Acute Humoral Rejection of Human Lung Allografts and Elevation of C4d in Bronchoalveolar Lavage Fluid

Geraldine G. Millera,∗, Luis Destarac, Adriana Zeevic, Kenneth McCurrye, Aldo Iaconoc, John J. Murraby, Deborah Crowed, Joyce E. Johnsona, Mathew Ninanf and Aaron P. Milstoneb

Divisions of a Infectious Diseases and b Allergy/Pulmonary and Critical Care, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA
c University of Pittsburgh Medical Center, Pittsburgh, PA, USA
d DCI Laboratory, Nashville, TN, USA
Departments of e Pathology and f Surgery, Vanderbilt University School of Medicine, Nashville, TN, USA
∗ Corresponding author: Geraldine G. Miller, geraldine.miller@vanderbilt.edu

Antibody-mediated rejection is well established for renal allografts but remains controversial for lung allografts. Cardinal features of antibody-mediated rejection in renal allografts include antibodies to donor human leukocyte antigen (HLA) and evidence for antibody action, such as complement activation demonstrated by C4d deposition. We report a lung allograft recipient with circulating antibodies to donor HLA who failed treatment for acute cellular rejection but responded to therapy for humoral rejection. To address the second criteria for antibody-mediated rejection, we determined whether complement activation could be detected by measuring C4d in bronchoalveolar lavage fluid (BALF) by ELISA. Airway allergen challenge of asthmatics activates the complement pathway; therefore, we used BALF from asthmatics pre- and post-allergen challenge to measure C4d. These controls demonstrated that ELISA could detect increases in C4d after allergen challenge. BALF from the index patient had elevated C4d concomitant with graft dysfunction and anti-donor HLA in the absence of infection. Analysis of BALF from 25 additional lung allograft recipients showed that C4d concentrations >100 ng/mL were correlated with anti-HLA antibodies (p = 0.006), but were also observed with infection and in asymptomatic patients. The findings support the occurrence of anti-HLA-mediated lung allograft rejection and suggest that C4d measurement in BALF may be useful in diagnosis.

Key words: Antibody, HLA, humoral rejection, lung transplantation

Abbreviations: BALF, bronchoalveolar lavage fluid; BOS, bronchiolitis obliterans syndrome; HLA, human leukocyte antigen; PRA, panel reactive antibody; IVIG, intravenous immunoglobulin; CMV, cytomegalovirus; PTLD, post-transplant lymphoproliferative disease; EBV, Epstein–Barr virus; RSV, respiratory syncitial virus; MBL, mannose binding lectin.

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Introduction

The pathogenic role and prognostic significance of anti-human leukocyte (HLA) antibodies are well established in hyperacute, acute, and chronic rejection of renal and cardiac allografts [reviewed in (1)]. In lung transplantation, antibody-mediated hyperacute rejection has been described in four cases (2–5) as well as increased early ventilatory requirement in patients with pre-transplant panel reactive antibody (PRA) > 10% (6). Evidence for the prognostic significance of anti-HLA antibodies in chronic rejection of lung allografts is provided by five reports demonstrating a correlation between anti-HLA antibodies and bronchiolitis obliterans syndrome (BOS) (6–10). Despite these findings for hyperacute and chronic rejection, clear evidence for antibody-mediated acute rejection in lung transplantation has been lacking. St. Martin (11) performed staining for IgG, IgM, and C3 on 107 biopsies from 55 lung transplant recipients. Despite the large number of specimens, no fluorescent staining for IgG, IgM, or C3 was observed. The authors concluded that humoral rejection could not be demonstrated in lung allografts (11). Subsequently, Gammi reported that pre-transplant PRA > 10% had no effect on the frequency of acute rejection, patient survival, or development of BOS (12). In another investigation, Lau retrospectively reviewed the effects of positive PRA on outcomes for 200 lung transplant recipients (6). Despite worsened early graft function and increased BOS, PRA > 10% was not associated with an increased frequency of acute rejection (6). Thus, these studies did not show evidence for either increased rejection, a distinctive clinical or pathological picture of graft dysfunction, or worsened outcome associated with positive PRA.

