Analysis of bronchoalveolar lavage from human lung transplant recipients by flow cytometry


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Bronchoalveolar lavage (BAL) cells and peripheral blood leukocytes (PBL) from 24 lung transplant recipients were analysed for leucocyte subsets and expression of cell surface antigens. Total and differential white cell counts were performed on BAL, and lymphocyte subsets were evaluated in both BAL and peripheral blood. Measurement of immunofluorescence by flow cytometry was used to assess the percentage of: T cells (CD3+); T-helper cells (CD4+); T-cytotoxic/suppressor cells (CD8+); and activated lymphocytes (HLA-DR+). Lymphocyte subsets in BAL demonstrated marked differences to those in blood. A lower percentage of CD3+ and CD4+ lymphocytes were found in BAL, whereas CD8+ cells were more prevalent in BAL than in PBL. The mean CD4:CD8 ratio was significantly lower in BAL (1:1) than in blood (2:1:1). In the absence of pulmonary infection, there was a trend for a lower CD4:CD8 ratio in BAL associated with acute rejection (1:1:1) and obliterative bronchiolitis (1:1), when compared to the group with no evidence of rejection (1:4:1). In the absence of pulmonary rejection, pulmonary infection was associated with a marginally lower CD4:CD8 ratio in BAL (0:7:1), than when infection was absent (1:4:1). This difference was more evident in cases of cytomegalovirus (CMV) infection with a mean CD4:CD8 ratio of 0:3:1, compared to 1:5:1 in the absence of CMV disease (P<0.05).

Introduction

Methods to monitor rejection of the pulmonary allograft have evolved in parallel with the clinical application of heart–lung transplantation (HLT) (1), single lung transplantation (SLT) (2) and double lung transplantation (DLT) (3). Transbronchial biopsy (TBB) is generally accepted as the most accurate technique for assessing both allograft rejection and opportunistic pulmonary infection (4). The recent standardization of histological grading of lung rejection has aided in the management of these patients (5). However, TBB is an invasive procedure with an associated morbidity.

Bronchoalveolar lavage (BAL), a relatively non-hazardous procedure, provides a high yield of cells present in the lungs, and is often used as an adjunct to TBB for diagnosing pulmonary infection in this group (6). Although BAL generally remains less specific than TBB in diagnosing rejection (7), sophisticated techniques including immunohistochemistry (8) and biological assays such as the primed lymphocyte reaction (9) have been developed to define further the immunological processes involved in rejection of the pulmonary allograft. However, these methods are protracted and hence not ideal for clinical practice.

The fluorescence activated cell sorter (FACS) (10) allows the rapid analysis of diverse cell populations for expression of many different cell surface antigens, and thus may provide BAL with greater clinical application. FACS analysis, or flow cytometry, has been used for studying BAL fluid in experimental lung transplantation (11), and in other pulmonary diseases including fibrosing alveolitis (12) and sarcoidosis (13), in which all the cellular components of BAL were evaluated, including alveolar macrophages. Although lymphocytes comprise a small percentage of the total cells in BAL from normal subjects (14), attention has been focused on this cell population in lung transplant recipients, as they are primarily implicated in mediating allograft rejection (15). Of particular interest in clinical transplantation...
are T cells (CD3+), T-helper cells (CD4+), T-cytotoxic/suppressor cells (CD8+), and activated T cells (HLA – DR+) (16). In this study we have analysed lymphocyte subsets in BAL fluid and peripheral blood from human lung transplant recipients and correlated these with clinical, histological and microbiological data.

Materials and Methods

Patients

Bronchoalveolar lavage (BAL) was performed on 43 occasions in 15 HLT, seven SLT and two DLT recipients, at the same time as transbronchial biopsy (TBB). The latter was performed for either acute clinical deterioration associated with a fall in pulmonary function, or as routine surveillance for graft rejection, as described previously (17).

