Activation of Eosinophils in the Airways of Lung Transplantation Patients*

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Eosinophils are important inflammatory cells involved in liver and renal allograft rejection. The role of these cells is less well defined in lung allograft rejection. Eosinophils may be activated in lung rejection and release cytotoxic eosinophil cationic protein (ECP). Other states of disease in lung transplant recipients, such as cytomegalovirus (CMV) and bacterial infection, may also be associated with activated eosinophils. We postulated that ECP may be detectable and elevated in the airway lavage samples obtained from lung transplant patients and may contribute to disease pathogenesis.

**Methods:** Fifty BAL samples were collected from 38 lung transplant patients. Their most recent pulmonary function test results within 1 week of collection were noted. The samples were analyzed for the concentration of ECP, WBC count and differential cell count, and total protein level. The results were analyzed to identify the presence of disease or abnormal lung function associated with a positive ECP test. Student's t test was used and a p value of <0.05 was considered significant.

**Results:** We found that ECP levels were elevated in 36% (n=14) of the patients. Those patients with a positive test result were more likely to have acute rejection, CMV disease, or the presence of a cultured pathogen in BAL compared to patients with a negative test result (p<0.01)

**Conclusions:** The presence of BAL ECP is associated with disease in lung transplant patients. Since ECP is directly cytotoxic, it may contribute to disease pathogenesis.

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**Abbreviations:** CMV=cytomegalovirus; ECP=eosinophil cationic protein; FEF$_{25-75\%}$=forced expiratory flow between 25% and 75% of FVC; IL=interleukin; POD=postoperative day

Eosinophils have been identified as important inflammatory cells involved in liver and renal allograft rejection following transplantation.1,2 Cytokines known to be produced by T lymphocytes, such as interleukin-3 (IL-3), are involved in the proliferation and activation of eosinophils. A murine model of lung transplantation has provided evidence of increased numbers of eosinophils in BAL fluid during the early phase of acute rejection.3 Increased numbers of eosinophils have been identified in lung transplant allografts during acute rejection as well.4 Eosinophil cationic protein (ECP) is released from eosinophils during activation and degradation and is a well-established marker of eosinophil activation. This highly charged protein is known to contribute to bronchospasm and direct epithelial cell and microvascular injury in a number of disease states, including asthma.5,6 ECP has been associated specifically with acute liver rejection and has been detected in liver biopsy specimens from patients undergoing allograft rejection.1

ECP may be involved in allograft lung rejection and possibly in other disease states, such as infection and obliterative bronchiolitis, that afflict lung transplant patients. In this study, the level of ECP was measured in the BAL fluid of lung transplant patients to investigate whether ECP is detectable and related to findings observed by BAL culture and transbronchial biopsy histologic analysis. Since ECP is a cytotoxic protein and a specific marker for eosinophil activation, we postulated that if ECP is found in the BAL of lung transplant patients, it may contribute to disease pathophysiology.

**Materials and Methods**

*Subjects*

There were 12 single lung recipients, 21 heart-lung recipients, and 5 bilateral lung transplant patients studied. Fifty BAL samples were obtained on separate occasions. The indications for bronchoscopy included routine surveillance, clinical suspicion of
infection, rejection, obliterative bronchiolitis, or malignancy. The mean (±SD) age was 38±14 years. The mean postoperative day (POD) of the subjects studied was 488 days. There were 31 female and 7 male subjects. The study was approved by the institutional review board at Stanford. The study took place over 8 consecutive months (1995 to 1996). All patients were maintained on a regimen of triple immunosuppression, including azathioprine, cyclosporine, and prednisone. In addition, they received treatment with an antilymphocytic preparation in the early postoperative period.

Collection of Sample

Fifty BAL samples were uniformly collected and processed from 38 patients on separate occasions. BAL was performed by previously described methods. Three 30 mL aliquots of sterile saline solution were instilled into a segmental bronchus through a flexible bronchoscope placed in a wedged position. The fluid was aspirated and collected into a sterile container. The fluid was sent for routine bacteriologic, viral, acid-fast bacilli, and fungal cultures, cytopathology, and cellular count and differential. An aliquot of 3 to 5 mL was reserved at −20°C for further analysis. Transbronchial biopsy specimens were obtained as clinically indicated after BAL collection using an alligator forceps under fluoroscopic guidance. The histologic analysis was performed on formaldehyde-treated tissue.

Analysis

ECP Measurement: The BAL fluid was centrifuged at 4°C at 500×g for 10 min. The supernatant was removed and frozen at −20°C for further analysis. The CAP colormetric system (Pharmacia; Kalamazoo, Mich) was used to measure ECP levels in the BAL fluid. All samples were analyzed in duplicate and reported as micrograms per liter. A negative control and positive control were used for each set of samples. Total protein measurements were made on each sample using a protein assay reagent kit (Pierce; Rockford, Ill).

