Activation of Eosinophils and Fibroblasts Assessed by Eosinophil Cationic Protein and Hyaluronan in BAL* 

Association With Acute Rejection in Lung Transplant Recipients

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Lung transplantation has become an accepted therapy for end-stage lung disease. Acute rejection of the transplanted lung still remains a major clinical problem since it decreases graft survival. Eosinophil cationic protein (ECP) from activated eosinophils, hyaluronan (HYA) from fibroblasts, and circulating intercellular adhesion molecule 1 (ICAM-1) have been associated with acute rejection in kidney and liver grafts. We investigated whether these, as well as other molecules, were increased in acute rejection of lung allografts. Serum and BAL fluid from 38 bronchoscopies performed in 9 single lung, 2 bilateral lung, and 4 heart-lung transplant patients were studied. Differential cell counts were made from the BAL fluid. Levels of ECP, myeloperoxidase (MPO), and HYA were used as indirect markers for activation of eosinophils, neutrophils, and fibroblasts, respectively. In addition, levels of circulating ICAM-1, VCAM-1, and E-selectin were analyzed.

Twenty-two episodes with acute rejection were diagnosed. Of these, 7 were minimal, 13 were mild, and 2 were of moderate character. We found increased levels of ECP and HYA in BAL fluid during mild acute rejection of the allograft. Numbers of eosinophils were also increased. Activation of neutrophils or neutrophil numbers were not significantly increased. Levels of circulating ICAM-1, VCAM-1, and E-selectin did not differ between the groups. This retrospective study shows that measurements of ECP and HYA can give information about the inflammatory process present during acute rejection in patients who have undergone lung transplants. Analysis of eCMS, however, appears to be of limited value as markers for acute rejection. (CHEST 1996; 110:89-96)

Key words: cell adhesion; eosinophils; hyaluronan; lung transplantation

Abbreviations: cICAM-1=circulating form of ICAM-1; CMV=cytomegalovirus; ECP=eosinophil cationic protein; EOS=eosinophil granulocyte; HYA=hyaluronan; ICAM-1=intercellular adhesion molecule 1; IL=interleukin; MPO=myeloperoxidase; PMN=polymorphonuclear granulocyte; RIA=radiolmmunoassay; TBB=transbronchial biopsy; VCAM-1=vascular adhesion molecule 1

In the last decade, lung transplantation has become an accepted therapy for end-stage lung disease. Improvements in organ preservation, surgical technique, immunosuppressive treatment, and infection control have all contributed to increased graft and patient survival.1 However, acute rejection of the transplanted lung still remains an important clinical problem as it increases the risk of developing chronic rejection and obliterative bronchiolitis.

Clinically, rejection is diagnosed by transbronchial biopsy specimens taken both at regular intervals and during clinical deterioration. Histopathologically, it is characterized by perivascular mononuclear infiltration and lymphoctic bronchitis/bronchiolitis.2 In addition, infiltration of eosinophil granulocytes has been observed during rejection Both in pulmonary as well as in kidney and liver allografts.2-5 Blood and urinary eosinophilia have been monitored as signs of acute rejection in human liver and kidney transplant allografts,5-8 and eosinophils in BAL fluid were increased during lung rejection in a murine model.9

Specific markers reflecting activation of inflamma-
tory cells and fibroblasts are possible to measure in the fluid phase. Cytotoxic molecules reflecting eosinophil activation such as eosinophil cationic protein (ECP) are found in acute rejection of the liver, and eosinophil major basic protein in rejection of the kidney. Hyaluronan (HYA), a large polysaccharide considered to reflect fibroblast activation, is increased in acute rejection of transplanted solid organs and associated with presence of interstitial edema in the graft.

Intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1), glycoproteins belonging to the immunoglobulin supergene family, are expressed on endothelial cell surfaces and upregulated by cytokines during inflammation. ICAM-1 is known to aid in the adherence and transendothelial migration of leukocytes, while VCAM-1 is associated with eosinophil adhesion. E-selectin (formerly called endothelial-leukocyte adhesion molecule 1), a single-chain glycoprotein, causes the initial leukocyte rolling on the vessel wall in areas of inflammation prior to the manifestation mediated by ICAM-1 and VCAM-1. All three CAMs have been found in acute rejection of the heart, and ICAM-1 has been found in rejection of the liver. Circulating forms of CAMs (cCAMs) are thought to originate from adhesion molecules expressed on activated cells and may reflect inflammatory activity. High levels of cICAM-1 have been found in bile during rejection of liver and in serum during kidney rejection.

In the present study, we investigated the pattern of inflammatory cells in BAL fluid, their corresponding activation markers in serum and BAL fluid, and the levels of circulating CAMs from patients who have undergone lung transplantation. The aim of the study was to investigate whether any of these variables were increased during acute rejection.

**MATERIALS AND METHODS**

**Design**

Retrospective analyses of serum and BAL fluid samples were performed. Differential cell counts of eosinophil granulocytes (EOS), polymorphonuclear granulocytes (PMN), macrophages, and lymphocytes were made from the BAL fluid. The presence of ECP, myeloperoxidase (MPO), and HYA in serum and BAL fluid were used as indirect markers for activation of EOS, PMN, and fibroblasts, respectively. In addition, levels of circulating ICAM-1, eVCAM-1, and E-selectin were analyzed.

Subjects

Fifteen patients undergoing single lung (n=9), bilateral lung (n=2), or heart-lung (n=4) transplantation were studied during an interval of 2 to 60 weeks. For patient data, see Table 1. The serum and BAL fluid samples were obtained for a different study that had been approved by the ethical committee of University of Göteborg. All subjects gave their written and oral consent. Donors and recipients were matched for cytomegalovirus (CMV) serologic status. All organs were harvested in a similar fashion.

**Immunosuppression**

Patients received immunosuppressants preoperatively with a loading dose of cyclosporine (6 to 8 mg/kg) and azathioprine (4 mg/kg) orally. Postoperatively, rabbit antithymocyte globulin (Pasteur Merieux; 2.5 mg/kg) was used for induction immunosuppression on 3 to 5 consecutive days. Methylprednisolone was given IV (500 mg at perfusion followed by 125 mg every 8 h for 3 days) and continued by enteral administration of prednisone, 0.5 mg/kg/d, tapered to 0.2 mg/kg/d, 1 month postoperatively.

Cyclosporine was given between 12 and 24 h after transplantation by continuous IV infusion at a dosage adjusted to maintain a serum level of 300 to 400 µg/L (radioimmunoassay [RIA] specific for whole blood; Cytotrac; Inctar; Stillwater, Minn). As soon as enteral function was normalized, cyclosporine was administered orally. Azathioprine was given orally once a day (2 mg/kg) and adjusted to maintain an absolute number of blood neutrophils of 3 to 5x10^9 cm^3.

Episodes of acute rejection were treated with methylprednisolone (1 g IV) for 3 days, and antithymocyte globulin therapy was reinstituted if rejection was resistant to steroid treatment.

**Postoperative Follow-up**

Transbronchial biopsies (TBBs) and BAL were performed routinely at 2 and 4 weeks after transplantation, and thereafter monthly for the first 3 months. Additional bronchoscopy with TBBs and BAL were performed after 6, 9, and 12 months postoperatively, or whenever indicated by clinical parameters such as dyspnea, hypoxemia, decline in FEV1 values, radiographic infiltrate, or unexplained fever. Repeated TBBs and BAL were performed approximately 4 weeks after treatment of rejection episodes. Patients were anesthetized for bronchoscopy with propofol IV, and supplemental 100% oxygen was delivered nasally at a rate of 4 to 5 L/min with blood oxygen saturation continually monitored with a pulse oximeter (Ohmeda; Louisville, Ky). Flexible fiberoptic bronchoscopes (Olympus; Lake Success, NY) of various models were used.