Patients ranged from 13-53 years (mean, 36.2 years), with 11 males and 13 females. All received maintenance triple immunosuppression therapy consisting of oral cyclosporin A (CyA), administered (mean dose, 9.3 mg kg −1 day −1) to maintain plasma levels at 150-300 ng ml −1 (radioimmunoassay method) azathioprine (mean dose, 1.7 mg kg −1 day −1) and prednisone (mean dose, 0.4 mg kg −1 day −1). In addition, an anti-lymphocyte preparation (rabbit anti-thymocyte globulin – supplied by C. Bieber, Specific Antisera, Fremont, CA, U.S.A. [n=12]; or OKT3 – Ortho Pharmaceuticals, Raritan, NJ, U.S.A. [n=12]) was given in the early post-operative period as prophylaxis for graft rejection. High dose intravenous methylprednisolone (1 g day −1 × 3), followed by a reducing oral prednisone course (commencing at 1 mg kg −1 day −1) was administered for episodes of acute rejection. Post-transplant survival of the cohort ranged from 0.5-100.5 months (mean, 13.4 months).

Bronchoalveolar Lavage (BAL)

Fibre optic bronchoscopy, BAL and TBB were performed on patients after sedation and application of topical anaesthesia to the upper airways. The flexible fibre optic bronchoscope (Olympus, Tokyo, Japan) was wedged into a subsegmental bronchus of either the right middle or left lingular lobe, and warmed, pH-corrected normal saline (0.275 ml of 8.4% sodium bicarbonate in 500 ml of normal saline) was instilled in 3 × 60 ml aliquots. BAL fluid was aspirated into a siliconized glass container, using gentle suction (200–300 mmHg) avoiding trauma to the bronchial mucosa. A sample of BAL fluid (15 ml) was removed for microbiological and cytological examination. After BAL, TBB was performed from both lower lobes.

BAL fluid was initially filtered through four layers of surgical cotton gauze, the cells pelleted by centrifugation at 320 RCF for 5 min, washed twice in the cell medium RPMI-1640 (Irvine Scientific Products, Santa Ana, CA, U.S.A.), and re-suspended in 10 ml of RPMI-1640. Total and differential white blood cell counts were performed, the former on a Coulter counter (Coulter, Hialeah, FL, U.S.A.), the latter manually on a Wright–Giemsa stained cytocentrifuge preparation.

A volume of the resulting suspension, containing at least 1.5 × 10⁷ white cells, was diluted in 10 ml of phosphate buffered saline (PBS), and layered on top of 4 ml of ficoll-hypaque lymphocyte separation medium (LSM – Organon Teknika, Durham, NC, U.S.A.). This was centrifuged at 750 RCF for 30 min to separate mononuclear cells, as previously described (18). The cell layer was removed, washed in 10 ml PBS on three occasions (centrifuged at 750, 150 and 320 RCF, respectively for 10 min each) and suspended in 1 ml of PBS.

Monoclonal Antibody (MAB) Staining of BAL and Peripheral Blood Lymphocytes

One hundred µl (approx 1 × 10⁶ cells) of the above prepared BAL cells were aliquoted into individual test tubes for each mAb. In addition, 100 µl of heparinized whole blood (approx 0.7 × 10⁶ white cells), collected at the time of bronchoscopy, was aliquoted into a duplicate set of tubes, and washed twice in PBS. The mAb panel, and the amounts of each used, were as follows: OKT3 (0.3 µg) and OKT4 (0.3 µg – Ortho Diagnostic Systems, Raritan, NJ, U.S.A.) for CD3 and CD4, respectively; RPAT8 (0.3 µg – supplied by Dr G Averson, DNAX Research Institute, Palo Alto, CA, U.S.A.) for CD8; and L243 (1 µg – American Type Culture Collection, Rockville, MD, U.S.A.) a pan MHC class II (HLA – DR) marker.