Clinical Respiratory Parameters: The most recent FEV1 and forced expiratory flow between 25% and 75% of FVC (FEF25-75%) predicted values obtained within 1 week of the BAL collection and measured PaO2 (mm) from arterial blood were recorded for each of the patients studied.

Histologic Analysis

The laboratory processing and staining for transbronchial biopsy samples has been described previously. All biopsy specimens were read by a trained lung pathologist and rejection scores were based on the accepted International Society of Heart Lung Transplantation criteria.

Statistical analysis was performed using Student’s t test, five number summaries, and simple regression analysis. Analysis was performed on all 50 samples and then reanalyzed to include only first samples on each patient. A p value of <0.05 was considered to be statistically significant. A software program (Statworks; Philadelphia) was used and the analysis completed by a trained statistician.

Results

Of the 50 BAL samples analyzed from 38 different patients, 14 BAL samples from 14 patients were found to have elevated levels of BAL ECP. A level of >18 µg/L was defined as a positive result based on the optical density readings of the negative control. Known standard positive controls were measured with each set of patient samples tested and analyzed to establish a standard curve. The mean and median values of BAL ECP level among the patients with positive test results were 67 µg/L and 38.5 µg/L, respectively.

The measurements of airflow obstruction and hypoxia (FEV1, FEF25-75%, and PaO2) were not significantly different between the positive and negative groups. The median POD was not significantly different between the positive and negative group. The positive group had a median POD of 104 and the negative group had a median value of 103 days.

The mean BAL cell count for all the patients studied was 238 cells per cubic millimeter. The cellularity did not differ substantially among the patients studied. In the cellular differential, none of the patients studied had an elevated absolute number of BAL eosinophils (range, 0 to 2%). There was a mean of 71% mononuclear cells and 14% polymorphonuclear cells among all samples analyzed. There was no correlation between a particular cell count and the ECP level. The median total protein BAL level for those patients with a positive ECP BAL test was 411 µg/mL, which was significantly higher than the negative group, which demonstrated a median total protein level of 110 µg/mL (p<0.002, Table 1).

Among the 14 patients with high ECP levels, 12 had a clinical indication for a biopsy to diagnose

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<th>Table 1—Five Number Summaries*</th>
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<tr>
<td>POD</td>
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<tr>
<td>+</td>
</tr>
<tr>
<td>−</td>
</tr>
<tr>
<td>BAL cells (mm³)</td>
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<td>−</td>
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*Five number summaries for POD and cell counts in BAL. A comparison in 25th, 50th, and 75th percent quantile values between the positive and negative groups. There was no significant difference between the groups.
suspected rejection or infection. Of the 12 transbronchial biopsy samples obtained among these 14 patients, 33% (n=4) had acute rejection (1, grade 1a, and 3, grade 2). Fifty percent of patients (n=7) had pathogens noted on BAL culture.

Two BAL cultures grew scant fungus and five cultures grew Pseudomonas. There were three patients with pneumonia and one with posttransplant lymphoproliferative disease. Only three samples (25%) had normal findings on biopsy specimens (Table 2).

The finding of abnormal culture and/or biopsy results among this group was significantly higher than that in the negative ECP group (p<0.01). In comparison, among the patients with negative ECP results, 87% had no evidence of rejection or any cytopathologic abnormality noted and 78% (28/36) had normal culture results. The mean value of BAL ECP among the seven patients with biopsy specimen-proven acute rejection was 41.6 μg/L (Table 3). This was significantly higher than in those patients with no rejection diagnosed by transbronchial biopsy specimen who had a mean BAL ECP concentration of 12.7 μg/L (p<0.05). There were 19 samples and 14 patients who had evidence of cytomegalovirus (CMV) infection proven by shell vial, culture, and/or biopsy specimen. The mean ECP level was elevated at 30.6 μg/L among these patients.

**Discussion**

This study demonstrates that among the 38 lung transplant patients studied, there was activation of eosinophils present in the airways of 14 patients (36%) as indicated by high levels of BAL ECP. This was usually associated with ongoing allograft rejection or infection with a respiratory microbial pathogen.

The percentage of BAL eosinophils was not, however, elevated in any of the samples examined. This indicates that migration of the cells after activation may occur and that ECP-related damage can occur in the absence of airway eosinophilia. The other cellular BAL counts also did not differ among the patients with high vs low levels of ECP.

The physiologic parameters of FEV1, FEF25-75%, and PaO2 were not significantly lower among the patients with elevated ECP, although in asthma, lower FEV1 values have been correlated to higher ECP levels.10 The total protein levels of those patients with ongoing disease and elevated ECP levels were higher in comparison. This indicates that a degree of alveolar permeability may be present in these patients as part of their disease process.11 Among the patients with CMV infection, as defined by a positive BAL culture, positive shell vial, or biopsy result, there was a notable elevation of the mean ECP concentration for the group. This may indicate that CMV pulmonary infection may be associated with eosinophil activation.