In contrast to those reports, Badesch described five patients with a distinct histopathology termed pulmonary capillaritis (13). The histology included neutrophilic infiltration of the alveolar septa, fibrin thrombi occluding the
septal capillaries, fibrinoid necrosis of the septa, and alveolar hemorrhage. The authors speculated this is a form of acute vascular rejection, but neither antibody deposition nor anti-HLA antibodies in serum were demonstrated. Magro recently reported similar histopathology (14,15). Patients had clinical graft dysfunction, and C1q, C3, and C4d were detected in septal lesions. None of the patients had anti-donor HLA antibodies, and the authors postulate that the lesions reflect humoral rejection due to anti-endothelial cell antibodies. The contribution of preformed and de novo anti-HLA antibodies to lung allograft dysfunction was documented in a recent study at the University of Pittsburgh (16). The frequency of severe acute cellular rejection (grade A3 and higher) was significantly increased in patients who had pre- and post-transplant anti-HLA antibodies.

Here we describe a patient who developed acute graft failure despite conventional therapy for biopsy-proven cellular rejection. Circulating antibodies specific for donor class I and class II HLA were present concomitant with graft dysfunction. Activation of complement within the lung was demonstrated by elevation of C4d in bronchoalveolar lavage fluid (BALF) in the absence of infection. Graft failure resolved after plasmapheresis and intravenous immunoglobulin (IVIG). The data show that acute humoral rejection secondary to anti-HLA antibodies occurs and can cause severe lung allograft dysfunction.

Materials and Methods

Source of samples for analysis of circulating anti-HLA antibodies and C4d in bronchoalveolar lavage fluid
All studies involving human subjects were approved by the Institutional Review Boards (IRBs) at Vanderbilt University Medical Center and the University of Pittsburgh. This study was a retrospective analysis of plasma and bronchoalveolar lavage fluids. Patients at Vanderbilt who had undergone lung transplantation were asked for consent to store samples of BALF, plasma, serum, and lung tissue as part of a tissue bank for future research (IRB #000397). These were collected at the time of surveillance bronchoscopy or for clinical deterioration. The specimens used in this study were from this bank of samples and included all specimens for which there was a paired simultaneous plasma and BALF sample. These were stored at −70°C from the time of collection. A total of 22 paired samples of BALF and plasma were available from 12 transplant recipients at Vanderbilt over approximately 2 years. During the period in which these samples were collected and stored, 21 patients were transplanted. The 12 patients from whom these samples were obtained did not differ substantially from the total population. Samples from the University of Pittsburgh were from patients enrolled in a randomized trial of aerosol cyclosporine prophylaxis. Residual material that could be used for this study was available from 18 paired BALF and plasma samples from 14 patients. The 40 pairs of samples from 26 patients comprise this study. Vanderbilt patients received induction with basiliximab (Simulect, Novartis, USA) (20 mg on day 0 and post-opertative day 4). Immunosuppression consisted of cyclosporine beginning orally prior to transplant, intra- and postoperative intravenous methylprednisolone, followed by oral steroids, cyclosporine, and azathioprine. Patients at the University of Pittsburgh were randomized to aerosol cyclosporine or placebo. The maintenance immunosuppression consisted of tacrolimus, azathioprine, and oral steroids. Transbronchial biopsy specimens were graded for acute rejection according to ISHLT criteria.

BALT from seven mild to moderate allergic asthmatics was also used for this study. These BALF had been obtained previously for other studies (Vanderbilt IRB #6862). Residual BALF remaining after completion of those studies had been stored at −80°C until used here. Segmental airway allergen challenge was performed as previously described (17). Briefly, after topical lidocaine anesthesia, the bronchoscope was inserted into the airways and a control BAL was performed in either a lingula or right middle lobe segment using 50 mL of saline. In the opposite segment, allergen to which the subject was skin prick test positive was instilled. Twenty-four hours later, the antigen-challenged segment was lavaged. The BALF was filtered and stored at −70°C until tested.

