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TASK GROUP REPORT

Technical recommendations and guidelines for bronchoalveolar lavage (BAL)

Report of the European Society of Pneumology Task Group on BAL
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Bronchoalveolar lavage (BAL) is a useful and safe method for sampling cellular and biochemical components from bronchoalveolar lung units. Today, BAL is performed in centres worldwide, and its role as an important research tool is unquestioned [1, 2]. Although a large number of investigators have described the clinical usefulness of BAL in various diseases of the lung and have suggested the value of BAL for diagnosis and monitoring, there is no general consensus whether, and to what extent, BAL can be used clinically for routine diagnostic purposes. In particular, differences in technical procedures and in processing the BAL fluid so far appear to be one of the main obstacles to universal acceptance of BAL as an established clinical tool. In order to develop reasonable technical guidelines on how to perform and process BAL as well as to make clinical recommendations about the value and indications of BAL, the Executive Committee of the European Society of Pneumology (SEP) set up a European Task Group on BAL. This is the first report of the group, focusing on technical guidelines and recommendations for performance of BAL and processing the material. In the past year members of the group have tried to collect and review the available literature. Personal experiences of group members are also included.

Fiberbronchoscopy

Bronchoalveolar lavage (BAL) is performed predominantly under local anaesthesia with a flexible fiberscope. A recent international survey has shown that about 93% of centres which perform BAL use local rather than general anaesthesia [3]. However, BAL performed under general anaesthesia appears to yield similar results [4]. BAL can also be performed safely in patients with an oral or nasotracheal tube in position by inserting the fiberscope through the tube, provided its diameter is greater than 6 mm. Mechanical ventilation can be continued throughout the entire procedure if necessary.

Most centres use a standard size fiberscope with a sucking channel of about 2 mm. Although small diameter fiberscopes can operate in more distal segments of the bronchial system, the risk of bronchial collapse increases. On the other hand when using large diameter fiberscopes the instrument cannot be advanced as far and more bronchial than alveolar constituents are found in the BAL samples. Optimal recovery is accomplished by occluding the bronchial lumen with the bronchoscope. The tip of the bronchoscope is therefore advanced into a bronchial segment until a wedge position is reached. Coughing should be prevented because it can cause trauma, contamination with blood and loss of instilled fluid.

Premedication

Most centres use sedating compounds such as diazepam or meperidine together with atropine as premedication for fiberscopy. Atropine is intended to minimize vasovagally induced bradycardia as well as to decrease airway secretion. The influence of atropine on the yield of BAL cells has only been preliminarily addressed [5] demonstrating that atropine pretreated patients have a higher BAL return in comparison to a control group. The effect of atropine on the composition of noncellular components of BAL remains unclear.

Local anaesthesia

Local anaesthesia is usually accomplished by local application of lidocaine: 1) spray aerosol for treatment of the nasal, oral, pharyngeal and laryngeal area; 2) direct instillation *via* the bronchoscope for anaesthesia of the trachea, carinae and bronchi. Lidocaine present in the airways should be removed prior to instillation of the lavage fluid, since it may influence cell viability.

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Safety of BAL

BAL is a safe procedure. Minor side-effects include coughing during lavage, fever and chills some hours after lavage (which can usually be treated by use of simple antipyretics), transient alveolar infiltration in the dependent lung segment 24 h after the procedure, transient deterioration of lung function parameters such as vital capacity, forced expiratory volume in one second (FEV_1), and decrease of arterial oxygen tension (Pao_2). Consequences of saline lavage are expressed more in patients with underlying pulmonary diseases than in healthy volunteers. Most of the reported side-effects are closely related to endoscopic technique, location and extent of lavaged lung area, and the volume and temperature of instilled fluid (summary in table 1). Lung epithelial permeability, when using a volume of 250 ml instilled fluid in the middle lobe, is not altered when measured 24 h after BAL [16].

Supplemental oxygen delivery, ear-oximetry and ECG-monitoring are strongly advised in patients with severe underlying disease or other critical condition. Patients with mild asthma have been successfully lavaged [10]. However, patients with a history of bronchial asthma should be handled with special caution and careful monitoring is advised [9, 17]. The following are recomended,

1. Supplemental oxygen with a nasal prong should be administered throughout the entire procedure;
2. Premedication with aerosolized beta-agonists;
3. Ear-oximetry and ECG-monitoring.

Table 1. – Consequences and side effects of BAL

Side-effect	Occurrence	Reference
Alveolar infiltration	Rarely seen*	[6, 7]
Crackles	Within 24 h over dependent areas**	[8, 9]
Wheezing	In hyperreactive patients up to 1–2 weeks	[10]
Fever	10–30%, some hours after BAL**	[6, 7, 11]
Lung function impairment	Transient decrease of FEV_1, VC, PEF, Pao_2** $^{\$,\$\$}$	[9, 11–15]
	No effect on lung epithelial permeability 24 h after BAL	[16]
	Transient rise of $Paco_2$ in patients with COPD$^{\$,\$\$}$	[4]
Bronchospasm	Rarely in normoreactive, more frequently in hyperreactive patients	[6, 9, 10]
Pneumothorax	Only when transbronchial biopsies are performed	
Bleeding	Insignificant	[6]

*: risk increases with size of instilled lavage fluid volume and numbers of lavaged segments; **: risk increases with volume of instilled lavage volume; $^{\$}$: more likely in hyperreactive patients or in patients with severe underlying infiltrative lung diseases; $^{\$\$}$: supplemental oxygen prevents hypoxaemia during BAL, warmed saline at body temperature protects from bronchospasm and increases BAL recovery; BAL: bronchoalveolar lavage; FEV_1: forced expiratory volume in one second; VC: vital capacity; PEF: peak expiratory flow; Pao_2: arterial oxygen tension; $Paco_2$: arterial carbon dioxide tension; COPD: chronic obstructive pulmonary disease.

Site of lavage

A standard site of sampling should be used unless the shadowing is not generalized throughout the lungs but localized. In general, the middle lobe or the lingula are used as standard sites for BAL. From these lobes, about 20% more fluid and cells are recovered than from the lower lobes. [7]. From an anatomical point of view, the middle lobe is the most convenient lobe to be lavaged. The lower lobes are difficult to occlude or wedge with the bronchoscope, and more lavage fluid is necessary to obtain a satisfactory recovery. A survey among BAL centres revealed that 98% of the centres use a standard site and 84% of the latter use the middle lobe as their preferred site [3].

Inhomogeneity of lung involvement may also occur in diffuse interstitial lung disease. Several studies have addressed the interlobar variation of BAL cell differentials in interstitial lung diseases by performing a BAL on the right side and on the left side in the same patient and analysing the two sites independently [18, 19]. In patients with sarcoidosis, interlobar correlation was excellent for percentages of BAL T-lymphocytes. PETERSON *et al* [19] found, that discrepancies between both sides could be attributed to an uneven distribution of lesions in the chest radiograph.

In idiopathic pulmonary fibrosis (IPF), percentages of neutrophils showed a good interlobar correlation ($r=0.79$) between middle lobe and lingula; however, 35% of patients showed a discrepancy greater than 10%

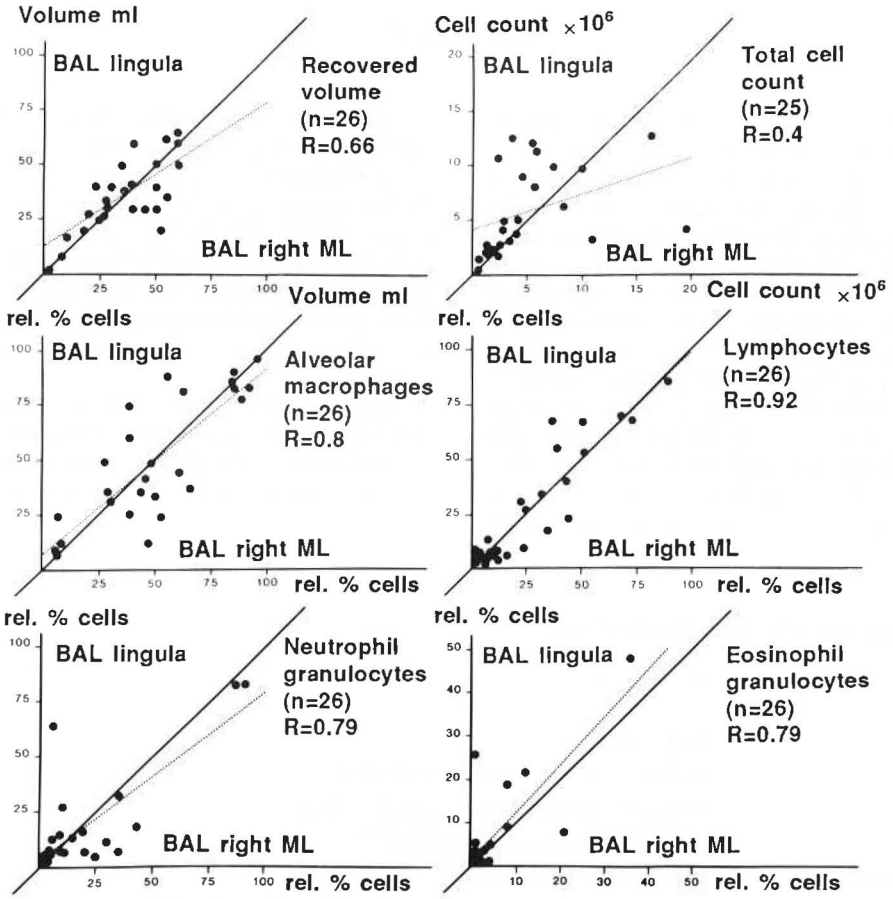

Fig. 1. – Comparison of interlobar correlation between right middle lobe (horizontal axis) and left lingula (vertical axis).