The histopathologic diagnosis of rejection was determined by TBB and graded according to the international working formulation for classification and grading of pulmonary rejection. BAL analysis included direct microscopy for CMV, Pneumocystis carinii, fungi, and mycobacteria. In addition, immunofluorescence microscopy for P carinii, Legionella pneumophila, and respiratory syncytial virus was performed, and polymerase chain reaction for CMV, cultures for bacteria, including Legionella and mycobacteria, fungi, and viruses were analyzed.

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**Table 1—Clinical Data of Patients Who Have Undergone Transplants**

<table>
<thead>
<tr>
<th>Preoperative Diagnosis</th>
<th>n (M/F)</th>
<th>Surgical Procedure</th>
<th>Mean Age, yr (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emphysematous disease</td>
<td>9 (3/6)</td>
<td>Single lung</td>
<td>47 (36-56)</td>
</tr>
<tr>
<td>Eisenmenger's syndrome</td>
<td>3 (3/0)</td>
<td>Heart-lung</td>
<td>18 (16-21)</td>
</tr>
<tr>
<td>Primary pulmonary hypertension</td>
<td>2 (1/1)</td>
<td>Bilateral lung</td>
<td>37 (33-41)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>1 (1/0)</td>
<td>Heart-lung</td>
<td>39</td>
</tr>
</tbody>
</table>

Clinical Investigations

90
Collection of Samples

Serum was separated from a 10-mL venous blood sample according to Ahlstedt et al.29 and frozen at −70°C for later analysis of inflammatory markers.

BAL fluid was obtained by performing an infusion of 7×20 mL warmed sterile phosphate-buffered saline solution into a segmental middle lobe bronchus with the bronchoscope in a wedged position. The fluid was aspirated, collected in a sterile siliconized container, and immediately transported on ice to the laboratory. Cellular components were sedimented by centrifugation at 4°C, 500×g for 10 min, and the supernatant was removed and frozen at −70°C. Before later analysis of inflammatory markers, an additional 10 min of centrifugation at 10,000×g was performed. Cytocentrifuge slides (Shandon Southern Products Ltd; Runcorn, UK) were made from 100-µL aliquots of the resuspended cell pellet. Slides were immediately fixed in 96% alcohol and stained with May-Grünwald-Giemsa. In addition, cell types were identified on a morphologic basis. Percentages of eosinophils, macrophages, and lymphocytes were calculated by counting 200 cells using a standard light microscope. All samples were analyzed in a blinded manner.

TBB specimens were taken after collection of BAL fluid. Four to six biopsy specimens were taken under fluoroscopic guidance from different sites within one lung using alligator forceps, immediately placed in 10% buffered formalin, and sent for histologic analysis.

Analysis of Inflammatory Markers and cCAMs

RIA analysis of ECP37 and HYA38 (Pharmacia Diagnostics AB; Uppsala, Sweden) was performed according to the instructions of the manufacturers. MPO39 and albumin were analyzed in BAL fluid only. Soluble ICAM-1, soluble VCAM-1, and soluble E-selectin were determined as single determinations using commercial enzyme-linked immunosorbent assays (R&D Systems Europe Ltd; Abingdon, Oxon, UK). The serum samples were diluted 1:20 and the BAL samples 1:4 in sample diluent. The assay was standardized against a purified soluble form of recombinant ICAM-1 or E-selectin. The intercoefficients and intracoef- ficients of variation of the enzyme-linked immunosorbent assays used in the study were investigated and accounted for in the study instructions issued by the manufacturers.

Statistical Evaluation

Results are expressed as arithmetic mean and SD. Kruskall-Wallis nonparametric test was used to analyze the significance of differences in data among the four groups of rejection. Mann-Whitney U test was then used for the comparison between individual groups. Spearman rank correlation test was used to examine possible associations between cells and inflammatory markers, and p values <0.05 were considered to be statistically significant.

RESULTS

There were no deaths during the study period. Bronchoscopies with concurrent TBB and BAL were performed on 38 different occasions. Eleven patients were studied at least twice (mean, 3.1; range, 2 to 5 per patient), 2 to 60 weeks after transplantation. In four patients, samples were from one occasion only, and on seven occasions BAL fluid, but not serum samples, was taken. There were 22 histopathologically diagnosed rejection episodes (58%), and of these 7 were minimal (A 1), 13 were mild (A 2), and 2 were rejection episodes of moderate (A 3) character. No episode of severe rejection was diagnosed.