Measurement of anti-HLA antibodies
Flow cytometric detection of anti-HLA antibodies and specificity testing. Flow PRA (One Lambda, Inc., Canoga Park, CA, USA) was performed according to the manufacturer’s instructions. Briefly, the assay uses pools of beads coated with purified class I or class II HLA from lymphoblastoid cell lines from 30 donors. Serum (20 μL) was mixed with 5 μL beads of each class, incubated for 30’, washed with PBS, then stained with FITC-conjugated anti-human IgG for 30’. The beads were washed, fixed, and analyzed on a Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA, USA). Positive samples were analyzed for specificity with the flow PRA specificity system using four pools of class I or class II beads coated with antigens from eight different Epstein–Barr virus (EBV) transformed cell lines.

ELISA detection of anti-HLA antibodies and specificity testing. Plasma or serum was screened for anti-HLA antibodies by ELISA according to the manufacturer’s instructions (LATTM, One Lambda Inc.). Briefly, diluent, control serum, or patient samples were added to plates coated with purified HLA antigens. The plates were incubated for 60 min followed by addition of enzyme conjugated anti-IgG secondary antibody. After 40 min, substrate was added, the trays were incubated for 10–15 min and reactions were stopped. Samples that were positive in the screen were analyzed for specificity by LATTM ELISA using a Bio-Tek ELX800 plate reader (Bio-Tek, Analytical Instruments, Golden Valley, MN, USA). Optical densities were analyzed by One Lambda software. All tests were duplicated and the positive cut-off calculated as 20% of average positive serum control and 10% of average positive IgG control.

C4d ELISA on plasma and BALF
ELISA for C4d was performed using the C4d Fragment Enzyme Immunoassay (Quidel, Mountainview, CA, USA). Plasma or serum samples were diluted 1:70 or 1:80 and assays were performed according to the manufacturer’s instructions. Protein concentration of BALF was determined using the Bio-Rad protein assay and samples (Bio-Rad, Hercules, CA, USA) were assayed undiluted and at 1:10 dilution. Optical density was read at 405 nm and the concentration was determined from the curve generated with the manufacturer’s standards.

Statistical analysis
Analysis of mean C4d in pre- and post-allergen challenge BALF was performed by Student’s t-test. The relationship between C4d in BALF and circulating anti-HLA antibodies was analyzed by Fisher’s exact test. A p-value <0.05 was considered significant.

Results

Case report
The index patient was a 58-year-old Caucasian male who underwent single lung transplantation for idiopathic pulmonary fibrosis. The patient’s HLA was A24, 68; B7, 39;
Despite negative cultures, graft function did not improve with negative endotracheal and blood cultures after 10 d. The infection was treated aggressively and he defervesced because of (Table 1). Plasmapheresis was begun but halted after 2 days. PRA was positive at 50% for class I and 53% for class II. Positive for class I and class II antibodies and the tested for anti-HLA antibodies. The screening ELISA was negative for class II and positive at 13% for class I. The patient remained ventilator dependent with dense infiltration to tacrolimus and mycophenolate mofetil. The patient was subsequently treated with steroid refractory rejection. There was no improvement with methylprednisolone (1 g/d for 3 d); however, hypoxia necessitated mechanical ventilation. Computerized tomography revealed extensive consolidation throughout the transplanted lung (Figure 1A). At discharge, FEV1 was 2.2 liters. Follow-up bronchoscopy with transbronchial biopsy (post-transplant day 138) revealed no rejection or infection and normal histology of the graft. Cultures of the BALF were negative for bacteria, fungi, and viruses. At the same time, a rebound in anti-HLA antibody occurred and both flow PRA and ELISA screening were positive. Specificity testing demonstrated donor-specific anti-HLA antibody to DQ2 only. The patient has been treated with monthly IVIG, daily prednisone, tacrolimus, and mycophenolate and remains well with stable FEV1.