All antibodies were conjugated with fluorescein isothiocyanate (FITC), except L243, which required a second step stain with FITC-conjugated goat anti-mouse antibody (1–25 µg – TAGO, Burlingame, CA, U.S.A.). The mAb was incubated with cells for 15 min at room temperature, and washed in PBS prior to the second step antibody when required. After two further washes in PBS, BAL cells were fixed in 0.5% paraformaldehyde. The peripheral blood samples were firstly treated with whole blood lysis (Coulter, Hialeah, FL, U.S.A.) to remove erythrocytes, and washed twice in PBS before fixing in 0.5% paraformaldehyde.
FLOW CYTOMETRY

Immunofluorescence of the stained BAL and peripheral blood lymphocytes was measured on a FACScan (Becton Dickinson, Mountain View, CA, U.S.A.). Subsequent generation of graphics and analysis of the lymphocyte subsets were performed on a VAX computer using software ('Electric desk') developed by the shared FACS facility at Stanford University School of Medicine. This was locally accessed and displayed on a Macintosh IIx personal computer (Apple Computer, Cupertino, CA, U.S.A.). Results of the analyses (i.e. CD3+, CD4+, CD8+, and HLA-DR+) were described as percentages of total lymphocytes. To assist in comparison between the different diagnostic categories, CD4:CD8 ratios were calculated.

DIAGNOSIS OF PULMONARY REJECTION AND INFECTION

Pulmonary allograft rejection was categorized into three groups; nil rejection (NR), acute rejection (AR) and obliterative bronchiolitis (OB). A diagnosis of acute rejection was made in the presence of histological changes on TBB, i.e. grades 1-4 of the International Society of Heart and Lung Transplantation (ISHLT) nomenclature (5), and/or when chest X-ray infiltrates cleared, or an acute fall in pulmonary function tests reversed, on augmented immunosuppression therapy (e.g. intravenous steroids or anti-lymphocyte preparation). Obliterative bronchiolitis was diagnosed histologically, i.e. category C of the ISHLT nomenclature (5) and/or when irreversible chronic lung obstruction on pulmonary function monitoring was evident (a forced expiratory flow at 25-75% of vital capacity [FEF25-75] less than 30% of predicted normal for more than 2 months) (19). Patients with OB remained in this category unless intervening acute rejection was diagnosed.

Pulmonary infection (INF) was diagnosed in the presence of clinical symptoms (viz cough, auscultatory changes and pulmonary infiltrates on chest X-ray), a positive BAL culture, and improvement after appropriate antimicrobial therapy. Infectious agents were classified into pathogenic bacterial, viral or other. A diagnosis of CMV pneumonitis (infection) required the presence of a clinical syndrome, histological identification of viral inclusions, together with isolation of CMV from either BAL or TBB. CMV colonization indicated isolation of the virus, but without the clinical or histological criteria.

STATISTICAL ANALYSIS

Data were analysed using Statview II software (Abacus Concepts, Berkeley, CA, U.S.A.) on a Macintosh IIx personal computer. Comparison of BAL and peripheral blood results, and analysis of infection data, were made using Student's t-test. Comparison of results in the different rejection categories (viz NR, AR, and OB) was made by analysis of variance, with probability calculated by the F and Fisher PLSD tests. A P value of less than 0.05 was considered significant. Pulmonary rejection data analysis was determined in the absence of pulmonary infection and, conversely, pulmonary infection data were analysed after excluding pulmonary rejection. As the clinical diagnosis in individual patients varied at different points, repeated BAL procedures in the same patient were considered as separate events.

Results

A total of 43 BAL and 38 peripheral blood samples were collected from 24 lung transplant recipients. BAL volume return averaged 59.3% of input, with the total white blood cell count ranging from 0.1-33 x 10^5 ml^-1 (mean, 3.3 x 10^5 ml^-1).

FLOW CYTOMETRY

BAL cells and PBL were analysed by flow cytometry in parallel. Lymphocyte populations were easily distinguishable in peripheral blood samples by their characteristic size (forward scatter) and granularity (orthogonal scatter) on the FACS probability graph [Fig. 1(a)]. Although this population was less clearly isolated in BAL samples, a definite lymphocyte population could also be observed and their percentage calculated [Fig. 1(b)]. After gating the lymphocytes, fluorescence for each mAb, and the relative number of positively stained cells were determined. Accordingly, the percentage of the various phenotypes in BAL and peripheral blood could be evaluated [Fig. 1(c) and 1(d)].

