1] The majority of physicians would use these areas for patients with diffuse disease.

For most sarcoidosis, lavage in one area may provide as much information as lavage in another area.[10] On the other hand, significant differences have been seen between different lobes for idiopathic pulmonary fibrosis[10] in advanced sarcoidosis.[35]

In a study of patients with possible interstitial lung disease associated with scleroderma, Clements et al performed lavage in 18 patients. Lavage was performed in the lower lobe and either the right middle lobe or the lingula. In nine patients, there was agreement between the two areas lavaged. In four patients, the lavage was abnormal only for the lower lobe sample. In addition, the authors found that there was agreement between the ground glass seen on high-resolution computed tomographic (HRCT) scan and finding of abnormal BAL in the lingula or middle lobe, but not for the lower lobes.[36] This was not surprising because basilar lung disease often contains a mixture of ground glass and honeycombing (Fig. 3). The honeycombing represents an area with

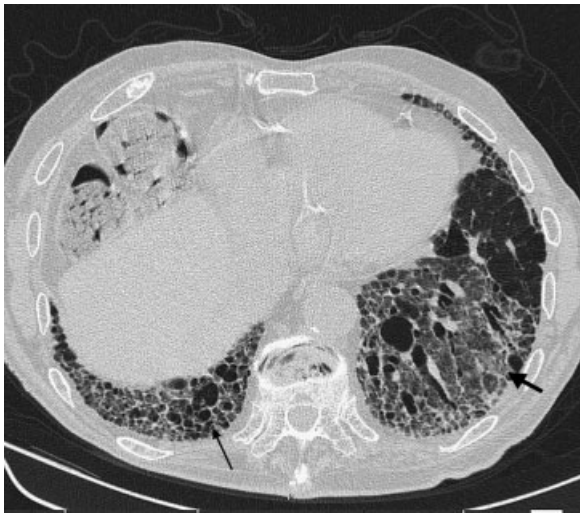

Figure 3 High-resolution computed tomographic scan of the lower lobe of a patient with both honeycombing (small arrow) and ground glass (large arrow).

collapsible lung and poor return. Therefore, lavage from these areas poorly samples the alveolar space. Others have also noted that ground glass on HRCT and BAL findings can be discordant in fibrotic areas of the lung.[37]

In evaluating a patient to undergo lavage, it may be wise to follow the rule of lavaging where the disease is. In evaluating BAL in patients with *Pneumocystis carinii* pneumonia, it was found that lavage in the upper lobes had a higher yield than the traditional right middle lobe or lingula.[11,38,39] This was found even though the volume returned was the same for the upper lobe as for the right middle lobe or lingula.[11,38] Therefore lavage can be done in the most affected areas of the lung. One should specify where the lavage is performed.

Number of Areas Lavaged

In the same way, the number of areas lavaged should be specified. For diffuse lung disease, one may wish to pool all the areas lavaged to get a higher number of cells and a more general response of the lung.[40] On the other hand, one may wish to compare the results of the BAL from one area to another. The number of areas lavaged should be standardized and specified. If the lavage aspirates are pooled, it should be specified.

Variability of Lavage Return

The percent of return is variable. As noted earlier, airway obstruction may affect results considerably. There are some conditions where the amount of return is immaterial to the value of the procedure. These include patients undergoing diagnostic procedures for malignancy and certain infections. In these circumstances, the finding of a cancer cell or *P. carinii* is considered diagnostic of disease. Whether the sample was a true representation of the alveolar space is immaterial.

For most circumstances, the lavage process is an attempt to give a good representation of the alveolar space. The effect of volume instilled has been shown to be important. The percent return is also important. If one aspirates less than 5% of the volume instilled, one is probably not getting an adequate alveolar sample. In one study of nonbronchoscopic lavage, it was shown that patients with a greater than 5% return of instilled fluid have a higher diagnostic yield.[41]

For safety reasons, some feel that the lavage should be stopped if the volume instilled becomes more than 100 mL larger than the aspirated volume. Because this will depend on the volume of the instilled aliquots, the bronchoscopist should be aware of the aspirated volume after each aliquot is instilled. If the difference between instilled and aspirated volumes becomes greater than 100 mL, one should stop lavage in that area. One may choose to perform a second lavage in a separate area if the patient is tolerating the procedure.

