MINIREVIEWS

Immunophenotyping of Bronchoalveolar Lavage Lymphocytes

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INTRODUCTION

The lung is continuously exposed to the external environment and its mixtures of complex antigens through the air we breathe. It is estimated that the resting human adult inhales 12,000 liters of air each day, while even mild physical activity can double or triple this amount (85). In addition to anatomical barriers, such as airway angulation, mucociliary clearance, and coughing, both humoral and cellular defense mechanisms play an important role in maintaining the viability of the host. One of the first lines of defense against particulate matter is mucociliary clearance and phagocytic activity of alveolar macrophages. Antigen entering the pulmonary tract encounters antigen-presenting cells comprised of alveolar and interstitial macrophages and effector T lymphocytes (reviewed in reference 1). This encounter leads to a complex sequence of events that results in cell migration and activation at a site of inflammation, with the subsequent development of lymphocyte functional effector activity. After many years of study there is still a paucity of information on the origin, half-life, fate, and specific function of pulmonary lymphocytes in health and disease (80).

Protective immunity against inhaled antigens is mediated by the lymphocytes that are localized to the surface of the respiratory tract. The compartments in the lung where lymphocytes are present are (i) the epithelium and lamina propria of the air-conducting regions, (ii) the bronchus-associated lymphoid tissue (BALT), which is found commonly in certain animals, i.e., rabbit and rats, (iii) the pulmonary interstitium and vascular beds, and (iv) the bronchoalveolar space. Lymphocytes present in the mucociliary epithelium of the trachea and bronchi are mainly CD8+ T cells. In the bronchial epithelium Fournier et al. (34) found 18 T cells per 100 epithelial cells but essentially no B cells. About 1% of these T cells express the γδ T-cell receptor (31). In contrast to the epithelium, the bronchial lamina propria contains more CD4+ than CD8+ T cells. The majority of these T cells express the memory marker of CD45RO (25). Also, this area shows more surface immunoglobulin-bearing lymphocytes (54). In the human, in contrast to rabbits and rats, BALT is present at birth but disappears in the adult lung. However, after certain stimuli such as cigarette smoking, BALT can develop in adults (90). In the whole human lung interstitium Holt et al. (51) calculated 10 to 109 lymphocytes, a number equivalent to the number of lymphocytes present in human circulating blood.

Lymphocytes in the bronchoalveolar space are the most easily accessible of the lymphocytes in the human lung. It has been estimated that the total number of these lymphocytes on the air side of the epithelium is between 2 x 109 and 4 x 109 (22, 54). This number represents about 5% of the total circulating lymphocyte pool in humans or about 5% of the size of the interstitial lung pool.

BAL

Much of our understanding of the role of pulmonary lymphocytes in host defense mechanisms and in disease comes from the study of lymphocytes recovered from the lung by bronchoalveolar lavage (BAL). BAL is the sampling of the lower respiratory tract by the instillation and subsequent aspiration of fluid (59). The technique recovers cells, soluble proteins, lipids, and other chemical constituents from the epithelial surface of the lungs. Clinically, BAL has been helpful in the diagnosis and differentiation of various types of lung diseases, including interstitial lung diseases, malignancies, and pulmonary infections (53). It also has been used in defining the stages of disease, its progression, and response to therapy. As a research tool, it is useful in the investigation of the cellular and humoral events occurring in lungs, especially in pulmonary diseases, and has aided in advancing hypotheses regarding disease pathogenesis and especially immunopathogenesis.

The European Respiratory Society and the American Thoracic Society have published guidelines and recommendations for fiber-optic bronchoscopy and BAL (4, 30). In brief, the technique of BAL generally involves the introduction of a flexible fiber-optic bronchoscope transnasally while the patient is in a semirecumbent position (59, 67). It is passed through the pharynx and vocal cords, into the trachea, and to the appropriate area of the lung. In localized disease, lavage of the involved lung segment is more likely to yield the best results, while in diffuse disease, the right middle lobe or lingula has been most commonly used because of the ease of access and the increased volume and cells recovered compared to other sites. Aliquots of sterile saline (generally 30 to 40 ml) are instilled through the bronchoscope, which is immediately and gently withdrawn. The total volume of saline instilled has been reported to range from 30 to 400 ml. In general, 20% is recovered after the first instillation of saline followed by 40 and 70% recovery in subsequent instillations. One-hundred milliliters of saline will sample the constituents of about 106 alveoli or about 1.5 to 3% of the lung and will recover about 1 ml of epithelial lining fluid. The total procedure takes less than 15 min.