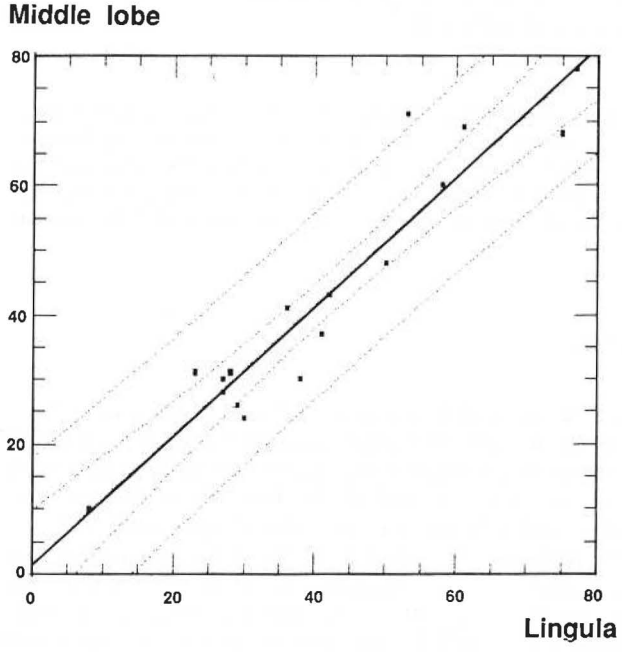

Fig. 2. – CD8+ T-lymphocytes (% of lymphocytes): interlobar correlation between right side (vertical axis) and left side (horizontal axis).

regarding the percentage of BAL-neutrophils between both sides [18]. Another study in 26 patients with homogenous lung lesions demonstrated a sufficient correlation of BAL differential cell counts between middle lobe and lingular. However, no correlation could be found for the total cell count [20] (fig. 1).

A further study addressed the question of whether the main lymphocyte subpopulations were also uniform at two different sites (middle lobe and lingula) in patients (n=17) with various interstitial lung diseases [21]. Staining with surface markers using the immunoperoxidase techinque [22] showed an excellent interlobar correlation for CD4+ T-lymphocytes (r=0.87), for CD8+ T-lymphocytes (r=0.95) and for Leu7+ cells (r=0.93) (fig. 2).

These observations indicate that in most patients with diffuse interstitial lung disease lavage at one site should give sufficient clinical information. In general, results obtained at one site should be representative for the whole lung. Depending on the nature of the disease and its heterogeneity, lavage of more than one site will reduce sampling errors. In patients with localized lesions, such as inflammatory infiltrates, malignant lesions etc, it is recommended that the area of greatest abnormality, as seen on the chest radiograph, should be chosen as the preferred site for BAL.

Sampling

Fluid used to perform lavage

The fluid used to perform bronchoalveolar lavage, must be a pyrogen-free saline solution (isotonic 0.9% NaCl, suitable for intravenous use). Lavage fluid should be warmed to body temperature (37°C) because it causes less cough and bronchospasm, less deterioration of lung function and, therefore, a better fluid recovery and increased cell yield of BAL in comparison to instillations of fluid at room temperature [7, 12]. However, most groups when performing BAL for diagnostic or research purposes have used fluid at room temperature [2, 23–28].

Methods to instil and recover the fluid

Saline fluid is instilled through the working channel of the fibreoptic bronchoscope as a bolus with a syringe, (the method usually employed), or by hydrostatic flow from a reservoir [25]. The fluid is usually recovered with the help of a suction trap to which mechanical suction is applied, is allowed to drain out under gravity [27], or is aspirated manually using the attached syringe [25]. When using mechanical suction (and hand suction) attention must be paid to ensure that excessive negative pressure is not applied avoiding collapse of the airways beyond the tip of the bronchoscope (which would impede fluid return), or trauma to the mucosal surface of the bronchus, causing bleeding and appearance of erythrocytes in the lavage. Negative pressures of 25–100 mmHg from normal clinical suction apparatus are usually appropriate. Occasionally, in patients with destructive lung disease, a few deep breaths can help the flow of fluid and increase the return volumes [23, 25]. After each instillation, the recovered fluid is collected in the syringe, in a sterile suction flask, in a plastic specimen trap, or in a siliconized vessel. The adhesion of macrophages to the walls must be avoided in order to prevent loss of mononuclear cells. Therefore, unsiliconized glass materials should not be used.

Volumes of fluid to be used

The greatest technical variation in carrying out bronchoalveolar lavage relates to the volume of fluid used. The volume infused by investigators usually ranges from about 100 ml to about 300 ml in each lung segment or subsegment lavaged. Much of the data on bronchoalveolar lavage cells and secretions derives from lavage performed with aliquots of 20 ml and a total lavage volume of 100 ml. Other research groups, however, think that 50–100 ml boluses, with a total volume of 300 ml are more appropriate, enabling more cells and proteins to be recovered for research purposes. In this context, the total number of cells collected seems to correlate well with the volume of fluid instilled and then recovered, especially when instilled lavage volumes are between 100 and 300 ml [26]. It has been demonstrated in nonsmokers that the proportion of alveolar cells in each bolus, when lavaged at the same site, was roughly uniform [29]. Data on proportions of bronchoalveolar cells reported by various groups performing lavage with different amounts of saline suggest that, at least in normals, the information about cell types obtained in volumes of 100–250 ml are comparable. Smaller volumes of instilled fluid carry the risk that a more "bronchial" washing component dominates the cellular picture. In this case the BAL cells are more likely to be characterized by the presence of increased numbers of neutrophils, particularly in the first two aliquots [30, 31].

The yield of cells is significantly dependent on the condition of the pre-alveolar airways. Recovery of fluid in the case of obstructive disorders of the airways may be markedly attenuated for example in chronic bronchitis [32], and asthma [30] and the recovered cells may show higher concentrations of neutrophils. Thus, the recovery of BAL fluid exerts an influence and seems to be responsible for the considerable intersubject variability seen in the recent study of ETTENSOHN and coworkers [33].

The problem of standardization of technique and data is more complicated when dealing with quantification of non-cellular components of the lavage fluid. In this context, if it appears that the concentration of soluble substances depends on the lavage volume, it is also clear that the concentration of soluble factors decreases, as the lavage volume increases, in a way that is not predictable by simple dilution [29, 34]. Alternatively, a different approach to obtain higher amounts of cellular and non-cellular components from the lower respiratory tract is to perform a 100 ml lavage in different sites of the lung of the same subject (usually three sites are lavaged, the lingula, a segment of the left lower lobe and right middle lobe) [35].

Although bronchoalveolar lavage is a safe technique, the incidence and importance of the side-effects appear different using small versus large lavage volumes (table 1). It has been demonstrated that patients receiving small volumes had only minor reduction of vital capacity, which returned to baseline within 24 h, and all other parameters were unchanged. In contrast, there was significant, although reversible, reduction in several functional parameters within 30 min after a large volume lavage [14]. It is unclear how long the instilled fluid must remain in the lung segment before being aspirated. A small delay of a few seconds, allowing the patient to breathe one or two times, may result in a better mixing in the lung segment between saline and cellular and non-cellular components of the alveolar surfaces. Lavage fluid is probably absorbed across the alveolar surfaces which suggests not waiting too long before retrieving it [8, 35].

Should the first aliquot of lavage be processed separately?

Several investigators have carried out cell differential analysis on sequential aliquots of the recovered lavage fluid. When using sequential boluses of 60 ml it has been demonstrated, that in nonsmokers the proportions of alveolar cells in each bolus are roughly uniform [29]. However, in smokers the first "wash" is different from all subsequent boluses [2]. Characteristics of the first "wash" are the relatively high proportion of epithelial cells and polymorphonuclear leucocytes and fewer macrophages, suggesting a more "bronchial" than "alveolar" sampling. Using small volumes, however, the percentage of fluid returned from the first "wash" and the number of cells recovered is generally low. In the absence of airway inflammation, the cellular and acellular components contribute little to the total lavage and, therefore, have only a small influence on the overall results. In contrast, when the subjects undergoing bronchoalveolar lavage have a disorder affecting the bronchial portion of the airways, or if there are obvious signs of bronchial inflammation at bronchoscopy, then the analysis can be heavily influenced by the contribution of the bronchial airways and the same workers then recommend that the first sample of bronchoalveolar lavage should be collected and analysed separately [2, 31, 37]. Other workers believe, that such samples are useful in indicating bronchial inflammation, but think it unlikely that alveolar components can be accurately differentiated.

Mucus filtration

Many workers consider that the bronchoalveolar fluid must be strained through a sterile gauze to trap large mucous particles [8, 23]. Nylon mesh or a very loose single layer mesh of cotton gauze is usually appropriate and, especially in smokers and patients with bronchial inflammation prevents mixture of the mucous particle with the cell pellet after centrifugation. The process of straining the recovered fluid, even through a single layer of cotton gauze, may cause loss of cells and other components of the lavage (see section "Dust and Minerals"); however, the full effect has not yet been clearly investigated. Other workers consider, that filtration should be avoided for routine diagnostic purposes because of the risk of loss of cells and other components and instead they remove the surface layer of mucus after the first centrifugation of the lavage sample [24]. Filtration of BAL fluid preferentially removes bronchial epithelial cells and, therefore, should be avoided if changes in this cell type may be of importance [34].

Total and differential cell counts

After measuring the recovered volume and performing total cell counts, the normal method of processing the cells from the BAL specimen for differential counting is to prepare cytospins. Preparation of a set of at least six slides is recommended. After air-drying one is stained with an appropriate differential white cell stain; May-Grünwald-Giemsa or Diff-Quick stains are preferably used. However, since Diff-Quick underestimates the number of mast cells, the May-Grünwald-Giemsa stain is recommended.

The remaining air-dried preparations are stored, unstained, at room temperature, so that special staining can be performed if requested. Routinely, the slides are not only viewed for the differential cell counts but also screened at low magnification (objectives of x10, x25) for the presence of unusual morphological features, *e.g.* dust particles, microorganisms, tumour cells, haemosiderin laden macrophages, or acellular aggregates suggestive of alveolar lipoproteinosis [38]. When unusual features are detected, special stains can be undertaken, *e.g.* PAS, iron, or silver stains. The macroscopic appearance of the BAL fluid can also give a preliminary suggestion of the diagnosis, *e.g.* a milky aspect is suggestive of alveolar lipoproteinosis and an aliquot can be immediately fixed for electron microscopy for final confirmation. A dark-orange colour may be present in haemosiderosis and other types of alveolar bleeding. This should be confirmed by iron stain.

Total cell counts

The total cell yield is normally assessed in the well-mixed original fluid or after the first centrifugation. Usually, a haemocytometer (Neubauer, Fuchs-Rosenthal) is used for cell counting. Some investigators prefer an automated method such as a Coulter counter. They should take care that the number of cells is not underestimated because the size of the macrophages may be outside of the "window" settings used. The total cell count can be expressed both as the absolute number of cells recovered per lavage, or as the concentration of cells per ml of recovered lavage fluid. Screening of cell viability may be performed by the Trypan blue exclusion test. Washing procedures may result in a considerable loss of cells. After two or three centrifugations, the average loss of cells represents 22%, 32% or 34% of the original number present, according to different authors [39–41].