Our findings provide one of the first lines of evidence that eosinophils are often activated in lung transplant recipients, during episodes of rejection or infection, and are consistent with findings reported in a recent study of 15 lung transplant patients with acute rejection.12 Based on the BAL profiles obtained in a murine model of allograft rejection, eosinophils may be present in increased numbers early in acute rejection.3 There was also a demonstrated increase in the number of neutrophils 3 days after transplantation in the murine model. In our study, there was no appreciable difference in POD between the negative and positive ECP groups. POD therefore cannot account for the difference in BAL ECP levels between these patient groups. Neutrophils are another cellular source of ECP, although the intracellular concentration is 1,000-fold lower, compared to eosinophils. Nevertheless, it is possible that if neutrophils are present in relatively high number during infection and rejection, they also may serve as a source of the ECP observed in the BAL.13 Our study did not find a correlation between BAL neutrophil count and ECP level. The cellular source of the ECP in lung lavage fluid is more likely to be from eosinophils. Our results also indicate that, not only in rejection, but in some states

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value, μg/L</th>
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<tbody>
<tr>
<td>Acute rejection</td>
<td>41.6</td>
</tr>
<tr>
<td>CMV infection</td>
<td>30.6</td>
</tr>
<tr>
<td>CMV + biopsy</td>
<td>33.3</td>
</tr>
<tr>
<td>No rejection</td>
<td>12.7</td>
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*Mean ECP levels of patients studied. The mean ECP level was not elevated in patients with no rejection and elevated in those with CMV infection or rejection.

**Table 2—Comparison of Positive and Negative ECP**

<table>
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<tr>
<th>ECP (+)</th>
<th>ECP (-)</th>
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<tbody>
<tr>
<td>% Patients Studied</td>
<td>% Studied</td>
</tr>
<tr>
<td>BAL culture (pathogens)</td>
<td>50 (7/14)</td>
</tr>
<tr>
<td>CMV infection</td>
<td>50 (7/14)</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>33 (4/12)</td>
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*Patients with a positive BAL test result are more likely to have rejection or infection. p<0.02. NS =not significant.

<sup>1</sup> Test was also performed using just first samples of the 38 different patients; no change in p values was observed.
of infection, there is an associated elevation of BAL ECP among the lung transplant patients studied.

The importance of eosinophils in liver and renal allograft rejection is well recognized. Patients with severe liver rejection in the first month after transplantation often demonstrate blood eosinophilia and marked eosinophil infiltration of portal tracts. The presence of major basic protein, another degranulation product of eosinophils, was also detected in the tissue of these patients.1 In renal allograft rejection, eosinophilic infiltration was reported in one study to occur in 93% of the patients with chronic renal allograft rejection.2 In other studies of renal transplant recipients, the presence of eosinophils in higher numbers among those with rejection suggests a role for this inflammatory cell in organ rejection.

The importance of ECPs in the lung transplant patient is less clear, but if present in a subset of patients, it could potentially contribute to lung injury. ECP is known in vivo to injure respiratory epithelia. The histopathologic changes noted on direct exposure to cationic proteins included excessive shedding and desquamation of bronchial epithelia to the level of the lamina propria. Cilia were also noted to be damaged in the presence of ECP. This damage to lung tissue occurred in vitro at doses as low as 10 μg/mL.14,15 Initial damage to the epithelia then would potentially predispose an individual to future infection with bacterial and viral pathogens.

Potential secretagogues for eosinophil activation and subsequent degranulation have been elucidated.16 Granulocyte-macrophage colony-stimulating factor, IL-5 and IL-3, and tumor necrosis factor-α were found in one study to cause significant release of granular proteins from eosinophils. In another study of 31 lung transplant patients, the transcript for IL-5 was detected in approximately 50% of the BAL samples collected.17 IL-5, in particular, is known to act as a potent and specific eosinophil differentiation and degranulation factor in humans. The potential relationship between the cytokines present in the airways of lung transplant recipients and the activation and recruitment of eosinophils should be further studied. Cytokines and several bacterial and viral pathogens can also act to increase superoxide production by eosinophils.16 This, in turn, could augment injury to the lung by mechanisms of lipid peroxidation and DNA damage.18 The cascade of inflammatory mediators that have been described previously in lung transplant recipients may act to recruit and activate eosinophils.17 Degranulation products such as ECP and major basic protein may be part of the pathogenesis of lung allograft rejection and infection. Further studies are needed to determine the clinical usefulness of BAL ECP as a marker of allograft rejection.

References