CELLS AND PROTEINS RECOVERED FROM BAL

The cells recovered from the lung by lavage are much more heterogeneous than the cells obtained from peripheral blood. The major cell populations include macrophages, neutrophils, eosinophils, erythrocytes, and lymphocytes (67, 100). Less frequently, mast cells, plasma cells, ciliated squamous epithelial cells, Langerhans cells, megakaryocytes, erythroid precursors or immature myeloid cells, alveolar type I and II epithelial cells, and endothelial cells are recovered. During inflammation

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and injury in the lung, epithelial cells increase significantly. Pulmonary macrophage size can range from 8 to 30 μm or larger, while BAL fluid lymphocytes can be larger than their peripheral blood counterparts depending on the condition of the lung and especially if they are activated (23). Common nonpulmonary materials recovered from the BAL fluid include nonpathogenic fungi, talc, carbon pigments, ferruginous bodies, hair, mineral fibers, pollen granules, starch granules, and vegetable cells (100). All of these cells and substances can confuse the light scatter profiles when specific populations of cells such as lymphocytes are analyzed by flow cytometry.

Most normal serum proteins are present in BAL fluid including immunoglobulins, albumin, α₁-antitrypsin, and α₂-macroglobulin (53). In addition, complement, carcinoembryonic antigen (CEA), transferrin, fibronectin, collagenase, lipids, and prostaglandins are all detected in the BAL. They arrive there as a result of either local synthesis (e.g., surfactant), active transport (e.g., immunoglobulin M), or passive transudation (e.g., albumin). With some exceptions, proteins with a molecular mass greater than 300,000 Da are not present in BAL fluid.

Over 80% of the cells recovered from normal individuals are macrophages, with smokers showing more than 90% macrophages (7). Lymphocytes account for roughly 10% of the cell types, with the remaining cells being neutrophils, basophils, and eosinophils (generally less than 1%). The subsets of T lymphocytes in the normal adult human lung closely parallel those seen in the peripheral blood; i.e., roughly 65 to 75% are CD3⁺ cells, 40 to 45% are CD4⁺ cells, and 20 to 25% are CD8⁺ cells. The B cells are fewer, generally under 5%. Analysis of the T-cell repertoire from the lungs of normal individuals indicates a largely polyclonal pattern corresponding to that found in peripheral blood (12). Yurovsky et al. showed that the pulmonary T-cell repertoire is diverse in normal subjects and that the repertoire changes over time, which may reflect environmental exposures (122). Ratjen et al. studied the lymphocyte surface markers of BAL fluid in 28 children ranging in age from 3 to 16 years without bronchopulmonary disease (86). The distribution of total T and B cells was similar to that reported for adults; however, there was an increase in the CD8 subset of T cells giving rise to a lower CD4/CD8 ratio (0.7 ± 0.4 [mean ± standard deviation]) than that for adult BAL cells.

**PROCESSING OF BAL FOR CELL COUNTS, DIFFERENTIALS, AND IMMUNOPHENOTYPING**

Unfortunately, there is no consensus on the processing of BAL samples for cell counts, differentials, and immunophenotyping, and as a consequence, there is a myriad of different methods described in the literature. If the BAL contains too much mucus (rare in individuals without inflammatory airway disease) or visible particulate material, it has been most frequently reported to be filtered through cotton gauze (59), although investigators have used nylon gauze (56), Dacron nets (44), 100-mesh grid (40), stainless-steel mesh (16, 38), and venous infusion filters (42, 43). One of the concerns with filtering BAL fluids is that there may be selective loss of cell populations or subpopulations to a filter, especially if the specimen contains activated cells. Our laboratory rarely filters BAL fluids unless the amount of mucus impairs our ability to analyze lymphocyte populations.