Cytocentrifuge preparations

There are different methods for making cytocentrifuge preparations. They can be made by spinning 400–1000 µl (depending on total cell number) of unconcentrated pooled BAL fluid. Other investigators

prefer to perform the cytospins after the first centrifugation (500 g, 10 min) of the native fluid. After the first centrifugation, the cells are resuspended in buffered medium, *e.g.* minimum essential medium (MEM), RPMI-1640 or Hank's balanced salt solution. A volume of fluid containing 5–20x10^4 cells is placed in the cytocentrifuge (usually a Cytospin-2-centrifuge is used). Cytocentrifugation speed (23–165 g) and time (4–10 min) varies considerably in individual laboratories [38–40,42]. These differences may affect the results of the differential cell counts. In one study the speed of 90 g yielded 33.2±25.3% lymphocytes *versus* only 26.7±22.1% lymphocytes at 23 g, ($p<0.0001$) [40]. Another study, using millipore filter preparations as "gold standard" for the recovery of lymphocytes, showed a relative recovery of 62±17% lymphocytes at a speed of 400 rpm, in comparison with a recovery of 82±12% lymphocytes at 800 rpm [39]. For this reason, a speed of 800 rpm or 90 g is recommended in order to approach the "ideal" lymphocyte count as closely as possible.

Another reason for the under-estimation of lymphocytes by cytospin techniques may be the addition of serum to the medium. It has recently been shown that addition of 10% autologous serum to Hank's balanced salt solution reduces the percentage of lymphocytes from 16.8 to 11.3 in otherwise identical cytospin conditions [43]. This was also the concentration of fetal calf serum which was used in the comparative study of cytocentrifuge *versus* millipore filter preparations; a comparison with serum–free medium was not undertaken [39]. We strongly recommend use of serum-free medium for cytospin techniques.

Cell smears

Although cytocentrifuge preparations are recommended as a routine procedure to obtain cell differential counts, preparations of cell smears can give additional information in terms of detecting tumour cells or *Pneumocystis carinii*, if these features are present in low numbers. However, preparation of hand-made cells smears needs considerable experience.

Millipore filter preparations

In addition to preparation by smear or by cytocentrifugaton, millipore filter preparations can be prepared from BAL specimens [39]. Most laboratories use 200,000 cells per filter and use gravity drainage. It is essential to keep the samples moist as drying will severely distort cytological features. Millipore filter preparations can be stained with a modified haematoxylin-eosin [39] or with a PAP technique [44]. The latter results in excellent preservation of nuclear and cytoplasmic detail. The similarity of findings on BAL and routine cytology stained by the PAP technique facilitates the diagnosis of cancer [44] and the detection of viral inclusions [45]. Such samples are also adequate for differential counting, but slight differences have been observed from cytocentrifuge preparations. Lymphocyte counts tend to be higher in the millipore filter preparations, as a fraction of the lymphocytes appear to be lost in the cytocentrifuge [39]. Conversely, a fraction of the neutrophils appears to be lost in the millipore filter during filtration [42]. It is likely that millipore filter preparations will be useful in a number of settings.

Counting of cell differentials

The differential counts are made by viewing with a light microscope, employing objectives of x40 or x100. At least 300–500 cells should be counted (random field counting method) in order to reach a good reproducibility. The spectrum of cells include alveolar macrophages, lymphocytes, neutrophils, eosinophils, mast cells and occasionally plasma calls. Ciliated or squamous epithelial cells should be registered but not included in the differential cell count. A high percentage of epithelial cells (>5%) is indicative of contamination of the alveolar samples by bronchial inflammatory cells. Interpretation of such BAL probes may be difficult, especially for neutrophils.

Definition of normal values, expression of results

There are no consistent quotations in the literature regarding normal values for the relative distribution of BAL cells. One problem is that in the data published so far there are only small numbers of normal patients/persons (mostly volunteers) available for comparison. The other problem is that cigarette smoking changes the pattern of cell distribution by increasing the number of neutrophils. Useful reference values are tabulated by COSTABEL and co-workers [46] and REYNOLDS [2].

For practical reasons the following percentages can be expected as normal within nonsmokers: lymphocytes <15%; neutrophils <3%; eosinophils <0.5%. Smokers usually have a decreased percentage of lymphocytes (<7%).

Although there are no clear indicators that the age of the patient undergoing lavage is of importance when looking at normal values of cell differentials, it has been demonstrated, in healthy nonsmoking volunteers at least, that the CD4/CD8 ratio in BAL lymphocytes increases proportionally with the age of the lavaged subjects [47].

Monoclonal assays

Special immunofluorescent (IF) and immunocytochemical techniques can be applied to better characterize the nature of some of the cells recovered from BAL, especially lymphocytes. These methods rely on the use of monoclonal antibodies directed against antigens present on the cell surface of lymphocytes and/or monocytes/macrophages. Monoclonal antibodies are basically used as a research tool in order to investigate the phenotypes of BAL cells. In addition, they can contribute to the diagnosis and clinical management of various interstitial lung disorders.

For IF technique the current method is staining and observation of viable cells in suspension. The cells are incubated either with a fluorochrome-labelled monoclonal antibody directly (direct IF technique) or with an unlabelled primary antibody first and then with a secondary labelled antibody directed against the primary antibody (indirect IF technique). Indirect techniques are more sensitive than direct techniques.

Advantages of IF techniques are:
1. Automatic counting by flow cytometry.
2. Double labelling easily possible to study the parallel expression of two different surface markers on a single cell.

Disadvantages of IF techniques are:
1. No permanent recording.
2. No morphological details.
3. Interference with autofluorescence of macrophages.

Peroxidase methods are most commonly in use for immunocytochemical techniques. Newer methods are streptavidin-biotintechniques or the use of alkaline phosphatase instead of peroxidase for labelling. A highly sensitive technique is the peroxidase antiperoxidase (PAP) technique, also called unlabelled antibody enzyme technique, where a PAP-immunocomplex is linked by a bridging antibody to the primary antibody.

Advantages of immunocytochemical techniques are:
1. Use of conventional light microscope,
2. Permanent recording of the reaction.
3. Excellent morphology.
4. Higher sensitivity than IF.

Disadvantage of immunocytochemical technique is:
1. Double labelling possible, but time consuming.

Handling of cell suspensions

The cells, recovered from the bronchoalveolar lavage, are washed three times with Hank's solution, resuspended in cell culture medium (*e.g.* RPMI-1640) and then counted in order to prepare a cellular suspension at the concentration of $10x10^6$ cells/ml. The viability of the cells is checked using the Trypan blue exclusion test. The percentage of dead cells must be less than 5%.

Provided highly specific monoclonal antibodies are available, it is usually not necessary to separate different cellular components before testing samples. However, a morphological control must always be performed during the final microscopic count since, for instance, CD4-related monoclonal antibodies may stain with surface antigens belonging to the macrophagic lineage and thus may alter the final cell counts [48].

Analysis by immunofluorescence

100 µl of the cell suspensions (1×10^6 cells) are incubated for 30 min at 4°C with the optimal amount of purified (indirect method) or conjugated (direct method) monoclonal antibody. The most widely used reagents are fluorescein-isothiocyanate (FITC, green)- and phytoerythrin (PE, red) - conjugated monoclonal antibodies [49]. The amount of antibody to be used for each determination depends on its original concentration; in general, 5–20 µl represent the right amount.

Following the incubation, 2ml of cold Hank's solution is added to the pellet and washed at 300 g for 10 min at 4°C three times. If the indirect method had been used, a fluorescent anti-mouse immunoglobulin is then added. As a control, to demonstrate nonspecific immunofluorescence, the same quantity of anti-mouse immunoglobulin is added to a sample of cell suspensions that had not been previously incubated with monoclonal antibodies. Following a 30 min incubation at 4°C, the sample is again centrifuged at 4°C at 300 g for three times. If the direct method is used, the control is represented by monoclonal antibodies without specifity for cells under study belonging to the same isotype as those utilized in the test and directly stained with fluorochrome. The pellet is then resuspended and the percentage of positive cells counted using a fluorescent microscope by evaluating at least 300—500 cells. It must be taken into account, that macrophages may show autofluorescence particularly in smokers.

Double fluroescence analysis

Using the double fluorescence technique [50] it is possible to identify two different determinants simultaneously expressed on the membrane of the same cell. This concept deals with the use of two different fluorochromes (usually FITC and PE). Both these fluorochromes are excited by UV radiation but they display a different spectrum of light emission (green for FITC and red for PE). With the simultaneous use of an FITC-conjugated monoclonal antibody and a PE-conjugated monoclonal antibody, and changing the filter during evaluation with the fluorescent microscope, it is possible to identify cell subpopulations:
a. Cells stained in red (PE+);
b. Cells stained in green (FITC+);
c. Cells that express both fluorescences (FITC+ and PE+);
d. Unstained cells (FITC- and PE-).

To rule out the possibilty that cells under study bind MoAbs *via* Fc-receptors, cell suspensions must be evaluated following incubation with gamma-globulin Cohn-fraction II and further washing.

Analysis by immunocytochemistry

A suitable method for monoclonal assay of BAL cells is the immunoperoxidase slide assay [51] which makes use of the PAP-technique. The method has been described in detail elsewhere [22]. The advantages of this method are as follows.

1. The number of cells needed to study one monoclonal antibody is very low (20,000 cells per reaction area). Thus, the number of lymphocytes for testing a battery of monoclonal antibodies is sufficient from a BAL specimen with a low percentage of lymphocytes such as those from normal smokers, and when low total lavage volumes of 100 ml are used.
2. The expensive monoclonal antibodies are applied in low amounts (only 20 µl of a dilution 1/50 or 1/100), leading to reduced costs for this method.
3. The cell morphology is excellent, much better than in air-dried cytospin immunocytochemical preparations, because the cells keep their three-dimensional shape and do not shrink due to the avoidance of air-drying, of centrifugation and of stronger fixatives like acetone. This allows accurate selection of different cell populations for counting on the basis of morphology.

The method in brief: 10 µl of cell suspension ($2x10^6$ cells/ml) are allowed to settle on each of 12 circular reaction areas of commercially available glass slides. The cells are not air-dried. Special care is taken in all preparative steps that the cells are always covered with fluid. To prevent the cells becoming dry, a humidified chamber is used. Attached cells are fixed with a very low concentration of glutaraldehyde (0.05%) to block Fc-receptors and preserve cell morphology. A gelatine-containing minimum essential medium (MEM) (with 0.2% gelatine, Merck, and 0.1% bovine serum albumin buffered with HEPES, pH 7.4) is used for dilution of antisera to avoid binding of immunoglobulins to the glass surface. The staining procedures are performed as listed. The incubation times for each step are short and last for only 5–10 min.