The recently revised Banff criteria for acute antibody-mediated rejection in renal allografts includes three cardinal criteria (18): (i) morphologic evidence of acute tissue injury; (ii) immunopathologic evidence for antibody action, such as C4d in peritubular capillaries; and (iii) evidence of circulating antibodies to donor HLA or other anti-donor endothelial antigens. The index patient demonstrated circulating antibodies specific for donor HLA A1, DR17, and DQ2 by both methods (Table 1). Plasmapheresis was begun but halted after 2 d because of Pseudomonas pneumonia and bacteremia. The infection was treated aggressively and he defervesced with negative endotracheal and blood cultures after 10 d. Despite negative cultures, graft function did not improve and he remained ventilator dependent. Therefore, plasmapheresis was instituted again (58th post-transplant day) for 5 consecutive days followed by IVIG 1 g/kg. Within 48 h, his chest radiograph began to clear and he was rapidly weaned from the ventilator. Computed tomography 5 d after completion of plasmapheresis and IVIG (68th post-transplant day) showed considerable clearing of the transplanted lung (Figure 1B). Flow PRA positivity declined but did not reach zero until the 86th post-transplant day. The CT scan showed complete clearing of the transplanted lung (Figure 1C). At discharge, FEV1 was 2.2 liters. Follow-up bronchoscopy with transbronchial biopsy (post-transplant day 138) revealed no rejection or infection and normal histology of the graft. Cultures of the BALF were negative for bacteria, fungi, and viruses. The patient responded to antibiotic treatment for P. aeruginosa with negative cultures, defervescence, and normal leukocyte count but remained ventilator dependent.

<table>
<thead>
<tr>
<th>Date</th>
<th>Post-operative day</th>
<th>Respiratory status</th>
<th>Flow PRA Class I</th>
<th>Flow PRA Class II</th>
<th>HLA ELISA Class I</th>
<th>HLA ELISA Class II</th>
<th>Donor specificitya</th>
<th>Biopsy</th>
<th>BALF C4d (ng/mL)</th>
<th>Culture and viral antigen assay results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/29/03</td>
<td>0</td>
<td>Good</td>
<td>13%</td>
<td>0%</td>
<td>NDb</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Negative bacteria, fungi, viruses</td>
</tr>
<tr>
<td>2/17/03</td>
<td>18</td>
<td>Hypoxia</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>A2–A3</td>
<td>P. aeruginosa, S. maltophilia</td>
</tr>
<tr>
<td>3/11/03</td>
<td>42</td>
<td>Graft failure</td>
<td>50%</td>
<td>53%</td>
<td>Pos</td>
<td>Pos</td>
<td>A1 DR17 Q2</td>
<td>ND</td>
<td>ND</td>
<td>Negative bacteria, fungi, viruses</td>
</tr>
<tr>
<td>4/25/03</td>
<td>86</td>
<td>Good</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>45%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Negative bacteria, fungi, viruses</td>
</tr>
<tr>
<td>6/17/03</td>
<td>138</td>
<td>Good</td>
<td>28%</td>
<td>45%</td>
<td>ND</td>
<td>ND</td>
<td>DQ2</td>
<td>ND</td>
<td>ND</td>
<td>Negative bacteria, fungi, viruses</td>
</tr>
</tbody>
</table>

a Determined by both flow cytometry and ELISA.
b ND, not done.
c BALF had no growth on bacterial, fungal, viral cultures. Antigen assays for CMV, RSV, influenza A and B were negative.
d Endotracheal aspirate 3/10 grew Stenotrophomonas maltophilia. Negative fungal and viral cultures. BALF and blood grew Pseudomonas aeruginosa on 3/15. Fungal and viral cultures were negative. The patient responded to antibiotic treatment for P. aeruginosa with negative cultures, defervescence, and normal leukocyte count but remained ventilator dependent.
tissue was not available for analysis of C4d staining to test this possibility. Therefore, we wished to determine if the third criterion could be satisfied by measurement of C4d in BALF and whether its concentration is a useful indicator of immunoglobulin-dependent classic complement pathway activation.