The results are presented as sampling episodes with regard to characterization of rejection. Two episodes of bacterial infection, one episode with CMV pneumonitis, and one with respiratory syncytial virus pneumonitis were diagnosed.

Table 2—Mean and SD for ECP, HYA, MPO, Albumin, and cICAM-1 in BAL Fluid With Relation to Infection and Rejection

<table>
<thead>
<tr>
<th>Bronchoscopic Diagnosis</th>
<th>n*</th>
<th>ECP, μg/L</th>
<th>HYA, μg/L</th>
<th>MPO, μg/L</th>
<th>Albumin, μg/L</th>
<th>cICAM-1, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial infection</td>
<td>2</td>
<td>11 (9)</td>
<td>148 (99)</td>
<td>489 (401)</td>
<td>60 (50)</td>
<td>61 (33)</td>
</tr>
<tr>
<td>Viral infection</td>
<td>2</td>
<td>15 (18)</td>
<td>33 (24)</td>
<td>235 (250)</td>
<td>116 (62)</td>
<td>29 (16)</td>
</tr>
<tr>
<td>Rejection</td>
<td>22</td>
<td>19 (28)</td>
<td>197 (250)</td>
<td>420 (560)</td>
<td>83 (51)</td>
<td>53 (38)</td>
</tr>
<tr>
<td>No rejectionb</td>
<td>12</td>
<td>8 (17)</td>
<td>62 (53)</td>
<td>181 (250)</td>
<td>55 (34)</td>
<td>62 (35)</td>
</tr>
</tbody>
</table>

*p<0.01 vs no rejection (Mann-Whitney U test).

p<0.05.

Parentheses following numbers=1 standard deviation (SD).

Table 3—Mean and SD for ECP, HYA, and cCAMs in Serum With Increasing Severity of Rejection (in Four Episodes No Serum Was Sampled)

<table>
<thead>
<tr>
<th>Rejection Severity</th>
<th>n*</th>
<th>ECP, μg/L</th>
<th>HYA, μg/L</th>
<th>cICAM-1, μg/L</th>
<th>cVCAM-1, μg/L</th>
<th>cE-selectin, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>No rejection (A 0)</td>
<td>11</td>
<td>7 (4)</td>
<td>92 (64)</td>
<td>375 (235)</td>
<td>1,522 (542)</td>
<td>57 (18)</td>
</tr>
<tr>
<td>Minimal (A 1)</td>
<td>7</td>
<td>13 (5)</td>
<td>142 (112)</td>
<td>374 (84)</td>
<td>1,710 (324)</td>
<td>67 (16)</td>
</tr>
<tr>
<td>Mild (A 2)</td>
<td>11</td>
<td>13 (7)</td>
<td>36 (26)</td>
<td>280 (65)</td>
<td>1,422 (335)</td>
<td>52 (15)</td>
</tr>
<tr>
<td>Moderate (A 3)</td>
<td>1</td>
<td>8 (-)</td>
<td>460 (-)</td>
<td>410 (-)</td>
<td>2,250 (-)</td>
<td>96 (-)</td>
</tr>
</tbody>
</table>

n* = number of sampling episodes.

Parentheses following numbers=1 standard deviation (SD).
**Eosinophils in BAL**

![Graph showing eosinophils in BAL](image)

Figure 1. BAL fluid levels of ECP (μg/L) in and percentage of eosinophils in no rejection (A 0), minimal (A 1), mild (A 2), and moderate (A 3) acute rejection. Mean values and SD are presented. Asterisk = p<0.05; two asterisks = p<0.01; n.s. = nonsignificant.