Although at least 5% of the instilled volume must be aspirated to be considered a true alveolar sample, smaller volumes may still identify diagnostic material. However, one needs to interpret the results of small volume returns as not necessarily true BAL samples.

Correcting for Bronchoalveolar Lavage Dilution and the Reporting of Acellular Components

This has been the topic of a specific ERS report.[6] One aspect of that report was an analysis of the limitations of the currently available markers of BAL dilution.[42]

During lavage the instilled fluid is mixed with the endogenous fluid in the alveoli, the epithelial lining fluid (ELF). The aspirated fluid contains a mixture of the instilled fluid and ELF. Determining the percentage of ELF, and hence the concentration of these constituents in the lung lining fluid, has been estimated by using a dilution marker. However, the alveolar space is also in contact with a vascular space. Water and solutes are in equilibrium between the two spaces. Fig. 4 shows the dynamic process that occurs during the lavage process, where solutes can transfer into the alveolar space. This process leads to the uncertainty of any measurement of the concentration of any material in the alveolar space.[3]

The most common endogenous marker has been urea.[43] The blood urea and ELF urea are assumed to be the same. Knowing the concentration of urea in the blood and in the aspirated fluid, one can calculate the dilution. This does provide a relatively good marker of dilution in many conditions. However, the amount of urea in the lung rises during the lavage process (Fig. 4). The dwell time during the bronchoscopy itself affects the measurements.[44] Conditions that increase epithelial permeability, such as ARDS and pneumonia, will also affect the results using this internal marker. Water has been shown to rapidly pass into the lung during the lavage process.[45] Thus it is unclear whether any internal marker can be used to measure all the changes that occur during lavage.[46]

The use of an external marker has also proved frustrating. Methylene blue was shown to be a relatively useful marker for some diseases.[47] Unfortunately, methylene blue is also taken up by the cells in the airway, reducing the concentration in the aspirated fluid. To date, all external markers have been taken up to a varying degree by the biologically active cells, such as macrophages.

One method has been to report per mL of aspirated fluid.[42] Using this correction method has allowed clinicians to quantitate the number of bacteria in the alveolar space and to therefore diagnose bacterial pneumonia.[48,49] Although this technique may fail to detect mild differences between groups, it should be able to detect changes that are 100-fold or greater.

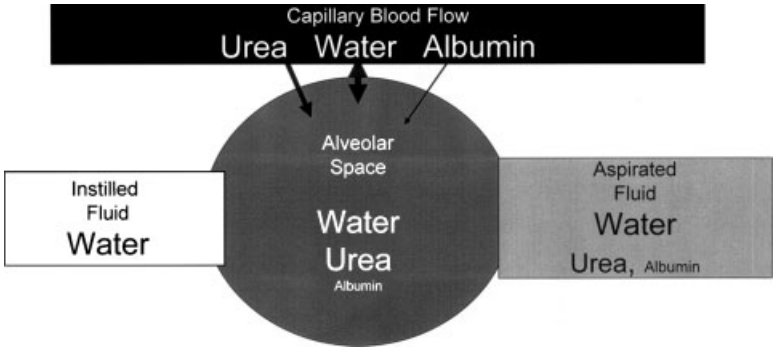

Figure 4 The various compartments involved during the lavage process. The instilled fluid contains only water and sodium chloride. The concentration of sodium chloride is the same in all four areas, so no change. The concentration of urea is similar initially in the capillary blood and alveolar space. The concentration of albumin is much higher in blood than in the alveolar space. During the lavage process, water passes freely between the capillary and alveolar spaces. Because the instilled fluid contains no urea or albumin, the lavage process dilutes the urea and albumin in the alveolar space. During the instilled time, urea in the capillary blood passes easily into the alveolar space, whereas albumin crosses more slowly into the space. The aspirate fluid contains urea from both the alveolar and the capillary compartments. Adapted from Baughman.[3]

The Storage of Fluid

The storage of BAL samples is crucial for the subsequent measurement of certain markers. Cells stored at 4°C can be analyzed up to 24 hours after the procedure without significant changes in the count and differentials.[50] Certain proteins may be temperature sensitive and the samples may need to be stored at −80°C. The handling of many of the proteins measured in the lung lining fluid is detailed elsewhere.[6]