C4d in BALF from subjects with asthma

Prior studies in animal models and human asthma have shown that complement is activated within the lung and C3a and C5a can be detected in BALF after allergen challenge (19–22). In these studies, pre-challenge C3a in BALF was low or absent and rose significantly after challenge. No increase occurred in normal subjects and minimal or no change was observed in asthmatics after saline challenge. Because generation of C3a required the presence of both antigen-specific IgE and antigen in the airways, this reflects activation of the classic pathway via immune complexes. Therefore, we anticipated that C4d should be present in BALF after segmental airway allergen challenge in asthmatics and such BALF could serve as positive controls for the sensitivity of C4d detection. Pre- and post-allergen challenge BALF that had been obtained previously for other studies were used to measure C4d. As shown in Figure 2, C4d levels were less than 100 ng/mL in 5 of the 7 asthmatic subjects pre-challenge and significantly increased by threefold to more than 20-fold (mean sixfold) post-allergen challenge in 6 of the 7 subjects (p < 0.001). The results indicated that C4d could be detected in BALF and quantitative changes reflected complement activation in the lung by antigen-antibody complexes. C4d levels in serum showed no change after allergen challenge (data not shown).

C4d in BALF from lung transplant recipients correlates with the presence of donor-specific anti-HLA antibodies in plasma

Based on the above results, we performed a retrospective analysis of BALF from lung transplant recipients. We tested two BALF from the index patient and 38 from 25 other patients. These samples were chosen based upon availability of a simultaneous plasma or serum sample.
Humoral Rejection of Human Lung Allografts

Figure 2: C4d concentration (ng/mL) in bronchoalveolar lavage fluid (BALF) from asthmatics pre- and post-segmental airway allergen challenge. BALF was obtained from mild to moderate asthmatic subjects as described in Materials and Methods either immediately before or 24 h after segmental airway challenge with an antigen to which they were skin-prick test positive. C4d was measured by ELISA.

for measurement of anti-HLA antibody. The presence of anti-HLA antibody was determined by a screening ELISA and positive samples were further characterized for donor specificity by a subsequent ELISA as described in Materials and Methods. Individual data for all patients who had elevated C4d, anti-HLA, or both are shown in Table 2. Summary data for all samples and patients are shown in Table 3. Thirteen out of 40 BALF (from 8 patients) had C4d levels >100 ng/mL. Ten out of 40 plasma or serum samples (from 6 patients) were positive for anti-HLA antibodies. Specificity for donor HLA could be determined for five of the six patients, and four of five had donor-specific antibodies (Table 2). Overall, there was a strong correlation between C4d >100 ng/mL and the presence of donor-specific anti-HLA when analyzed either by samples or by patients (Table 3).

The findings in the index case (Table 1 and pt. V166, Table 2) and two other patients with multiple samples (V161, P512, Table 2) are noteworthy. For the index patient, anti-HLA was negative on day 18 post-transplant; however, C4d in BALF was elevated. Donor-specific antibodies were present in the next available sample 24 d later. Bronchoscopy was not performed at day 86 when the patient was clinically improved and antibodies were absent after plasmapheresis and IVIG. BALF obtained approximately 50 d later showed that C4d was >100 ng/mL concomitant with reappearance of anti-donor class II antibodies. Whether the elevation of C4d at this time reflects persistence of complement activation or new elevation with reappearance of anti-donor antibodies cannot be determined.