BAL VERSUS PERIPHERAL BLOOD

 Significant differences (non-paired results) were noted in the distribution of lymphocyte subsets in BAL when compared to peripheral blood (Fig. 2). The percentage (mean ± SD) of: CD3+ lymphocytes was 63.6 ± 19.4% in BAL, compared to 75.8 ± 10.9% in blood (P=0.001); CD4+ lymphocytes was 26.5 ± 15.6% in BAL compared to 38.0 ± 20.9% in blood (P=0.006); CD8+ lymphocytes was 37.9 ± 15.6% in BAL compared to 38.0 ± 20.9% in blood (P=0.006); CD4-CD8 ratio in BAL was 1.1, compared to 2:1 in blood (P=0.008). These differences remained when paired results, i.e. BAL and peripheral blood from the same patient, were analysed (data not shown). However, there was no difference (P=0.6) observed in the...
percentage of HLA-DR+lymphocytes in BAL (15.5 ± 11.8%) compared to blood (16.8 ± 12.5%).

PULMONARY REJECTION

The diagnosis of pulmonary rejection and infection in the lung transplant recipients relating to the 43 BAL samples are shown in Table 1. After excluding those samples with infection, 14 showed no rejection (NR), six were associated with histologically proven acute rejection (AR: two with grade 1; three with grade 2; and one with grade 4 acute rejection), and four with obliterative bronchiolitis (OB). Although there were no differences in the total BAL cell count between the groups, there was a decreased percentage of macrophages, with an associated increase (P<0.05) in granulocytes in the AR group (Table 2).
There was also a higher mean percentage of lymphocytes in the AR and OB groups, but this did not reach statistical significance (Table 2).

The mean percentage of CD3+ lymphocytes in BAL remained relatively unchanged when comparing the three rejection categories (NR, 57.0%; AR, 61.0%; OB, 60.0%), as it did in peripheral blood (NR, 72.8%; AR, 78.5%; OB, 80.0%). The mean percentage of HLA-DR+ (i.e. activated) lymphocytes in BAL showed no significant differences when analysed for rejection (NR, 16.3%; AR, 15.0%; OB, 26.6%), although there was a trend for an increased percentage in the OB group. Analysis of PBL also failed to show any correlation (NR, 20.0%; AR, 22.3%; OB, 19.1%).

The mean CD4:CD8 ratio in BAL was lower in both AR (1:1) and OB (1:1), when compared to NR (1:4:1), whilst in peripheral blood, the mean CD4:CD8 ratio was marginally elevated in AR (2:1), and lower in OB (1:4:1), when compared to NR (1:7:1). However, these differences failed to reach statistical significance (Fig. 3).

**PULMONARY INFECTION**

After excluding cases with pulmonary rejection, pulmonary infection (INF) was diagnosed on 14 occasions. Pathogenic bacteria were isolated in 11 cases, viruses in five, and others in two (namely *Pneumocystis carinii* and *Legionella pneumophila*). Multiple organisms were noted in three cases. The mean white cell count in BAL was marginally higher when infection was present, with the percentage of macrophages significantly lower. Understandably, this was due to a relative increase in the percentage of granulocytes (Table 2).

The mean (± sd) percentage of CD3+ lymphocytes in BAL associated with INF was 67.9 ± 19.6%, compared to 57.0 ± 24.0% when pulmonary infection was not present (P=0.02). This trend was not detected in peripheral blood, where the percentage of

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<thead>
<tr>
<th>Table 1</th>
<th>Pulmonary rejection and pulmonary infection in lung transplant recipients</th>
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<tr>
<td>Rejection*</td>
<td>No. INF†</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>NR</td>
<td>14 (32.5)</td>
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<tr>
<td>AR</td>
<td>6 (14)</td>
</tr>
<tr>
<td>OB</td>
<td>4 (9)</td>
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<tr>
<td>Total</td>
<td>24 (55.5)</td>
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*NR, nil rejection; AR, acute rejection; OB, obliterative bronchiolitis.
†INF, pulmonary infection.
‡Number of patients and percentage of total.