**Inflammatory Markers and cICAMs in BAL and Serum**

The mean levels of BAL ECP and BAL HYA were significantly higher when all rejection episodes were compared to no rejection (p<0.01 and p<0.05; Table 2). The patients with mild acute rejection (A 2) had significantly higher mean levels of BAL ECP (mean, 25; SD, 33; p<0.01) and BAL HYA (mean, 266; SD, 299; p<0.05) compared to the group with no rejection (A 0). (BAL ECP: mean, 8; SD, 17; Fig 1; and BAL HYA: mean, 62; SD, 52; Fig 2). The patients with minimal acute rejection (A 1) had intermediate values (BAL ECP: mean, 12; SD, 18; BAL HYA: mean, 97; SD, 111), albeit not significantly different from group A 0 and A 2. The mean level of BAL MPO was raised in group A 2, but not statistically different compared with the other groups (Fig 3). The two patients with moderate acute rejection (A 3) had intermediate values of ECP, HYA and MPO (Fig 1-3). The BAL albumin levels were comparable in all four groups (Fig 4).

The levels of cICAM-1 in BAL fluid were not statistically different between the rejection groups (Fig 4). BAL VCAM-1 and BAL cE-selectin were all below the detection limit of 2 μg/L (data not shown). In serum, levels of all three cCAMs were measurable, but the mean levels did not differ statistically between the groups (Table 3). Rejection (n=22) compared to no rejection (n=12) showed no differences in levels of inflammatory markers, cCAMs, or albumin (Table 2). Episodes with bacterial and viral infection were few, and these results were analyzed separately from the rejection episodes (Table 2).

The serum levels of ECP, HYA, and cCAMs did not differ statistically between the groups (Table 3), but the mean serum ECP levels were significantly higher when the rejection episodes (mean, 12 μg/L; SD, 6 μg/L) were compared to no rejection (mean, 7 μg/L; SD, 4 μg/L; p<0.05).
**Differential Cytology in BAL**

The patients with mild acute rejection (A 2) had significantly higher mean percentage of eosinophils (p<0.05) compared to the group with minimal rejection (A 1) (Fig 1). Neither the mean percentage of neutrophils (Fig 3) nor the percentages of lymphocytes and macrophages were statistically different between the groups (data not shown).

**Correlation Between Inflammatory Markers and Cells**

BAL fluid levels of ECP correlated significantly with percentage of eosinophils in BAL (p<0.05; rho=0.43). BAL fluid levels of MPO correlated significantly with percentage of neutrophils in BAL (p<0.002; rho=0.55).

**DISCUSSION**

This retrospective study in patients who have undergone lung transplantation shows that activation of eosinophils and fibroblasts assessed by ECP and HYA in BAL fluid is present during mild acute rejection of the allograft. The percentage of eosinophils is also increased. Increased activation of neutrophils or changes in neutrophil percentage are not apparent. The findings are in agreement with earlier reports in which increased percentage of eosinophils and signs of eosinophil activation have been found both in acute kidney and liver allograft rejection. To our knowledge, this is the first study reporting increased ECP levels and allograft eosinophilia during acute rejection of human transplanted lungs.

The eosinophil can release potent cytotoxic granule products that have been associated with the cellular damage seen in a variety of inflammatory diseases, including bronchial asthma. Several studies have hypothesized that the eosinophil could act as an effector-cell in the inflammation observed during acute graft rejection. Pathogenetically, the eosinophilia could be triggered by release of cytokines such as interleukin-3 (IL-3) and IL-5 from activated helper T cells, and IL-1 from antigen-presenting macrophages in the allograft. Eosinophilia is found early in the rejection process, and it is reversed promptly with the standard high-dose steroid rejection treatment.

From our data, the levels of ECP in BAL fluid appear to reflect eosinophil activity better than serum levels. Increased serum ECP level was found during acute rejection compared to no rejection, but there were no differences between the subgroups as found in the BAL fluid samples. Our interpretation is that the eosinophil activation is largely a local process in the rejecting lung, rather than a systemic one.

**Neutrophils in BAL**

![Graph showing Neutrophils in BAL](image)

**MPO in BAL**

![Graph showing MPO in BAL](image)

**FIGURE 3.** BAL fluid levels of MPO (μg/L) and percentage of neutrophils in no rejection (A 0), minimal (A 1), mild (A 2), and moderate (A 3) acute rejection. Mean values and SD are presented; n.s.=nonsignificant.