Cell counts are made by counting an aliquot on a hemocytometer, for example, a Neubauer, Malassez, Burker, or Fuchs-Rosenthal counting chamber (59). One report indicates that Türk solution is used as an aid in counting (104). The use of an automated hematology analyzer has also been described (79, 81, 107, 109, 118). The problem with the latter is that these instruments are capable of distinguishing cell types in peripheral blood but are not so good at fluid analysis such as BAL analysis. Frequently, cell viabilities are performed by the Trypan blue dye exclusion technique (7).

For differentials of the leukocytes, a conventional-cytocentrifuge preparation or smears are air dried and stained with a hemato logic stain such as Wright-Giemsa, Diff-Quik, or May-Grunwald-Giemsa (7, 30). At least 200, but more frequently 500 to 1,000, cells are counted and classified as lymphocytes, neutrophils, eosinophils, macrophages, basophils, or epithelial cells. However, the imprecision of a differential has been repeatedly emphasized, especially when small numbers of cells are used (60, 94). As with blood, the enumeration of specific lymphocyte subsets in BAL is often dependent on a multiplet form, three-stage process. The final lymphocyte number is the product of the leukocyte count, the percentage of leukocytes that are lymphocytes, and the percentage of lymphocytes that have a particular subset marker. Thus, the resulting multiplication steps for determining the total lymphocyte count can have a magnified imprecision. There have been reports of selective loss of lymphocytes by cytocentrifugation or from smears due to the poor adherence of lymphocytes to glass slides, which is exacerbated during an aqueous staining process (72). Esterase staining is often employed to distinguish macrophages from lymphocytes (59, 64).

The overwhelming number of cells in the BAL are macrophages. Some investigators remove these cells prior to lymphocyte immunophenotyping by adherence to plastic in media such as RPMI, which is frequently supplemented with a source of protein, e.g., 10% fetal calf serum (36, 55, 58, 66, 73–75) for 30 min to 1 h or by the magnetic removal of ingested carbonyl iron (40, 88). Further depletion of alveolar macrophages has been reported with complement-mediated lysis and anti-CD11c (123) or passage through a nylon wool column (3). As mentioned above, one must be aware that activated T cells could also bind to plastics, etc. This is especially important since several studies have analyzed BAL lymphocytes for activation markers, i.e., CD69, CD25, and HLA-DR (5, 16, 19, 25, 71, 73, 74, 79, 92, 102, 108–110). For isolation of specific T-cell subset populations, one report used rosetting with neuraminidase-treated sheep erythrocytes followed by isolation by Ficoll-Hypaque gradient centrifugation (40).

**METHODS TO ENUMERATE LYMPHOCYTE POPULATIONS IN BAL**

Diseases and conditions for which the immunophenotyping of BAL fluid lymphocytes has been investigated include sarcoidosis (8, 9, 25, 27, 32, 33, 43, 44, 52, 55, 63, 71, 75, 87, 102, 120); hypersensitivity pneumonitis (6, 17, 27, 87); asthma (21, 38, 40, 50, 56, 61, 69, 79, 83, 92, 96, 99, 107, 109, 110, 119); infectious diseases including human immunodeficiency virus (2, 45, 57, 85, 111, 123), tuberculosis (3, 49, 97, 121), human T-cell lymphotropic virus type I infection (74, 101), and hepatitis C virus infection (62); lung transplantation (16, 88, 117, 118); collagen vascular diseases (36, 42, 114, 115); malignant lung diseases (35, 82, 89, 93, 98, 105, 116); allogeneic bone marrow transplantation (66); alcoholic liver cirrhosis (113); pulmonary diseases associated with eosinophilia (108); bronchiolitis (20, 28, 58, 73); radiation pneumonitis (41, 43, 91); and beryllium disease (77).

Several of these diseases show an increase in the lymphocytes recovered in BAL fluids. These include hypersensitivity pneumonitis, sarcoidosis, berylliosis, tuberculosis, various
has been demonstrated for lymphocytes in BAL fluids (106). Immunofluorescence microscopy and immunocytochemistry negative staining. As noted for the immunoperoxidase technique, examined by fluorescence microscopy. At least 200 cells which were positive for mouse monoclonal antibodies to specific lymphocyte subsets, carried out by an alkaline phosphatase–anti-alkaline phosphatase procedure except that the primary antibody is omitted. Positive antibodies has also been used to enumerate specific lymphocytes from non-peripheral blood fluids such as BAL. The absence of uniformity in lymphocyte immunophenotyping of BAL fluids has made comparisons between various reports difficult.