<table>
<thead>
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<th>Table 2</th>
<th>Total and differential white cell count in bronchoalveolar lavage from lung transplant recipients</th>
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<tbody>
<tr>
<td>Diagnosis* (number)</td>
<td>WBC† × 10⁶ ml⁻¹</td>
</tr>
<tr>
<td>NR/No INF (n=14)</td>
<td>2.89 ± 2.48</td>
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<tr>
<td>AR (n=6)</td>
<td>1.56 ± 1.48</td>
</tr>
<tr>
<td>OB (n=4)</td>
<td>0.72 ± 0.73</td>
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<tr>
<td>INF (n=14)</td>
<td>4.82 ± 8.43</td>
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*NR, nil rejection; AR, acute rejection; OB, obliterative bronchiolitis; INF, pulmonary infection.
†Results are shown as mean ± sd.
‡P<0.05 for AR compared to NR by analysis of variance.
§P<0.05 for AR compared to OB by analysis of variance.
¶P<0.05 compared to No INF by Student's t-test.
Fig. 3 Mean (SD) CD4:CD8 ratios in BAL and peripheral blood, by rejection grade. ( ■ NR, nil rejection; □ AR, acute rejection; □ OB, obliterative bronchiolitis) and presence of pulmonary infection ( △ INF).

Fig. 4 Sequential CD4:CD8 ratios in BAL from a DLT (Pt 1) and an HLT (Pt 2) recipient over the first 6 months post-transplant. Pt 1 suffered persistent pulmonary allograft rejection and infection, and was associated with a reduced CD4:CD8 ratio. Pt 2 remained relatively free of these complications and maintained a relatively normal CD4:CD8 ratio. ○, Pt 1; ■, Pt 2.

CD3+ lymphocytes was 77.6 ± 9.6% when associated with INF, and 72.8 ± 13.8% without.

Interestingly, there was a higher, although not significant, percentage of HLA-DR+ lymphocytes in BAL when infection was absent (16.3 ± 10.2%), compared to when present (13.3 ± 12.3%), and this trend was also noted in peripheral blood with 20.0 ± 14.0% HLA-DR+ lymphocytes observed in the absence of infection, compared to the lower 9.7 ± 2.3% found with infection (P=0.03). The CD4:CD8 ratio in BAL demonstrated a marginally lower ratio (mean, 0.7:1) in the presence of INF than in its absence (mean, 1.4:1, P=0.14), although this was not the case in peripheral blood (Fig. 3).

**CMV Pulmonary Disease**

Further analysis by patient’s CMV status was performed. After excluding for pulmonary rejection, CMV status was detailed as: infected (n=4); colonized (n=8); and nil (not colonized or infected, n=16). There was an elevated total BAL cell count (mean ± SD) in the infected group (10.39 ± 15.08) compared to the nil group (2.49 ± 2.47), and colonized (3.30 ± 2.74), although this just failed to reach significance (P=0.06).

The mean CD4:CD8 ratio in BAL was 1.5:1 in the nil group compared to 0.6:1 in the colonized group (P<0.05), and 0.3:1 in the infected group (P<0.05), indicating a relatively higher percentage of CD8+ (T-cytotoxic/suppressor) lymphocytes in the presence of CMV colonization and infection. A similar trend was also observed in peripheral blood (data not shown).

**Sequential Studies in Individual Patients**

The CD4:CD8 ratio in sequential BAL samples from an HLT and a DLT recipient over the first 6 months post-transplant are depicted in Fig. 4. These patients pursued very different clinical courses. The HLT recipient (Pt 2), apart from early mild acute allograft rejection, remained free from serious pulmonary rejection and infection. The CD4:CD8 ratio in this case remained stable at a relatively normal value (c. 1.6:1).

The DLT recipient (Pt 1) suffered more persistent allograft rejection and had numerous pulmonary infective episodes. The CD4:CD8 ratio in BAL in this case was consistently depressed at 0.2-0.3:1. This patient has subsequently developed OB.