The steps are:
Step 1: Incubation with monoclonal antibodies against various surface antigens.
Step 2: Rabbit anti-mouse immunoglobulin.
Step 3: Swine anti-rabbit immunoglobulin.
Step 4: Peroxidase-antiperoxidase complex from rabbit.
Step 5: Diaminobenzidine-H_2O_2, followed by postfixation with OsO4.
The slides are covered with glycerine and a coverglass.

To evaluate the reaction the slides are viewed under a light microscope with a magnification of 400–1,000 fold. Positively reacting cells are clearly recognized by a dark brown granular staining of the cell membrane. At least 200 cells (lymphocytes or monocytes/macrophages) of each reaction area are counted to determine the percentage of positive cells.

Reagents available for differentiation of mononuclear cells

Some terminology problems have arisen because of the endless numbers of monoclonal antibodies produced against antigens of the haemopoietic system and because monoclonal antibodies against the same antigen are often differently labelled when they are independently produced by different laboratories and/or companies. In order to overcome this problem, during three recent workshops on human leucocyte differentiation antigens (Paris 1982; Boston 1984; Oxford 1986), an attempt has been made to classify monoclonal antibodies directed against the same determinants into discrete families, the so called "clusters of designation" (CD). According to the above quoted workshops [52–54], the main characteristics of the most important CD of lymphocytes T, B, and cytotoxic cells (including NK cells), are summarized in tables 2–5.

To define interleukin-2 receptors (IL-2R) on different types of cells (activated T,B, NK and myelomonocytic cells), monoclonal antibodies belonging to the CD25 are available including anti-Tac, IL2R1, OKT26, Dako-IL2-R [53, 54].

Numerous studies have described markers of macrophage activation and a number of monoclonal antibodies have been produced against myelomonocytic

Table 2. – Clusters of designation for T-cells

Cluster	Reactivity	Specific Mo Ab
CD1 a, b and c	thymocytes (cortical)	Leu6-OKT6-T6-BMA 060
CD2	SRBC receptor	Leu5-OKT11-T11-9.6
CD3	pan-T (T3 molecule)	Leu4-OKT3-T3-BMA 030
CD4	helper/inducer (HLA-class II restricted)	Leu3-OKT4-T4-BMA 040
CD5	pan-T	Leu1-OKT1-T11-10.2
CD7	wide spectrum pan-T	Leu9-OKT16-WT1-3A1
CD8	suppressor/cytotoxic (HLA-class I restricted)	Leu2-OKT8-T8-BMA 081

Table 3. – Clusters of designation for B-cells

Cluster	Reactivity	Specificity Mo Ab
CD19	wide spectrum pan-B	B4-HD37-Leu12-OKpan B
CD20	pan-B	B1-Leu16-IF5
CD21	C3d receptor (cR2)	B2-OKB7-CR2
CD22	progression signal	Leu14-HD39-OKB22
CD23	Fc-IgE receptor	Leu20-MHM6-Blast 2
CD24	wide spectrum pan-B	BA1-OKB2
CD38	includes plasma cells	Leu17-OKT10-T10

CD37, CD39, CDw40, CD30, CD31 and CDw32 also display pan-B reactivity.

Table 4. – Myelo/monocyte correlated clusters of designation

Cluster	Reactivity	Specificity Mo Ab
CD11	C3bi-receptor and p150	OKM1,Mol (b)-LeuM5(c)
CD13	myelomonocytic lineage	MY7-OKM13-MCS2
CD14	monocytic lineage	MY4-LeuM3-UCHM1-Mo2-OKM14
CD15	myelomonocytic lineage	LeuM1-FMC10
CD33	myelomonocytic lineage	MY9-L4F3
CD36	monocytes and platelets	OKM5-5F1

antigens. These types of reagents are not, however, completely standardized since some problems have been encountered related to their production. In table 4 a tentative list of clusters of designation defining myelo/monocytic lineage is reported [54]. It is evident that there are only a few reagents that can discriminate between myeloid and monocyte antigens. In addition, reagents defining discrete steps of monocyte differentiation are still not available and an ontogenetic model is still lacking.

It seems appropriate at this point to mention that monoclonal antibodies against human leucocyte antigen (HLA) A, B and C (Class I-MHC) nonpolymorphic antigens and Class II nonpolymorphic antigens (including DR/DP/DQ) may be useful for defining monocyte/macrophage activation and these reagents have been used to characterize macrophages in different interstitial lung disorders [55–57]. Furthermore, other antibodies have been used to study pulmonary alveolar macrophages, including PAM-1 [58] and CB12 [59] monoclonal antibodies. However, the usefulness of these reagents and their specific reactivity is far from being precisely characterized.

The monoclonal antibodies currently used to routinely study BAL cells and their frequency in healthy subjects are summarized in table 6. Values refer to normal nonsmokers; they are taken from the standards used in

Table 5. – Monoclonal antibodies to define cytoxic lymphocytes

Cluster	Reactivity	Specific Mo Ab
CD 57	granular lymphocytes	HNK1 (Leu7)
CD16	Fc receptor IgG	NK15 (Leu11a)-3G8-VEP13 Ab8.28-B73.1(Leu11c)-OKNK
not assigned	NK cells, NK precursors	NK9
CD 56	non MHC-restricted cytoxic cells	N901-NKH1 (Leu19)
CD8	Class I MHC-restricted cytoxic cells	OKT8-Leu2-T8-BMA o81

Table 6. – Control values related to lymphocyte subpopulations recovered from the bronchoalveolar lavage as defined by monoclonal antibodies

Cluster of differentiation (available in the market)	Specificity	Controls (range) 10 nonsmokers
CD1 (Leu6, OKT6, T6)	thymocytes Langerhans cells/histiocytosis-X cells can be distinguished by morphological criteria	0–3%
CD2 (Leu5, OKT11, T11)	receptor for sheep red blood cells	82% (70–90)
CD3 (Leu4, OKT3, T11)	pan-T	75% (63–88)
CD4 (Leu3, OKT4, T4)	Includes class II-MHC restricted helper/inducer cells	53% (36–70)
CD8 (Leu2, OKT8, T8)	includes class I-MHC restricted supressor/cytotoxic cells	28% (15-40)
CD19 (Leu12, B4)	pan-B	5% (0–12)
CD25 (a-Tac, a-IL-2R, OKT26)	IL-2 receptor	2% (0–5)
HNK-1 (Leu-7)	granular lymphocytes	7% (1–14)

the laboratories of the authors of this chapter. In heavy smokers an increase of CD8 positive cells with a consequent reduction of the CD4/CD8 ratio can be observed [60].

The specific use of monoclonal antibodies and their practicability in different interstitial lung disorders is extensively reviewed by a wide range of authors [1, 2, 28, 60–64].

Flow cytometry

Flow cytometers fitted with fluorescence as well as light scattering detectors provide one of the most advanced systems for detecting and quantifying surface and intracellular markers on cells in suspension [65]. They have been widely applied in basic research to evaluate antigens expressed on cells using monoclonal antibodies, and have the advantage, over conventional light microscopy methods, that large numbers of cells can be rapidly analysed and simultaneous multiparameter quantitive measurements performed. In spite of their advantages, the use of flow cytometers in many fields of clinical investigation, including bronchoalveolar lavage, has been restricted by the expense of the instruments and the special training required to operate them. However, lower cost instruments have now become available and flow cytometry is increasingly becoming the method of choice over the more subjective, time consuming, manual methods for detecting and enumerating cell surface and intracellular markers.

Principle of flow cytometry

In brief, operation of a flow cytometer with fluorescence activated cell analysis and sorting capabilities involves injecting cell suspensions, under pressure, into the centre of a stream of sheath fluid in a flow chamber establishing a co-axial flow which constrains the cells to the centre of the stream so that they flow in a single line. After emerging from the nozzle of the flow chamber, the narrow stream passes through a laser beam focused on the centre of the stream and intersecting it at right angles. As cells pass through the laser beam they scatter the laser light and also emit fluorescent light if they have been labelled with fluorochromes (*e.g.* labelled monoclonal antibodies).

Light scattered in the forward direction over a narrow angle (approximately 2° or 3° on either side of the beam) is collected by a forward angle scatter detector and converted to electronic signals which give an indication of the size of the cell. Light scattered at right angles (90° scattered light) is also detected and gives an indication of the internal characteristics of the cell such as granularity. Fluorescent light emissions are collected by a "collecting lens" and wavelengths of different colours (related to different fluorochromes) can than be directed to separate detectors (photomultiplier tubes) by means of a dichromic reflector. The signals are processed by an electronics console, amplified, and then digitized and passed to a computer for storage and analysis. If cell sorting is required, the computer can be used to define the population required (by setting a "sorting window" or "gate"), then the nozzle assemby is vibrated by a piezoelectric transducer to break the stream into drops. Drops containing a cell required can be positively or negatively charged and then deflected to the right or left as they pass two charged deflection plates. The deflected drops can then be collected into suitable sample tubes.

Many different types of flow cytometer are available but the minimum requirement for most current applications is that the machine must be fitted with both forward angle and 90° scatter detectors and a minimum of two, or preferably three fluorescence detectors. For application with bronchoalveolar lavage (BAL) samples it is also important that the machine should have the capacity for logarithmic, as well as linear, acquisition of data and for non-rectangular "gating". Argon ion lasers may be tuned to produce beams of suitable wavelength to excite most commonly used fluorochromes, including fluorescein isothiocyanate (FITC) and phycoerythrin, which are frequently employed in studies using monoclonal antibodies.

Preparation of samples

Preparation of BAL cells for flow cytometric evaluation of cell surface markers involves collecting the cells from fresh BAL samples by low speed centrifugation, washing, resuspending at a suitable cell concentration (*e.g.* $1x10^7$ cells per·ml^{-1}), and then staining the suspension with monoclonal antibodies using direct or indirect immunofluorescence staining techniques as described in detail in the section on "Monoclonal Assays".

Slightly different modifications of staining procedure have been used by a small number of research groups who have currently reported work on flow cytometric analysis of BAL samples [66–69], but there are still insufficient data to recommend precise standardization of procedure. BAL cells are usually stained for flow cytometric analysis without the need for any prior separation of lymphocytes or other cell types. For satisfactory analysis of stained BAL samples by flow cytometry, it is essential to filter the stained samples (using a non-cellular adherent material such as nylon gauze), immediately prior to running them through the instrument, to remove all clumps of cells and any residual mucus which can interfere with accurate cell analysis and risks clogging of the nozzle of the machine. It is also most important that there is minimal contamination of the sample with erythrocytes since they overlap and interfere with accurate analysis of the lymphocyte population due to their similar light scattering properties. A method for overcoming this problem, recommended for use with BAL samples, has recently been reported [70]. When evaluating the results

of flow cytometric measurements of BAL samples it is also essential to selectively "gate" different cell populations for separate analysis because different types of cells in BAL have different amounts of background autofluorescence. Autofluorescence of lymphocytes is low and relatively constant, but autofluorescence of alveolar macrophages is much higher, especially in smokers, and can mask specific fluorescence on lymphocytes if the entire cell population is analysed.