CELLULAR ANALYSIS

In many cases, the most important information obtained from the BAL procedure is the analysis of the cellular population of the inflammatory cells retrieved by the lavage. This has been the case for interstitial lung diseases and infections.[51,52] Cellular populations have been used to differentiate between various interstitial lung diseases.[53,54] This is the subject of several other articles in this issue. However, there are limitations to using BAL cellular population to diagnose interstitial lung diseases.[55]

There are several factors that will affect the cellular population of the BAL sample. This is because different methods can be applied to measure the cells and these different methods can lead to different results. Table 2 summarizes some of the important steps in analyzing the cellular population of the BAL fluid.

Preparation of Cells for Analysis

The most commonly used method is the cytocentrifuge-prepared slide. A cytocentrifuge can concentrate the cellular sample without damage to the cells. It enhances sensitivity to detect infectious agents by as much as 30-fold.[56,57] For the dilute BAL sample, it has the advantage of being a rapid, easy method of concentrating cells onto a glass slide.

There are several conditions for the cytocentrifuge that can change the BAL differential cell counts. In a series of elegant studies De Brauwer and colleagues demonstrated that the speed and duration of the cytocentrifuge preparation affected the results.[58] Although the speed affected cell population, the change in mean percentage of lymphocytes varied from 19.8 to 23.3%. In examining clinical specimens for diagnosis, only one of 17 patients was misclassified using the group's computer program to analyze BAL cells.[53] The authors did not find the rate of acceleration affected BAL results.[58] Others have also felt that the changes due to different conditions of the cytocentrifuge were not large enough to be clinically relevant.[26,59] It was also shown that the cellular count

Table 2 Steps in Handling Cellular Population of Bronchoalveolar Lavage Fluid

Step	Usual Process	Others Used
Preparing cells for differential count	Cytocentrifuge	Millipore filter Smeared slide
Cellular staining	Wright-Giemsa*	May-Grunwald-Giemsa Papanicolaou
Number of cells	200	100–500
Reader of slides	Clinical laboratory	Research laboratory
Measurement of lymphocyte subpopulations	Flow cytometry	Immunohistochemistry

*May be modified (Diff-Quik, Sigma, St. Louis, MO).

was more reliable in the center of the cytocentrifuge specimen.[60] The percentage of lymphocytes was lower at the edge of the slide, suggesting cells may have been thrown off the slide.

Other methods for preparing the cells have led to changes in the differential cell count. The use of a Millipore filter (Millipore, Billerica, MA) to prepare the BAL sample has been studied.[61,62] The filter technique identifies a larger number of lymphocytes, which may be spun off during the cytocentrifuge procedure.[62] The Millipore filter method is time consuming and not widely performed. Another method is a direct smear of the slide, similar to that used for peripheral blood samples.[63,64] However, these samples are less concentrated, and therefore one is less likely to detect other conditions such as malignancy or pneumocystis.[65,66]

Cellular Staining

Several stains have been used to analyze the cells retrieved by BAL. Papanicolaou staining is the customary cytological stain and useful for detecting cancer and infection. It is not good at differentiating between inflammatory cells. Therefore the Wright-Giemsa and its variants have been used.[67] A modification of the Wright-Giemsa stain (Diff-Quik, Sigma, St. Louis, MO) is a rapid method allowing staining of the slide within a few minutes.[66] However, these techniques have their limitations. The cells must be adequately adhered to the slide prior to fixation.[68] Some cells are underestimated by this technique. Mast cells are better seen by toluidine blue staining.[69]

Number of Cells Counted

As for many situations, the higher the number counted, the better the sample reflects the whole population. In a study comparing differential cell counts, De Brauwer et al determined that between 300 and 500 cells counted provided a good representation of the number of nucleated cells for a BAL sample.[70]

For less common cells, a larger number of underlying cells may need to be counted. This would include mast cells. In studying BAL samples to quantitate the amount of *Pneumocystis jiroveci*, clusters of *Pneumocystis* were counted after every 100 nucleated cells were counted. For these less common events, at least 500 nucleated cells had to be counted to obtain a reliable reflection of the pneumocystis clusters.[71]