There are several important issues to be aware of in enumerating BAL lymphocyte populations by flow cytometry (48). While data can be obtained more rapidly by flow cytometry, often the heterogeneity of the cellular populations makes analysis difficult and can lead to the exclusion of cells of interest as well as the inclusion of unwanted cells. The light scatter patterns often show overlapping clusters of cells and debris, with specific lymphocyte populations being difficult to delineate. Cellular autofluorescence and nonspecific binding can be strong and can obscure or mimic specific staining of low-expression markers. If attention is not paid to the specific technical issues, the analysis can yield inaccurate results.

The technique of obtaining BAL can itself often lead to dead cells or naked nuclei. Generally, the viability of cells recovered from the BAL is greater than 80% but rarely exceeds 90% (7). In addition, BAL samples are frequently contaminated with erythrocytes. This can pose specific problems in lymphocyte analysis since erythrocytes show light scatter characteristics similar to those of lymphocytes, which can lead to an underestimation of the true lymphocyte percentages. While many reports fail to mention if erythrocytes are removed, several have indicated the use of erythrocyte lysing reagents including ammonium chloride (42, 108–110, 112) and commercial lysing reagents (29, 56, 57, 91, 107). Mild hypotonic lysis has also been used to remove erythrocyte contamination (5). However, any lysing method could lead to the release of cellular debris and interfere with the lymphocyte gating purity.

As flow technology has progressed, investigators have gone from a one-color approach to two- and three-color approaches for enumerating lymphocyte populations in BAL fluids and from an indirect-staining approach to direct staining. The use of isotype controls to distinguish positively stained cells from background staining has varied widely. After staining, most investigators fix the cells with 0.5 to 1% paraformaldehyde or formaldehyde prior to analysis. Commonly, 5,000 to 10,000 cells are counted; however, the number of gated events counted varies widely, ranging from several hundred cells (112) to 20,000 cells (58, 73–75).

The quality of the results from flow cytometry depends on the nature and quality of the lymphocyte gate. Gating becomes easier and less of a problem in analysis when there is an increase in the percentage (greater than 10%) and number of lymphocytes in the BAL. Most published studies have used a variety of methods to establish lymphocyte gates in analyzing BAL including light scatter only (i.e., forward scatter [size] by side scatter [granularity]), the combination of CD14 and CD45 along with light scatter to estimate the percentage of nonlym-
phocyte contamination of the gated area, light scatter with CD3 positive selection, the combination of side scatter and CD45, BAL lymphocyte gates defined by the light scatter characteristics of peripheral blood lymphocytes, and the use of commercial software programs. References for these methods are given in Table 1.

Several publications have used lymphocyte gating methods relying solely on forward and side scatter properties (Table 1). This can lead to inaccuracies in the data due to the exclusion or inclusion of nonlymphoid cells. Often, macrophages in the BAL may exhibit the same light scatter profile as the lymphocytes, in which case the errors would be made in determining the size of a particular lymphocyte population. Identification of lymphocytes within a forward by side scatter histogram with the use of CD45 and CD14 to aid in distinguishing lymphocytes from the various other nonlymphoid populations of cells improves the reliability of the results and also allows the purity of the gated population to be estimated. CD45 is expressed on macrophages and granulocytes but at lower levels than lymphocytes (68). However, under the best of conditions, the percentage of CD45 bright cells (or lymphocytes) and CD14-negative cells is frequently less than 75%. This is especially true when the percent and number of lymphocytes in the BAL fluid are low. The use of CD14 to identify alveolar macrophages in a gated lymphocyte population is limited by the fact that only 70 to 90% of alveolar macrophages express this marker (46).