Patient P512 had pre-existing donor-specific anti-HLA at post-transplant day 6 when a steroid bolus was given for presumed rejection. C4d was not elevated then but rose subsequently. Two consecutive samples, separated by 10 d, demonstrated circulating antibodies and complement activation in the lung (P512.2 and 512.3). Elevated C4d was still observed 3 months later (P512.4), but circulating antibodies were not present. The first sample pair from V161 was concordant for elevation of C4d and presence of anti-donor HLA antibody, whereas the second revealed persistence of circulating antibody but low C4d in the lung. These data show that the timing of complement activation in the graft and detection of circulating anti-HLA antibodies are not necessarily concordant. As discussed below, similar disparity is observed for C4d deposition in renal allografts and the presence of anti-HLA antibodies. When the overall pattern is considered, all four patients with donor-specific anti-HLA had C4d >100 ng/mL on one or more occasions compared with three out of 21 without donor-specific antibodies (Table 3, p = 0.0028).

Two patients also had multiple samples with elevated C4d but no anti-HLA antibodies (V152, V150, Table 2). Three out of the four elevated levels coincided with evidence of infection in the graft. As discussed further below, these findings suggest that complement activation in lung allografts can be observed under a variety of inflammatory conditions and will not be unique to anti-HLA-mediated rejection.

Discussion

The data on the index patient meet the recently modified Banff criteria for antibody-mediated acute rejection in renal allografts (18) and provide the strongest evidence to date for similar events in lung allografts. The frequency of humoral rejection mediated by HLA-specific alloantibodies in lung allograft recipients remains to be determined. Our finding that four of five patients for whom antibody specificity could be determined had donor-specific antibodies and all had elevated C4d in BALF on one or more occasions suggests that it occurs more commonly than is currently appreciated. Importantly, however, we found that not all patients with alloantibodies, even those with elevated C4d, had clinically evident graft dysfunction. Studies cited earlier (6–10) suggest these patients are at higher risk for development of BOS.

Our results show that C4d can be detected in BALF and reflects classic pathway complement activation. This approach differs from the immunohistochemical approach developed over the last decade in renal allografts (23–26). First, diagnosis of antibody-mediated rejection in renal allografts is dependent upon the location of C4d deposition. Only deposition in the interstitial peritubular capillaries of the allograft is diagnostic, because C4d normally is present in glomerular mesangium. BALF does not provide information on the anatomic site of C4d deposition. However, low C4d levels in pre-challenge BALF from asthmatics suggest
Table 2: C4d in BALF and circulating anti-HLA antibodies