Current applications of flow cytometry with BAL

The major current application of flow cytometry with bronchoalveolar lavage has been evaluation of BAL lymphocyte subsets and activation markers in sarcoidosis and extrinsic allergic alveolitis (hypersensitivity pneumonitis), using BAL cells labelled with monoclonal antibodies and stained by immunofluorescence methods [66–69]. The advantage of flow cytometry for studying mixtures of cell types, as occur in BAL samples, is that different cell populations can be identified on the basis of objective measurements of their forward angle (related to size) and 90° (related to granularity) light scatter characteristics and/or their reactivity with a specific monoclonal antibody. Each cell population (*e.g.* lymphocytes) can then be selectively "gated" so that other measured parameters such as T-cell subsets within that population can be accurately assessed. The manual methods for assessing T-cell subsets by immunofluorescence microscopy have the disadvantage that, although the total number of positive and negative cells can be counted, it is much more difficult to accurately select different populations of cells for counting on the basis of morphology. Evaluation of double stained preparations to detect cells simultaneously expressing more than one surface marker is also much more easily achieved using flow cytometry.

In 1982, Ginns *et al.* [66] reported analysing T-lymphocyte subsets in BAL samples from four patients with idiopathic pulmonary fibrosis (IPF) and six with sarcoidosis using monoclonal antibodies and flow cytometry. Fresh lavage samples were filtered through a single layer of sterile gauze and the cells collected and resuspended in phosphate buffered saline (PBS) (pH 7.4) at a lymphocyte concentration of $2x10^7$ cells·ml^{-1} then stained with monoclonal antibodies (OKT3, OKT4 and OKT8) at 4°C for 30 min, treated with buffered ammonium chloride to lyse erythrocytes, then washed and stained with FITC conjugated anti-mouse antigen at 4°C for 20 min. The cells were then fixed with 2% formaldehyde in PBS for 5 min at room temperature, then washed and kept in PBS prior to analysis. Analysis was undertaken using a Spectrum III flow cytometer (Ortho) and the lymphocyte population was "gated" on the basis of 90° *versus* forward angle light scatter prior to analysis of fluorescent emission. As a control for background staining, BAL cells incubated with control mouse ascitic fluid were used in place of monoclonal antibody and the control employed to check the purity of the lymphocyte "gate" was an aliquot of BAL cells stained with the monoclonal antibody OKTM1 which reacts with monocytes and granulocytes. The authors concluded that in IPF there is a lower proportion of OKT4+ cells and higher proportion of OKT8+ lymphocytes in BAL compared to blood, but in sarcoidosis there is an increase in OKT4+ and a decrease in OKT8+ cells in lung lavage. Thus, these results using flow cytometry are similar to those of other workers using immunofluorescence microscopy or immunocytochemical methods.

In 1982, Pacheco *et al* [67]. used flow cytometry to seek evidence of BAL lymphocyte activation by analysing the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content of BAL T-lymphocytes from four patients with stage I, and eight patients with stage II, sarcoidosis and from four patients with acute extrinsic hypersensitivity pneumonitis. They concluded that T-cells with an increased DNA content, indicative of proliferation, could be demonstrated in hypersensitivity pneumonitis and stage II sarcoidosis but not in stage I sarcoidosis. They used an acridine orange staining technique to detect DNA and RNA, and the stained cells were analysed to Cytofluorgraf H50 (Ortho).

In 1983 and 1984, Mornex and co-workers [68, 69] used flow cytometry to investigate BAL T-lymphocyte subsets and activation markers in a small number of patients with sarcoidosis and hypersensitivity pneumonitis. They employed fresh BAL samples filtered through sterile gauze and then stained aliquots of the total cells by immunofluorescence. They used the OKT series of monoclonal antibodies to detect T-cell subsets and used monoclonal antibodies to HLA-DR to detect "Ia like" antigens expressed on activated T-cells. The samples were analysed in a Cytofluorgraf H50 (Ortho) using light scatter measurements to "gate" the lymphocyte population. To determine the levels of background staining the controls were cells treated with FITC-labelled anti-mouse Ig alone, and the control employed to check the purity of the lymphocyte cluster was the Mo2 monoclonal (Coultronics) to detect contaminating monocytes. They concluded that T-Cells bearing markers of activation can frequently be detected in BAL but not in the blood of patients with these granulomatous lung diseases.

In conclusion, although most current reports on flow cytometric studies of BAL samples have been confined to lymphocytes, the techniques have considerable potential for investigation of surface and intracellular markers on other types of cells in BAL samples. The problems due to variable autofluorescence on macrophages can be adequately overcome for the analysis of many markers by appropriate "gating" and use of controls. The validity of flow cytometric analysis of BAL samples is indicated by the preliminary results on the BAL lymphocyte subsets which are consistent with those of earlier studies using immunofluorescence microscopy or immunochemical techniques. The need for expert operators trained in both flow cytometry and immunology must be emphasized.

Non-cellular components

In recent years, analysis of the soluble components of the epithelial lining fluid (ELF) has gained significant attention. Widespread research has been applied in this field. A variety of methods for preparation have been developed and various assays for a large number of the components of ELF have been employed (table 7). Today, the number of species detected and quantified in the lower respiratory tract continues to increase. While the clinical application for this technology remains uncertain at present, the current chapter is designed to give an overview and some preliminary recommendations for the types of analyses which have been undertaken. However, definite recommendations cannot yet be given.

Table 7. – Solute components detected in BAL fluid*

Solute	Approximate concentrations**
Total protein	70 $\mu g \cdot ml^{-1}$
Albumin	20 $\mu g \cdot ml^{-1}$
Immunoglobulins	
IgG	2.5–10 $\mu g \cdot ml^{-1}$
IgG1	1.6 $\mu g \cdot ml^{-1}$
IgG2	0.8 $\mu g \cdot ml^{-1}$
IgG3	0.06 $\mu g \cdot ml^{-1}$
IgG4	0.18 $\mu g \cdot ml^{-1}$
IgA	2.5–6 $\mu g \cdot ml^{-1}$
Free secretory piece	700 $ng \cdot ml^{-1}$
IgM	100 $ng \cdot ml^{-1}$
IgE	0.06–0.3 $ng \cdot ml^{-1}$
Alpha-1-antiprotease	1-2 $\mu g \cdot ml^{-1}$
Alpha-2 macroglobulin	0.04 $\mu g \cdot ml^{-1}$
Low molecular weight bronchial inhibitor	+
CEA	0.8 $ng \cdot ml^{-1}$
Transferrin	4 $\mu g \cdot ml^{-1}$
Fibronectin	30–150 $ng \cdot ml^{-1}$
Leucocyte elastase	+
Collagenase	+
Angiotensin convertase	+
Lipid polar	78 $\mu g \cdot ml^{-1}$
Lipid non-polar	45 $\mu g \cdot ml^{-1}$
PGE	200–2000 $pg \cdot ml^{-1}$
6 Keto F 1α	20-400 $pg \cdot ml^{-1}$
TXB	25–85 $pg \cdot ml^{-1}$
PGF 2α	30 $pg \cdot ml^{-1}$

*: data collected from BARTH 1987 [130]; BELL 1981 [131]; GADEK 1979 [132], 1981 [133]; LOW 1978 [134]; MERRILL 1980a [100], 1980b, 1981, 1982, 1985 [135–138]; RENNARD 1981 [139]; REYNOLDS 1974 [8], 1977 [25]; VILLIGER 1981 [140]; WARR 1977 [141]. **: concentrations are estimated for unconcentrated BAL fluid; CEA: carcinoembryonic antigen; PGE and PGF: prostaglandin E and F, respectively; TXB: thromboxane B. +: present in BAL fluid.

Origin of soluble components

Soluble BAL components may originate from various sources:

1. Passive transudation (*e.g.* albumin or orosomucoid);
2. Active transport (*e.g.* IgA, IgM);
3. Local production (secretory component).

Quantitation of epithelial lining fluid

Attempts to quantitate soluble components in BAL fluid are difficult because completely reliable denominators or reference substances are not yet available. The problem is that the saline used for lavage significantly dilutes the epithelial lining fluid that is sampled. Differences in lavage technique probably result in varying degrees of dilution, thus accounting for much of the variation amongst studies [2]. The simplest method for expressing the quantity of a substance in the recovered BAL fluid is by multiplying the concentration by the volume recovered.

Several so-called denominators have been suggested to function as a reference substance. Ideally this should be a substance which will maintain a regular static concentration within the alveolar space without being changed by the underlying disease. The main problem is that the recovered fluid represents a mixture of cells, of epithelial lining fluid and of other epithelial lining components. Most investigators use albumin as the denominator assuming that the albumin present in the epithelial lining fluid is diluted to the same degree as any species of interest. This could allow for comparison of results amongst study groups and amongst investigators. However, the concentration of albumin in the epithelial lining fluid is unknown since various diseases alter the integrity of the epithelial capillary membrane [23, 26]. This complicates the drawing of conclusions concerning comparisons between albumin ratios in BAL fluid obtained from patients with lung disease and values determined in normal subjects.

Alternatively, the use of potassium has been suggested in order to standardize protein concentrations in bronchial washings. This method has been criticized in the past because potassium may diffuse into the fluid during the lavage or can be released from lysed cells in the BAL fluid thus complicating the analysis [29].

Methylene blue has been suggested as an external marker of dilution [71]. However, methylene blue might be lost by diffusion or binding to pulmonary cells and the dilution of the markers by the ELF is very low, complicating its practical use [72]. Urea has also been proposed as an internal marker of dilution [73]. However, since urea can diffuse across the membrane its practical use as a marker of dilution strongly depends on the time lag between instillation and sampling of the BAL fluid ("dwelling time") and requires that the BAL be accomplished as quickly as possible [72].

In conclusion, there is general agreement that no ideal denominator or method of calculation exists to quantitate the dilution factor. Since the underlying disease may alter fluid recovery, all data should be expressed by stating the volume of instilled fluid, the volume of

recovered fluid and the percentage of recovery. Comparison to a standard substance may be necessary if: a) a substance is present in the serum and also derived from local lung production; and b) the percentage of the recovery is markedly reduced by the underlying disease.