**Discussion**

This preliminary study has identified flow cytometry as a useful technique in the rapid analysis of lymphocytes isolated by BAL from lung transplant recipients, and supplements the repertoire of diagnostic methods available for this patient group (20). For this study, numerous methods of processing BAL fluid were evaluated to permit satisfactory flow cytometry, and although other techniques have been advocated (21), we found the ficoll–hypaque separation method (11,18) the most simple and efficient. This method may be more appropriate when processing the more turbid, cellular BAL fluid, resulting from the associated pulmonary rejection and
infection that is often present in lung transplant recipients. In addition, we found no differences in the cell distribution obtained using the ficoll-hypaque separation technique when compared to the whole blood lysis method in peripheral blood.

A limiting factor to the use of flow cytometry to analyse BAL lymphocytes was the percentage of lymphocytes present in the sample. A minimum of 3–5% was required to enable a definite lymphocyte population to be determined, gated and analysed on the FACS plot. Following anti-thymocyte globulin or OKT3 therapy, lymphocyte populations were detectable within the first month post-transplant. In addition, flow cytometric analysis should be easily applicable to the investigation of the other cellular components present in BAL, especially as their role in the lung allograft becomes further delineated (22).

For these initial studies, we elected to use one colour immunofluorescence staining to analyse lymphocyte subsets in BAL and peripheral blood. The percentages of CD3+, CD4+ and CD8+ lymphocytes in BAL, and the resultant CD4:CD8 ratio, were significantly different to those found in peripheral blood. Although the mean CD4:CD8 ratio of 2:1:1 in blood was not remarkable, there was a reduced BAL ratio of 1:1. This varied to a mean ratio of 1:86:1 observed in BAL fluid from a normal population (23). These data, indicating an elevated CD8+ (T-cytotoxic) lymphocyte representation in the lung allograft, correlate with other reports of this phenomenon, particularly in association with rejection (8,24,25).

The high incidence (44.5%) of pulmonary infection observed in our patient group, and also seen in another series (26), confounds the attempts of using BAL as a method for diagnosing rejection. We have shown that flow cytometric methods applied to BAL may aid in the differentiation between the absence or presence of pulmonary infection, particularly in the case of CMV pneumonitis. However, to assess more accurately the power of this technique in diagnosing pulmonary rejection, a greater number of patients are required to allow further classification of the diagnostic categories, i.e. by rejection ± infection, rather than separately as in this study.

By its exposure to the environment, the pulmonary allograft will continue to be at risk of infection and recently much interest has centred on CMV disease as suggested in a recent study (31). Our data, demonstrating an elevated percentage of CD8+ (T-cytotoxic) lymphocytes in the lung allograft associated with both CMV colonization and infection, support this theory.

The decreased percentage of HLA−DR+ (activated) lymphocytes in both BAL and peripheral blood in patients with pulmonary infection was somewhat surprising. This may be explained by an increased number of immature, non-activated lymphocytes, recruited to deal with the infecting organism, or by activated cells being sequestered at the site of infection.

As the cellular components of BAL vary markedly between normal subjects (14), more useful diagnostic information may be obtained by sequential studies in individual subjects. In the two cases illustrated, we have demonstrated that different clinical courses may be associated with variable CD4:CD8 ratios over time. A persistently low CD4:CD8 ratio may be suggestive of a worse prognostic category. Certainly, sequential BAL studies such as these may be more helpful in the clinical setting than isolated studies.

In conclusion, flow cytometric analysis of BAL fluid may be safely employed in the management of lung transplant patients, improving the speed and efficiency of BAL processing. With a focus on bronchoalveolar lymphocytes only, we have shown data which may assist in the diagnosis of pulmonary rejection and pulmonary infection. The diagnostic yield may be enhanced by applying additional BAL data such as the relative distribution of macrophages and granulocytes. As the role of other cellular components within the alveolar space in the pulmonary allograft is appreciated, this technique may be more widely applied to enable a greater understanding of the immunological processes involved in rejection of the transplanted lung.

References

5. The International Society for Heart Transplantation: Yousem SA, Berry GJ, Brunt EM et al. A working