<table>
<thead>
<tr>
<th>Pt./Sample</th>
<th>C4d (ng/mL)</th>
<th>Anti-HLAa</th>
<th>Donor specificityb</th>
<th>TB Bx (ISHLT ACR)c</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>V166.1</td>
<td>222</td>
<td>– (+)d</td>
<td>A1 DR17 Q2</td>
<td>A3</td>
<td>Infiltrates, graft failure</td>
</tr>
<tr>
<td>V166.2</td>
<td>117</td>
<td>+</td>
<td>DQ2</td>
<td>A0</td>
<td>Post-pheresis, stable</td>
</tr>
<tr>
<td>V161.1</td>
<td>256</td>
<td>+</td>
<td>A68</td>
<td>A0</td>
<td>Surveillance</td>
</tr>
<tr>
<td>V161.2</td>
<td>36</td>
<td>+</td>
<td>A68</td>
<td>A0</td>
<td>Surveillance</td>
</tr>
<tr>
<td>V152.1</td>
<td>60</td>
<td>–</td>
<td></td>
<td>A0</td>
<td></td>
</tr>
<tr>
<td>V152.2</td>
<td>194</td>
<td>–</td>
<td></td>
<td>A0</td>
<td>Diffuse infiltrates</td>
</tr>
<tr>
<td>V152.3</td>
<td>192</td>
<td>–</td>
<td></td>
<td>A0</td>
<td>Suspected viral infection</td>
</tr>
<tr>
<td>V150.1</td>
<td>90</td>
<td>–</td>
<td></td>
<td>A0</td>
<td>Surveillance</td>
</tr>
<tr>
<td>V150.2</td>
<td>350</td>
<td>–</td>
<td></td>
<td>A0</td>
<td>Surveillance</td>
</tr>
<tr>
<td>P570</td>
<td>258</td>
<td>+</td>
<td>Unknownf</td>
<td>A1</td>
<td>CMV pneumonia 14 d previously</td>
</tr>
<tr>
<td>P567.1</td>
<td>10</td>
<td>+</td>
<td>No</td>
<td>A3</td>
<td>Eosinophilic infiltrate</td>
</tr>
<tr>
<td>P567.2</td>
<td>5</td>
<td>–</td>
<td></td>
<td>A3</td>
<td>Eosinophilic infiltrate</td>
</tr>
<tr>
<td>P546</td>
<td>152</td>
<td>+</td>
<td>B7(CREG)</td>
<td>A1</td>
<td>Bx, focal BALTh</td>
</tr>
<tr>
<td>P527.1</td>
<td>237</td>
<td>–</td>
<td></td>
<td>A1</td>
<td>Bx acute congestion, alveolar septal necrosis</td>
</tr>
<tr>
<td>P512.1</td>
<td>0</td>
<td>+1</td>
<td>B67i</td>
<td>NDj</td>
<td>6 d post txp, steroid bolus</td>
</tr>
<tr>
<td>P512.2</td>
<td>106</td>
<td>+</td>
<td>B67</td>
<td>B1</td>
<td>3 weeks post 512.1, eosinophilic bronchitis</td>
</tr>
<tr>
<td>P512.3</td>
<td>149</td>
<td>+</td>
<td>B67</td>
<td>B1</td>
<td>Eosinophilic bronchitis</td>
</tr>
<tr>
<td></td>
<td>139</td>
<td>–</td>
<td></td>
<td>A1</td>
<td>Late acute cellular rejection and OB</td>
</tr>
</tbody>
</table>

aAnti-HLA measured by screening ELISA (LAT-M).
bDonor specificity performed by ELISA and flow cytometry for samples V166.1 and V166.2 and by ELISA (LAT 1288) only for all others.
cTB Bx Transbronchial biopsy, ISHLT grade of rejection.
dFirst plasma sample 2/17/03 negative, second plasma sample 3/11/03 positive (see Table 1).
ePTLD, post-transplant lymphoproliferative disease.
fUnknown, sample not available for specificity testing.
gCMV, cytomegalovirus.
hBALT, bronchoalveolar lymphoid tissue.
iAnti-donor antibody present pre-transplant
jTransbronchial bx not done.

In contrast, the post-allergen challenge BALF data show that immune complexes, regardless of specificity, activate the classic complement pathway and generate C4d in the lung. This is analogous to finding C4d in glomerular capillaries, rather than peritubular capillaries, in patients with immune complex nephritis (27).

Our data are thus consistent with studies on C4d in urine of renal allograft recipients (28,29) and patients with lupus nephritis (30). Patients with acute steroid-resistant rejection had the highest mean levels of C4d (70–80 ng/mL) in urine. Patients with acute steroid-sensitive or chronic rejection had levels significantly higher than those with stable graft function which were only minimally different than normal controls. Only a small number of patients with other conditions such as infection were studied (28) and these had minimal increases in C4d. Not surprisingly, however, patients with active lupus nephritis also have elevated urinary C4d (30). These data and ours indicate that the concentration of C4d in fluids from sites with antigen-antibody complexes identifies classic pathway activation.