Concentration steps

Different approaches have been used to measure the components of epithelial lining fluid samples by bronchoalveolar lavage. These include chemical assays, immunoassays, bioassays and functional enzyme assays. The concentration of many species which are of interest is quite low in BAL fluid because of the dilution involved. Therefore, concentrating steps may be necessary. For this purpose methods of lyophilization, pressure filtration and chemical extraction have all been utilized. Each of them have advantages and disadvantages for their specific application. The disadvantage of lyophilization is that it also concentrates the salts present in the lavage fluid, often making a dialysis step necessary. Aggregation of certain protein species can also be a problem.

Pressure filtration is widely utilized because of its convenience; but can only be used in cases of relatively large molecular mass. Some of the proteins tend to stick to the filters used in the procedure.

Chemical (LIPID) extraction is useful for species like prostaglandins or surfactants. For some species oxidation can be a problem.

Osmotic dialysis is another method to be considered.

In conclusion, no method of specimen concentration should yet be regarded as a standard procedure.

Characterization of immunoassays for bronchoalveolar lavage

Radio-immunodiffusion is frequently used for measurement of proteins. Commercial kits are widely available. These kits are not sensitive enough to detect certain substances present in BAL fluid at very low concentrations. Therefore, a concentrating step is necessary.

Radio-immunoassays (RIA) and enzyme-linked immunoassays (ELISA) are more sensitive than radio-immunodiffusion. They are generally used to quantify non-protein species in bronchoalveolar lavage fluid, including prostaglandins and leukotrienes.

Laser-nepholometry is often sensitive enough to detect species of interest for albumin and immunoglobulins without prior concentration.

Functional assays have been utilized to quantify molecules of special pathophysiological interest and bronchoalveolar lavage fluid such as neutrophil chemotactic activity.

In conclusion, it is advisable to use unconcentrated BAL samples, since any concentrating step might diffuse the actual quantity of the species. ELISA or an RIA-test are often applicable in this setting etc.

General recommendations

1. A total volume not below 80–100 ml should be used for analysis.
2. The first aliquot recovered might be different and should be analysed separately. Remaining aliquots should be pooled.
3. The exact volume of instilled and recovered BAL fluid should be recorded.
4. BAL cells should be separated at an early stage.
5. Some substances may not be stable during storage.

In conclusion, it is emphasized that all approaches to quantitation of species in the alveolar lavage fluid are seen to have major restrictions. All molecular substances and their concentrations are largly dependent on a gradual exchange between several lung compartments. It must be borne in mind that any fluid instilled into the alveolar lumen initiates processes of response which are characterized by exchange of species between blood, alveolar space and interstitial tissue. As yet, there is no reliable method which can be recommended for estimation of absolute concentration of soluble BAL components. For the time being all investigative efforts have to be regarded as of laboratory and research interest.

Dust and minerals

The technique of bronchoalveolar lavage (BAL) has proved a sensitive method for detection of inorganic particles in the lungs relating to a wide range of occupational and environmental exposures [74–84]. The information can discriminate exposed from unexposed individuals, but the relationship between levels of different components and disease is still unclear for the majority of exposures, due to the small number of cases so far studied. Asbestos exposure has been most extensively investigated and evidence of a quantitative relationship between asbestos-body counts in BAL and the severity of interstitial disease has been obtained [75] confirming previous conclusions from studies of biopsy and post-mortem lung tissue [85, 86].

Three approaches employing BAL have proved useful:

1. Detection of certain types of particles during routine cytological screening of BAL cells is of clinical

value in suggesting that an occupational history should be carefully checked and the possibility of occupational disease considered.

2. Mineralogical analysis can then be used to identify the particles. This is especially useful in cases without a known history of exposure and also to clarify the situation in cases with a history of mixed dust exposure.

3. Quantification of particles is also useful for exploration of the relationship between the levels of dust in the lungs and the development of disease, in the hope that limits of diagnostic value might be defined.

Cytological appearance of particles

Particles are often observed by light microscopy in the conventional slide preparations of lavage cells used for routine cell counting. The presence of ferruginous bodies amongst the cells is an indication of exposure to dusts of various kinds, including asbestos, [75, 77, 87–89], talc [77, 80] and glass fibre. Examples of the appearance of these different types of ferruginous bodies in BAL cytocentrifuge preparations have been reported [38, 77]. Ferruginous bodies have been reported in BAL sample controls without occupational exposure [84], but their detection appears to require the screening of much larger volumes of fluid than used in conventional cytocentrifuge preparations where, in our experience, their presence has always led to disclosure of a relevant history. It is recommended that cytocentrifuge preparations for routine diagnostic purposes are prepared without pre-filtration to avoid the risk of loss of fibres and other components. Uncoated fibres, too small to form ferruginous bodies, are not readily detectable by light microscopy and are usually investigated by electron microscopy (see below). On the other hand, it should be emphasized that light microscopy is more sensitive than electron microscopy for detecting ferruginous bodies [75, 82–84]. Particles within the cytoplasm of alveolar macrophages are also frequently detectable by light microscopy in routine cytocentrifuge preparations. Smoking is the usual explanation, but in other instances the appearances may suggest exposure to inorganic dust. In particular, the presence of highly refractile particles can indicate exposure to crystalline and metallic particles including crystalline silica, coal dust, hard metal [76, 77, 81], albumin [78] and chromium-cobalt-molybdenum alloys used in dentistry [79].

Mineralogical analysis of dust particles

In routine clinical investigation, when dust particles are observed at the level of light microscopy, questioning of the patient is often sufficient to disclose a detailed history of exposure. However, methods to identify the particles are useful when there is no clear history of exposure related either to occupation or to hobbies, or when there is a history of multiple exposures. In research, since most dusts are heterogenic in their chemical composition, identification of components selectively retained in the lungs is also of value to indicate more precisely the components which may have greater relevance to disease.

Particles in lavage samples have mainly been analysed using electron microscopes fitted with energy dispersive X-ray spectroscopy systems. For analysis of fibres and ferruginous bodies, 5–25 ml of fresh BAL samples are usually treated with an equal volume of filtered sodium hypochlorite to digest the organic material. Aliquots of the suspension equivalent to 5 ml of BAL are then filtered onto polycarbonate Nucleopore membrane filters of 0.2–0.4 μm pore size and 25 mm diameter. The particles collected at the upper surface of the membrane are embedded in carbon film and transferred onto grids for examination by transmission electron microscopy [74, 75, 81] or scanning electron microscopy [87].

Analysis of uncoated fibres (fibres defined as having an aspect ratio of at least 3 : 1) or the cores of ferruginous bodies can then be undertaken using an energy dispersive X-ray spectrometer fitted to the electron microscope. This records the spectrum of the characteristic X-ray photons emitted from the elements in the specimen following their bombardment by the electron beam. This method achieves the rapid and simultaneous analysis of elements above atomic number 8 and the technique has been applied to identify different types of asbestos fibres in lavage samples [87], and also to explore the heterogeneity of fibres in BAL in cases exposed to dust [84].

Extraction methods have also been used by some investigators to analyse nonfibrous particles in BAL samples, [78, 79], but others have employed filter preparations of intact BAL cells coated for scanning electron microscopy [81] or BAL cell suspensions blocked and sectioned for transmission electron microscopy [77]. Using scanning EM and energy dispersive X-ray microanalysis, intracellular levels of silicon have been investigated in granite workers [81] and particles within alveolar macrophages relating to a wide range of exposures, including asbestos, hard metal, talc, silica and constituents of printing inks, have been identified and quantified using scanning transmission EM and energy dispersive X-ray microanalysis [77]. Crystalline and amorphous forms of particles can be distinguished by detecting electron diffraction.

These independent reports from various centres have all concluded that BAL provides a sensitive and specific method to detect and identify inhaled particles of many types.

Quantification of particles

Demonstration of dust in the lungs is an indication of exposure but is not evidence of disease. Quantification of asbestos bodies and fibres extracted from open lung biopsy and post mortem tissue has demonstrated that the lung tissue burden is increased in asbestos workers compared with that in the general population [90], and in patients with asbestosis the lung tissue fibre burden is even higher and appears to relate to the severity of the disease [85, 86]. BAL studies have also

shown that the mean ferruginous body count per ml of BAL fluid is higher in asbestos exposed subjects than in controls (determined by light microscopy using sodium hydroxide extracts of BAL spun onto cytocentrifuge preparations) [84] and that the mean total fibre count per ml determined by electron microscopy is also significantly greater than in controls [87]. DE VUYST *et al* [75] have also shown that asbestos body counts in BAL (sodium hypochlorite extracts on 0.45 μm Millipore filters counted by light microscopy) correlate with the type of disease being higher in asbestos-exposed patients with interstitial lung disease than in those with benign pleural disease or malignant mesothelioma. In addition it was shown [82] that BAL concentrations of ferruginous bodies are positively correlated with the lung parenchymal concentrations studied from material obtained by open biopsy or autopsy. A BAL containing more than 1 AB/ml is highly predictive of a lung tissue concentration exceeding 1,000 AB·g.

There is currently no known tissue level of particles above which development of disease in inevitable. However, quantification is usually on the basis of total fibre counts without enumeration of the different fibre types present. Technical difficulties, for example the presence of iron and calcium in the ferroprotein coat of bodies, can interfere with identification of some fibres so that accurate quantification on the basis of individual fibre types is difficult. An alternative method for quantifying the range of inorganic particles within alveolar macrophages in BAL appears less subject to such problems [77], but further work is needed to assess the value of this method in larger groups of exposed individuals with and without disease and in unexposed controls. Individual suspectibility of humans to the development of occupational lung disease may be influenced by many factors including individual difference in rate of clearance and degradation of different types of particles.

Identification and quantification of different types of particles retained in the lungs, using the opportunity offered by BAL to study large numbers of cases prospectively, has the potential to address this question.

BAL and asbestos-related malignancies

The value of BAL for investigation of the types of retained fibres associated with mesothelioma may be limited, because washings from the air-spaces are unlikely to reflect the types of particles which gain access to the pleural and peritoneal surfaces accurately. BAL may prove more useful for the study of fibre types in patients with asbestos-associated carcinoma but this also awaits further investigation.

Electron microscopic analysis

Electron microscopic study of BAL cannot be performed in routine diagnosis. The technique, as well as the analysis, is time consuming and must be saved for research projects or for a few diagnostic purposes such as diagnosis of alveolar proteinosis, histiocytosis-X, amiodarone pneumonitis, typing of tumours and mineralogical quantification [83, 87, 91, 92].

A sample of lavage cells containing at least $3x10^6$ cells (the cell pellet must be visible macroscopically) is centrifuged and the pellet promptly fixed in cacodylate buffered glutaraldehyde at a concentration of 2.5% for 1 h at 4°C. The cells are washed in buffered cacocodylate containing 1% saccharose and the cell pellet is postfixed in 1% osmium tetraoxide and embedded in epon. Between each step, the pellet is put back to suspension. This avoids differential sedimentation and cellular distortion. Ultra-thin sections are prepared and contrasted with uranyl acetate and lead citrate.