Reader of the Slide

Perhaps the most important issue is who is reading the slides.[72] Authors have noted that there can be a clinically significant difference in the cellular population with simply rereading the slides.[36] Some multicenter studies have tried to minimize this variability by using a core reading facility.[72,73]

Clinical laboratories often read the BAL cellular populations. It is not clear that the technicians have been trained to read these slides in a standard manner. There are some difficulties in appreciating the differences between macrophages and lymphocytes.[74] With training, these differences can be overcome. This would lead to a more standard reading of the cell population. In a comparison between a clinical hematopathology laboratory and a research laboratory, the correlation on lymphocyte percentage was significant but low ($R = .43$). In a comparison between two BAL research laboratories, correlation on these same slides was much better ($R = .91$). Part of the difficulty was training. After a 2-hour course on reading BAL cells, a hematopathology technician was able to improve her correlation with the BAL research laboratory ($R = .90$).[75] No standards exist for reading BAL samples. Therefore, the clinician must accept that inexperienced readers may influence the reading of BAL samples.

Measurement of Lymphocyte Subpopulations

Immunologic markers to measure T-lymphocyte subpopulations have been used to separate sarcoidosis from other interstitial lung diseases.[76,77] Although there is little question that some patients with sarcoidosis will have a high CD4:CD8 ratio, many patients do not.[55,78] It is also clear that some patients with extrinsic allergic alveolitis (hypersensitivity pneumonitis) will have a low CD4:CD8 ratio.[79] However, some cases of hypersensitivity pneumonitis will have normal or increased ratios.[80]

Table 3 Effect of Bronchoalveolar Lavage CD4:CD8 Ratio on the Diagnosis of Various Interstitial Lung Diseases

Diagnosis	Number of Patients	Odds Prior to BAL	< 0.5	0.5–3.5	> 3.5
Sarcoidosis	239	33.7	9.1	40.3	69.1*
Idiopathic pulmonary fibrosis	112	15.8	13.6	12.2	5.2†
Extrinsic allergic alveolitis	66	9.3	27.3†	17.2	12.5

*$p < .001$.
†$p < .05$.
Adapted from Welker et al.[77]

A recent analysis of a large cohort of patients evaluated for interstitial lung disease focused on 583 patients in whom a final diagnosis was made, including 239 cases of sarcoidosis. The authors determined the prebronchoscopic probability of a specific diagnosis based on clinical presentation and radiographic information. The BAL subpopulations were useful in enhancing the posttest probability of a specific diagnosis as indicated in Table 3.[77] This application of the Bayesian probability points out that BAL results are not usually viewed in isolation to other diagnostic features. This analysis also demonstrates the diagnostic value in some, but not all, situations.

The enumeration of lymphocyte subpopulations relies on monoclonal antibodies. The detection of these antibodies is often performed using immunofluorescent markers and flow cytometry.[81] The original scans were limited to only one stain. The investigator relied on forward and side scatter to separate lymphocytes from macrophages. The forward scatter determined size, the side scatter the presence of granules (such as seen in macrophages). The number of markers that can be stained at one time has increased as three- or four-color flow cytometers become more widely used.[82] A single sample can then be stained for a pan T marker as well as both CD4 and CD8 receptors.

An alternative is to perform immunohistochemistry staining on cytocentrifuge-prepared slides. This allows the reader to identify the characteristics of the cell (lymphocyte vs macrophage) and then comment on staining. This technique has been widely used by some groups.[77] Its major limitation is the number of cells that can be counted. Flow cytometry is often performed on thousands of cells, whereas immunohistochemistry usually counts 200 to 500 cells.

In addition to CD4:CD8 ratio, immune markers have been used to identify other cells in the BAL specimen. The CD1 marker stains Langerhans cells. The finding of more than 5% CD1 cells is highly diagnostic of Langerhans cell histiocytosis.[83,84] Malignant cells from leukemia or lymphoma have also been detected by immunohistochemistry.[85–87]

CONCLUSION

The utility of BAL relies a great deal on the ability to obtain the sample correctly. One limitation of BAL over the years has been the apparent resistance to standardizing the technique. Although there are some conditions in which the technique is not crucial, there are several conditions in which a standard approach to lavage will enhance its value. For most of the studies published, a standard technique has been used by the investigator. However, the major limitation has been transferring those results to other centers.

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