This phenomenon may explain the observations in patients V152 and V150 (Table 2). Four of seven BALF had C4d >100 ng/mL although none of the sera had

Table 3: BAL C4d vs. circulating donor-specific anti-HLA. (A) BALF C4d vs. circulating donor-specific anti-HLA in 39 paired samples, (B) BAL C4d vs. circulating donor-specific anti-HLA in 25 patients

<table>
<thead>
<tr>
<th>Anti-HLA</th>
<th>C4d &gt; 100</th>
<th>C4d &lt; 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>BAL C4d (ng/mL)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>C4d &gt; 100</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>C4d &lt; 100</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>B.</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>BAL C4d (ng/mL)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>C4d &gt; 100</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>C4d &lt; 100</td>
<td>4</td>
<td>21</td>
</tr>
</tbody>
</table>

aFisher’s exact test.
anti-HLA antibodies. Three of these coincided with bacterial or viral infection. The highest C4d (V150.4, Table 3) was observed after respiratory syncytial virus (RSV) pneumonia and treatment with anti-CD20 antibody (Rituximab, Biogen Idec, Cambridge, MA, USA) for post-transplant lymphoproliferative disease (PTLD). Autopsy confirmed the presence of both necrotic EBV lymphoma and RSV antigen in the lungs by immunohistochemistry (data not shown). Thus, elevated C4d in these cases may reflect activation of the classic pathway via immune complexes of antibodies and their cognate bacterial, viral, or fungal antigens.

An alternative mechanism for activation of the classic complement pathway by infectious agents is via mannose binding lectin (MBL) [reviewed in (31)]. The lectin domain of this pattern recognition molecule binds to sugars on the surface of multiple bacterial, fungal, and viral species. MBL bound to microbial antigens activates an associated protease, MASP-2, which expresses enzymatic activity identical to C1 esterase. Thus MBL functions in a C1- and antibody-independent fashion resulting in the sequential cleavage of C4 and C2. Whether activation is initiated by MBL or IgG complexes, the frequency of infection in lung transplantation suggests that elevated C4d in BALF will be a less specific marker of anti-HLA-mediated humoral rejection than it appears to be in the kidney when assayed by immunofluorescence.

Previous studies show that C4d staining in peritubular capillaries and circulating anti-donor antibodies are not completely concordant (25,32,33). Several possibilities have been proposed to account for this. They include absorption of alloantibodies by the graft; differences in antigen expression between capillary endothelial cells and the targets used to assess presence of circulating alloantibodies; differences in kinetics of degradation or loss of antibodies in the graft vs. the circulation; and presence of non-MHC antigens on endothelial cells that are targets for antibodies. Despite the occurrence of discordant results, alloantibody production is demonstrated in the majority of cases where C4d deposition is documented. Equally important, the presence of C4d heralds significantly worse short- and long-term renal function and survival (23,25,32–34) even in groups where alloantibody is not concordant with C4d. Because complement activation per se is injurious to grafts, we suspect the same finding will be true for elevations of C4d in BALF.

Our findings differ somewhat from recent studies (14,15) that report detection of C1q, C3, C4d, and IgG by immunofluorescence in lung allografts with humoral rejection. In one report (15) none of the patients had a positive PRA; however, they improved after plasmapheresis and post-pheresis biopsies showed decreased complement deposition (15). The authors attributed complement deposition and effects of plasmapheresis to antibodies directed at non-MHC endothelial cell antigens. Some of the BALF grew a variety of organisms including CMV, Pseudomonas, Candida, and Aspergillus. Thus, as discussed above, the observed complement activation may have been related to infection. A second study in noninfected patients (14) also found no anti-HLA in any patient despite positive C4d immunofluorescence in biopsies classified as humoral rejection and BOS. The uniform absence of donor-specific anti-HLA antibodies in these reports contrasts with results for C4d deposition in other solid organ allografts and the results found here. The reasons for this discrepancy are unclear.

In summary, we describe a patient in whom clinical and laboratory data provide strong evidence for anti-HLA-mediated rejection. C4d can be measured in BALF and was elevated in approximately 30% of samples from lung transplant recipients. Half of these were concomitant with donor-specific anti-HLA antibodies, but were not clearly correlated with clinical status. Prospective studies are needed to assess the true frequency of antibody-mediated rejection in lung transplantation and the significance and utility of C4d elevations in BALF.

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References

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