BAL in lung cancer and other malignancies

Bronchoscopy is traditionally the most effective means for obtaining a morphological diagnosis of lung cancer. In centrally located tumours, direct forceps biopsy and transbronchial needle puncture usually yields excellent results especially in terms of staging.

In peripheral lesions, which are outside the usual range of access for bronchoscopy, the diagnostic yield by means of brush or catheter biopsy is significantly lower. Bronchial washings with small amounts of fluid have for a long time been successfully used to obtain a cell yield from peripheral lesions. In small and peripheral malignant lesions, in particular, bronchial washings or BAL can give valuable additional results to plain brushes or collection of bronchial secretion.

At the present time, a few studies have reported successful utilization of BAL in patients with primary lung cancer [44, 93, 94], in patients with pulmonary Hodgkin's disease [95, 96], and with non Hodgkin's lymphoma [97] in patients with metastatic pulmonary spread of non-lung cancer [98, 99]. In conclusion, so far no standardized procedure or protocol has been developed for this particular purpose. However, some practical recommendations can be given:

1. The fibrescope should be inserted in the dependent segmental bronchus where the lesions can be located. Fluoroscopic guidance is recommended.

2. Bronchial washing should be performed with a varying amount of fluid dependent on the percentage of recovery. Fluid recovery in these patients largely depends on specific tumorous alterations of the airways such as stenosis or partial atelectasis. At least 2 or 3 aliquots of 20 ml saline should be instilled and sucked back.
3. Bronchial washings immediately after brush biopsy can improve the yield of malignant cells. However, contamination with blood is usually the consequence.
4. A plastic catheter inserted through the fibrescope can be used in order to better and selectively reach the tumour. Again, fluoroscopic guidance is recommended.
5. In the case of multiple disseminated lesions within a lobe, or within several lobes, the usual procedure for BAL can be applied. Cellular and fluid yield, however, is different in different lobes. The best yield of fluid can be expected by using the middle lobe or the left anterior upper lobe. A smaller recovery is usually observed when lavaging the upper lobes. Recovery can be improved by letting the patient cough during the sucking procedure. Transbronchial lung biopsy may accompany BAL procedure when investigating disseminated lung lesions.
6. Bronchial washing can be used in selected patients as part of a "mapping procedure" in a particular clinical setting to confirm a diagnosis (*e.g.* the sputum of a patient shows malignant cells, but chest radiography is negative). Segmental bronchi can be selectively lavaged in a consecutive order. The recovered aliquots should be collected in separate vessels with separate labels. A maximum of 500 ml saline should be used in this procedure for a single patient.
7. Cells are prepared by the usual techniques and cytospin preparations can be stained with Papanicolaou and May-Gruenwald-Giemsa. Specific morphological changes of malignancy are usually examined more accurately using the Papanicolaou stain than the Diff-Quick stain [44]. In the case of low numbers of cancer cells it is helpful to use in addition smear preparations or sections of cell pellets.
8. There is little experience with detection of tumour markers in lavage fluid. Measurement of carcinoembryonic antigen (CEA) levels has been proposed [100], but no definite recommendation can be given as yet.

Techniques for infectious agents

Bronchoalveolar lavage (BAL) is the preferred method for obtaining specimens from lower airways and the lung in immunocompromised patients with pulmonary infiltrates for the following reasons: a) it samples a representative area of the lung; b) it has a high yield for *P. carinii* and other parasites, viruses, fungi and bacteria.

Safety of BAL in immunocompromised patients

With an appropriate technique BAL has a minimal bleeding risk even in patients with low platelet counts. As immunocompromised patients with extensive pulmonary infiltrates are at risk of developing respiratory failure blood gas analysis prior to bronchoscopy, continuous monitoring of the ECG and administration of supplementary oxygen during and after BAL procedure are necessary. Continuous monitoring of oxygen saturation or percutanous oxygen tension is suggested. In patients with respiratory distress and severe hypoxaemia despite supplementary oxygen BAL should only be performed if subsequent mechanical ventilation of the patient can be instituted.

Detection of P. carinii

Currently BAL is the method with the highest yield for detection of *P. carinii* with a sensitivity exceeding 90% using appropriate techniques [27, 101, 102]. *P. carinii* can be detected by the following methods: Wright-Giemsa stain (*e.g.* Diff-Quick), Gram-Weigert stain, Toluidine-blue stain, silver stain (Gomori-Grocott). In addition, monoclonal antibodies are now commercially available to identify *P. carinii* .

In order to increase the yield of *P. carinii* in BAL the following technical considerations are recommended:
1. BAL should be performed with at least 100 ml of NaCl 0.9%, and preferably more than 200 ml if the patient is not at risk of respiratory failure. The volume used in children should be reduced according to the vital capacity.
2. Bronchoalveolar lavage fluid should not be filtered through gauze as *P. carinii* is often found in mucous material otherwise excluded from analysis.
3. At least six slides with more than $2x10^5$ cells per slide should be screened before a negative report for *P. carinii* is recorded.
4. Wright-Giemsa (*e.g.* Diff-Quick) or Gram-Weigert stains should be used to screen for *P. carinii*. If these preparations are negative a silver stain (Gomori-Grocott) or a Toluidine-blue stain is recommended. If a silver stain (Gomori-Grocott) is performed, a quality control slide known to contain *P. carinii* and a slide without *P. carinii* should be processed in the same batch.

BAL is not an adequate method for monitoring the success of treatment in AIDS patients with *P. carinii* pneumonia as *P. carinii* was found in BAL of AIDS patients three weeks after treatment and was observed to be independent of the clinical outcome [103]. However, in these patients *P. carinii* cannot usually be found in BAL 10–15 days after treatment has been successfully employed. Thus, in such patients a second BAL might be useful when the clinical situation does not improve.

Detection of cytomegalovirus and other viruses

To detect cytomegalovirus (CMV) infection of the lung, various diagnostic means have been proposed. Direct cytological examination particularly of PAP stained preparations, can reveal viral inclusions characteristic of CMV or herpes [45]. For detection of CMV, direct cytological evaluation performed on either cytospin preparations or smears stained by Wright-Giemsa or Papanicolaou has a poor sensitivity compared to direct antigen detection by either immunofluorescence or immunochemistry [104]. In addition viral cultures and DNA-probe analysis can also be applied [105, 106]. Although the latter has the best diagnostic yield it is expensive and not yet available to all laboratories. These methods should be used if Wright-Giemsa and Papanicolaou stains are negative.

The clinical significance of detection of CMV in BAL has yet to be established. CMV pneumonitis has been a major problem following allogenic bone marrow transplantation. Whilst the significance of CMV in BAL of AIDS patients is more controversial, it is also felt to be a pathogen in these patients [107]. Other viruses can be detected in BAL with appropriate culture techniques.

Detection of Mycobacteria

It is possible to detect Mycobacteria in BAL either by appropriate culture techniques or by direct staining with Ziehl-Neelsen or Auramin-Rhodamin on cytocentrifuge preparations.

Detection of fungi

Fungi such as *Candida, Aspergillus, Cryptococci, Nocardia* and *Histoplasma* can be identified on cytocentrifuge preparations or on concentrated smears using silver staining, Gram-Weigert staining or periodic-acid-schiff staining. Direct detection of the antigen in BAL using monoclonal antibodies is possible for some of these agents (*e.g. Cryptococci*).

Detection of bacteria

It has recently been proposed that BAL may be an efficient method for diagnosis of bacterial infections of the lung [108]. By quantitative culturing of bronchoalveolar lavage fluid colonization and contamination of the specimen can be differentiated from infections of the lower respiratory tract. As the region sampled by BAL is considerably larger than the area sampled by protected brushes, quantitative cultures of bronchoalveolar lavage fluid in combination with blood cultures may in the near future be the methods of choice for investigation of pulmonary infiltrates compatible with bacterial pneumonia in the immunocompromised host. When performing cultures, appropriate care must be taken to detect *Legionella* sppf direct fluorescence assays being particularly helpful.

Detection of other microorganisms

Occasionally other microorganisms (*e.g. Toxoplasma, Cryptosporidia*) can be visualized on direct examination with Wright-Giemsa and Grocott staining.

Technical recommendations for BAL in immunocompromised patients

BAL for detection of infectious agents is performed and processed using the same techniques as for patients with interstitial lung diseases. To further enhance the recovery of fungi and cells infected by CMV an aliquot of the cell pellet, recovered after the first centrifugation, can be embedded in paraffin and processed like a histological specimen or used to prepare smears. In summary the following specific recommendations are given when BAL is performed in immunocompromised patients:

1. Prior to BAL, blood gas analysis should be obtained. During BAL, supplemental oxygen should be administered and ECG-monitoring should be continuously applied.
2. BAL in adults should be performed with at least 100 ml NaCl 0.9%, and preferably more than 200 ml unless respiratory failure is expected.
3. A sufficient number of cytocentrifuge slides containing more than $2x10^5$ cells should be prepared.
4. Filtering through gauze should be avoided.
5. The following stains should be used: Wright-Giemsa, Ziehl-Neelson and optionally Gomori-Grocott, Gram-Weigert, Papanicolaou, perdiodic-acid-schiff, Toluidine-blue.
6. According to the clinical circumstances cultures for Mycobacteria, viruses, bacteria and fungi are recommended.

Bronchoalveolar lavage in lung transplantation

Combined heart-lung transplantation (HLTx) offers a successful method treatment for patients with severe pulmonary vascular disease [109] and end-stage chronic lung disease [110], including cystic fibrosis [111]. Single lung transplantation (LTx) has been used to treat cryptogenic fibrosing alveolitis [112]. However, the main complicating event remains the rejection of the transplanted organ. Rejection usually starts in the lung (obliterative bronchiolitis) rather than in the transplanted heart. From studies in animals [113], and post-mortem and open lung biopsy material in humans [114, 115], the characteristic histological abnormality of acute rejection appears to be circumscribed perivascular lymphocytic infiltrates. Extension of these infiltrates into alveolar septa and bronchiolar walls occurs later and finally alveolar space inflammatory exudates are seen

[111]. Transbronchial lung biopsy (TBB) shows that these changes will resolve after treatment with augmented immunosuppression [116–118].

Potential applications of BAL in HLTx

Experience of BAL in HLTx is limited [111]. BAL not only samples a larger volume of lung than sampled with TBB but also a large number of cells are retrieved, offering a unique means of studying the cell-mediated response of the recipient to the donor lung. There are three levels of management in which BAL would be helpful.