Brandt et al. (10) assessed lymphocyte subsets by flow cytometry with a tricolor staining procedure. They adapted a procedure originally published by Terstappen et al. (103) in which the fluorescent DNA dye LDS 751 was used to exclude damaged cells and debris. Lymphocytes are identified by their CD45 expression, side scatter, and cellular integrity. Comparing this procedure to an immunocytochemical method, the authors found a strong correlation with the percentages of CD3+, CD4+, and CD8+ cells. Weak or no correlation was found between CD25+ and CD56+ cells, probably due to the low number of these cells counted with the immunocytochemical method.

Dauber et al. (24) used the combination of a common leukocyte antigen CD45 and side scatter to gate on BAL lymphocyte populations obtained from normal individuals and allograft recipients. Using this gating procedure, they were able to exclude events that were not leukocytes including erythrocytes (not staining with CD45) and cellular debris that would normally fall into the forward by side scatter gates. In addition, they used an additional fluorescent marker to identify specific T-cell subsets. When the authors examined the correlation between this CD45/side scatter gating method and the traditional forward/side scatter method or an immunocytochemical analysis, they found a good correlation to the immunocytochemical method for CD3+, CD4+, and CD8+ cells. However, as expected, the forward/side scatter analysis did not correlate well with the immunocytochemical method. The advantage of using CD45 and side scatter to gate on lymphocytes is that large lymphocytes are not excluded from analysis, since forward scatter is not used to select the cells. However, as the authors point out, selecting lymphocytes by CD45 expression and side scatter can underestimate high side scatter, large granular lymphocytes expressing CD16. By expanding the side scatter gate, one runs the risk of including CD45-positive macrophages in the analysis. Another potential problem is the presence of CD45-positive degranulated neutrophils in the BAL fluid. These cells, which may have arisen as a result of activation upon passage into the alveolar spaces, have side scatter characteristics, which allows them to fall within the lymphocyte gate.

The use of three-color analysis with gating on CD45+ and side scatter as proposed for whole-blood analysis may offer an acceptable alternative to two-color analysis of BAL lymphocytes (78). In this procedure, lymphocytes are identified by their side scatter and bright CD45 expression, with the subsets of T cells identified by CD3 and CD4 or CD8. The combination of CD3 with CD4 or CD8 ensures that these markers are measured only on T cells. In a limited number of BAL samples examined in our laboratory, three-color analysis, e.g., CD45/CD3/CD4 and CD45/CD3/CD8, showed good correlation with two-color analysis of BAL lymphocytes, e.g., CD3/CD4 and CD3/CD8, when CD45/CD14 was used to gate on the lymphocyte populations (47).

Padovan et al. compared flow cytometry to the conventional peroxidase-antiperoxidase method for the immunophenotyping of BAL cells obtained from patients with various interstitial lung diseases (81). Comparable results were obtained for CD3+, CD4+, CD8+, and CD57+ cells. The authors found that HLA-DR-positive lymphocytes could be measured more reliably by flow cytometry than by the immunoperoxidase method due to the fact that the continuum of expression for HLA-DR with a shift in fluorescence intensity was more easily detected by flow cytometry than by the subjective measure of immunoperoxidase.

**SUMMARY AND CONCLUSIONS**

In summary, analysis of lymphocytes in BAL fluid has been important in our understanding of the pathophysiology of pulmonary diseases and has been useful in diagnosing various pulmonary inflammatory conditions. Standard methods used in the enumeration of lymphocytes from peripheral blood by flow cytometry may not always be applicable to the analysis of BAL in part due to the potential for heterogeneous populations of cells and nonpulmonary materials present. The development of a standardized procedure for the processing and immunophenotyping of BAL lymphocytes will make comparisons between various reports easier.

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**TABLE 1. Gating methods used for BAL lymphocyte immunophenotyping**

| Method | Reference(s) |
|--------|--------------|
| Light scatter (forward by side scatter) | 2, 5, 29, 32, 44, 55, 57, 58, 70–74, 79, 93, 98, 101, 107, 108, 117, 118, 123 |
| Light scatter with CD45/CD14 or CD14 alone | 25, 38, 42, 50, 56, 87, 91, 112 |
| Light scatter with CD3 selection | 61, 95, 119 |
| CD45 and side scatter | 40, 24, 47, 104 |
| Comparison to PBL | 92 |
| Commercial software | 83 |

*PBL, peripheral blood lymphocytes.*

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