Our findings of increased levels of HYA in episodes of mild acute rejection are slightly different from the recently published results by Rao et al. They reported elevated HYA levels in BAL fluid from lung transplant recipients with moderate and severe rejection compared to clinically stable recipients, whereas patients with mild rejection had intermediate levels. However, they did not immediately discard the cellular compo-
Increased levels of HYA, which have been found in patients with kidney and liver transplants during acute rejection, are associated with the interstitial edema seen in the allograft tissue. It is known that HYA, a molecule with unique water-binding properties, may be produced by activated fibroblasts in response to macrophage-released mediators such as tumor necrosis factor-α, tumor necrosis factor-β, and IL-1. It is presumable that HYA and possibly fibroblast activation can be involved in the edema present in rejecting lung allografts.

No significant increase in levels of MPO was found, even if a trend toward increasing values with severity of rejection was present. The incidence of bacterial infection during the study period was too low to evaluate if it affected the levels of MPO. Patients with chronic bronchitis, an inflammatory airway disease characterized by neutrophil activation, have increased levels in bronchial lavage. Chronic, but not acute, lung rejection has been associated with increased numbers of neutrophils, but the activation status of the cells involved is not known.

Notably, the almost consistent pattern in our study of increasing ECP and HYA levels with more severe rejection is disturbed by the two episodes of moderate rejection (A 3) with intermediate values. However, the low number of A 3 episodes makes it difficult to evaluate the activity level for both eosinophils and fibroblasts for these episodes. The level of immunosuppression was comparable between the groups.

We found circulating forms of ICAM-1, cVCAM-1, and cE-selectin in serum, but only cICAM-1 was present in BAL fluid. This is consistent with earlier findings from patients with obstructive chronic bronchitis, reflecting the fact that VCAM-1 and E-selectin are expressed on endothelial cells, whereas ICAM-1 can be found also on airway epithelial cells. In transplantation, increased expression of CAMs has been shown in tissue samples during rejection. Only cICAM-1 of the circulating forms has been studied in connection with rejection, and conflicting reports exist regarding the value of this molecule as a marker. One study on patients who had undergone liver transplantation found evidence of local release of cICAM-1 in bile during rejection, but serum levels were not specific since they were elevated also in infection. A second study on heart transplant recipients found no correlation of serum cICAM-1 with endomyocardial biopsy scores, although high levels were associated with poor survival. In contrast, a study on kidney transplant recipients found increased serum cICAM-1 levels in connection with acute rejection, and irreversible graft rejection showed persistently elevated levels. In our study, we found no relationship between either cCAM in serum or in BAL fluid and

**Figure 4.** BAL fluid levels of cICAM-1 (μg/L) and albumin (mg/L) in no rejection (A 0), minimal (A 1), mild (A 2), and moderate (A 3) acute rejection. Mean values and SD are presented; ns = nonsignificant.
degree of rejection, and levels of eCAMs therefore appear to be of limited value as markers for acute rejection in lung transplant recipients. The similar albumin levels signal that plasma leakage is not a prominent factor during rejection.

Elevated levels of ECP and HYA are not unique findings in rejection of transplanted organs, but they can also be found in a variety of inflammatory disorders. However, it appears from our results that measurements of ECP and HYA in BAL fluid in addition to TBB can give complementary information about the quality of the inflammatory process present during acute rejection. However, the individual values in our study partly overlap, which makes the interpretation of a single sample unsuitable for diagnostic purposes. Therefore, the role of ECP and HYA for monitoring lung- and heart-lung transplant recipients will have to be evaluated in larger prospective studies.

ACKNOWLEDGMENT: The authors thank Anders Thylen, MD, and Gunnar Martensson, MD, PhD, at the Department of Pulmonary Medicine, for assistance in specimen sampling, Christer Kjellström, MD, at the Department of Pathology, for reevaluating all the biopsy specimens, and Ingrid Enander, PhD, Pharmacia Diagnostics, for her benevolent supply of ECP RIA kits.

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