Diagnosis of opportunistic lung infections..BAL has an established role in the diagnosis of both *Pneumocystis carinii* pneumonia [119] and cytomegalovirus pneumonitis [120], (see also "Techniques for infectious agents").

Diagnosis of rejection. The allograft response is a T-cell lymphocytic mediated reaction. The T-cell subsets involved in the process are as yet unknown, but work using immunocytochemical techniques is in progress. In rejection BAL shows an increased number of lymphocytes in absolute numbers but not in percentage counts. Similar changes in absolute lymphocyte numbers are not seen in opportunistic infections.

Determination of the lymphocyte reactivity in HLTx. Acute lung rejection in experimental animal models is associated with altered lymphocyte reactivity, measured by two types of assay. Spontaneous proliferation of BAL lymphocyte mediated cytolysis of donar spleen cells [121, 122]. Similar changes have now been observed in human HLTx patients [123–125].

The value in these studies is the identification of patients at special risk of developing obliterative bronchiolitis [125] despit augmented immunosuppression.

Technique of BAL in HTLx patients

A standard BAL procedure is followed in HLTx patients [126]. The patients undergo fibreoptic bronchoscopy (FOB) routinely at 10 days after surgery, before discharge home, and then at three and six months, and annually. FOB is also performed when patients develop respiratory symptoms, a fall in spirometry and if the chest radiograph shows pulmonary shadows [116]. Four transbronchial biopsies (TBB) are obtained at FOB, but the BAL is performed prior to biopsy.

The BAL is performed with physiological saline. Aliquots of 20 ml are instilled into a segmental bronchus, with the fibreoptic bronchoscope wedged in position. Further procedure follows the recommendations as described in previous chapters. The sample is divided into two, one for differential count and immunocytochemistry, and one for studies of lymphocyte reactivity.

Differential cell count and immunochemistry

The unfiltered sample is placed in a cytospin, the pellet is then stained in a standard fashion and a differential cell count performed. Values for cell types are expressed in absolute numbers and, percentages of total counts. Preliminary data suggest that absolute lymphocyte counts in BAL are markedly increased when the patient is experiencing acute lung rejection confirmed by biopsy.

The cytospin pellet will be subjected to T-cell subset analysis. Preliminary data suggest that in acute rejection confirmed by biopsy T8 cells predominate. Interestingly, one HLTx patient with sarcoidosis has shown recurrence of sarcoid granuloma in the lungs associated with a predominance of T8 cells in the lavage.

Studies of lymphocyte reactivity

The BAL cells are cytospun, washed and resuspended in RPMI-1640 containing HEPES. Unseparated cells are cultered in this form. The remaining cells are separated into purified lymphocytes and macrophages by the following technique.

Lymphocytes are obtained from unseparated cells by incubation with carbonyl iron for 90 min at 37°C with rotation, followed by passage through a high magnetic field to remove phagocytic cells. The supernatant is applied to a discontinuous Ficol-Hypaque gradient and after centrifugation the lymphocyte enriched layer is removed, washed and resuspended in RPMI. BAL macrophages are isolated by Percoll gradient centrifugation.

Donor spleen cells are obtained from 2–3 cm^3 blocks of spleen, obtained at the time of procurement of the donor heart and lungs. The splenic tissue is passed through a fine mesh sterile stainless steel strainer into approximately 100 ml RPMI-1640 containing HEPES, treated with carbonyl iron to remove phagocytic cells, purified by discontinuous Ficol-Hypaque gradient and prepared for cryopreservation in liquid nitrogen.

Spontaneous proliferation. The unseparated BAL cells are assessed by culture for 72 h in RPMI-1640 containing 10% FCS gassed with 5% CO_2 at 37°C. During the final 18 h the cultures are pulsed by 1 μCi ^{3}H-thymidine (Amersham International) harvested and counted by liquid scintillation.

Non-specific activation of T-cells. This is assessed by staining purified BAL lymphocytes with fluorescent labelled monoclonal anti-IA and anti-IL2 receptor antibodies. The labelled lymphocytes are counted, plotted and analysed by fluorescent activated cell sorter/analyser to assess proportions of lymphocyte receptors.

Secondary allogenic lymphocyte proliferation. This is assayed by incubating $3x10^4$ purified BAL lymphocytes with $1x10^5$ irradiated (2000/rads) or mitomycin treated stimulator cells. The allogenic stimulator cells can be

either: a) cryopreserved spleen cells; or b) purified BAL macrophages.

Cell mediated lympholysis (CML). The BAL lymphocytes can be tested for cytolytic activity towards allogenic target cells in the CML assay. Lysis is measured by release of labelled chromium in the supernatant of $1x10^4$ target cells (cryopreserved donor spleen lymphocytes).

In conclusion the use of BAL in HLTx is still in its infancy. However, the technique combined with TBB in HLTx offers a powerful diagnostic and scientific tool for investigation of the mechanisms and the effect of treatment rejection.

Freezing and storage

Instead of evaluating bronchoalveolar lavage components immediately after the procedure, it is sometimes more convenient to save part of the fluid for further studies. Both cellular and non-cellular components of bronchoalveolar lavage can be frozen and stored. After centrifugation (500 g, 10 min), to remove bronchoalveolar cells from the bronchoalveolar lavage fluid, the cell pellet is resuspended in Hank's balanced salt solution or other tissue culture media (without Ca^{++} and Mg^{++} for certain assays) at the desired cell density for morphological or functional studies and the fluid is generally frozen and kept for subsequent use [23].

Storage and freezing of bronchoalveolar fluid

The fluid can be stored as it is or after centrifugation. There are different techniques to concentrate the fluid:

a. Pressure filtration using an Amicon apparatus (Amicon EC-20, Amicon Corporation, Lexington, Massachusetts) and a UM 2 membrane (molecular weight cutoff, 2,000 daltons).

b. Dialysis against water with subsequent lyophilization. It should be remembered that all procedures using pressure filtration may result in loss of proteins which can be different using different membrane types [1, 23, 25]. Using UM membranes there is approximately a 10% loss, due to sticking of the proteins to the membrane itself or to the container.

The volume of fluid should be reduced about 20–50 fold and then dispensed as aliquots into plastic-capped tubes for storage [2]. It is most convenient to divide the sample into aliquots first, to obviate the need for repeated freezing and thawing in the future. If assays are to be analysed within 3 months a storage temperature of -20°C is acceptable. For longer periods of storage a freezing temperature of -70°C is necessary to preserve enzyme activity and protein integrity [2].

Storage and freezing of bronchoalveolar cell suspension

To store and freeze bronchoalveolar cells, the cell pellet is resuspended in RPMI-1640 with 20% FCS and 10% DMSO (Dimethylsulphoxide) at a concentration of 1–10 x 10^6 cells·ml^{-1}. The cell suspensions are rapidly frozen and stored in liquid nitrogen. DMSO should be avoided in case it is planned to perform molecular studies (*e.g.* mRNA studies).

When needed for analysis, the cells are warmed in a thermostat bath to 4°C, and then immediately resuspended in 10-fold greater volumes of fresh medium and washed 3 times with the same medium to remove the DMSO. After washing, the cells can be used for phenotype characterization or for biological studies. However, since with this technique only approximately 50% of the cells are alive and available for functional studies, before culturing cells that have been frozen, separation of the dead cells through Hypaque-Ficoll centrifugation is recommended. In addition, the possibility of selective losses of cell subpopulations during freezing must be considered. For these reasons it is strongly recommended, whenever possible, to use fresh BAL samples when looking for phenotypes and functional properties of mononuclear cells.

Transport of bronchoalveolar lavage fluid

Bronchoalveolar lavege (BAL) is usually performed to obtain alveolar lining fluid, cells from the peripheral bronchial tree and from alveoli or infectious agents such as bacteria, fungi and *Pneumocystis carinii.* Depending on which of these components of bronchoalveolar lavage (BAL) fluid is to be analysed appropriate care has to be taken when BAL fluid is sent to different locations for analysis.

Analysis of cells in BAL fluid

The majority of studies concerning quantitative analysis of cells in BAL fluid were performed in such a way that the specimens were processed within a couple of hours after they were obtained. Experience shows that storing cells at 4°C in a siliconized tube will reduce the viability (Trypan blue) from 87.2±3.1% to 71.8±4.8%

(mean and SD) within 12 h while there is no significant change as far as the cell differentials are concerned. Storing cells at 4°C in media such as MEM or RPMI-1640 with penicillin and streptomycin will enhance viability significantly [127]. Storage up to 24 h is possible without impairment of the total cell count, viability, differential cytology, and surface marker analysis of lymphocytes [46].

If BAL fluid is worked up within 4 h it can be kept at ambient temperature without effecting cell counts or causing bacterial growth [128]. If kept at 4°C without addition of culture media any growth of bacteria or fungi is minimal. Thus, adding antibiotics does not seem to be necessary. If kept in culture media penicillin and streptomycin should be added.

Thus, it is suggested, that for research purposes analysis of cells should be performed as soon as possible after recovery. An interval of up to 4 h seems to be tolerable. Further delays are only possible if appropriate studies are available showing that the delay of cell preparation does not affect the aim of the study.

In case assays assessing the functional capabilities of cells from BAL fluid are intended the specimens should be sent to the laboratory within 12 h unless they are separated and resuspended in appropriate media such as MEM or RPMI-1640 containing penicillin and streptomycin. Temperature to be kept during transport is dependent on the type of assay.

A recent study demonstrated that for clinical routine purposes shipping of cells from BAL is possible in an appropriate medium. Within 24 h at ambient temperature an analysis of cell proportions including lymphocyte subsets such as CD3+, CD4+, and CD8+ is possible [129].

Analysis of supernatant

If the supernatant is to be analysed for proteins, enzymes or immunoglobulins which are not secreted or released by cells in BAL fluid the specimen should be sent to the laboratory within 24 h. If mediators, enzymes, proteins or immunoglobulines are studied which are secreted or released by cells an immediate separation of cells and supernatant is mandatory. Appropriate care should be taken to provide adequate conditions of *e.g.* pH and temperature, to facilitate the further analysis. Usually the supernatant can be frozen and kept at -20°C or -70°C. It is important to recognize that depending on the type of substances which are subject to analysis there are many which may be unstable therefore requiring further preservation.

Infectious agents

If BAL fluid analysis for infectious agents such as bacteria, mycobacteria or fungi is intended, it should be handled in the same way as other specimens such as sputum, pleural effusions or urine. For detecting *P. carinii* in BAL fluid no special precautions are necessary. BAL fluid should be kept in a siliconized tube, preferably at 4°C. The analysis should be performed within 24 h.

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