Anti-Human Leukocyte Antigen Antibodies, Vascular C4d Deposition and Increased Soluble C4d in Broncho-Alveolar Lavage of Lung Allografts

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**Background.** The hallmark of humoral rejection is the presence of subendothelial C4d in the allograft. A simultaneous determination of vascular C4d with soluble C4d in broncho-alveolar lavage fluid (BAL) and circulating anti-human leukocyte antigen (HLA) antibodies (HLA-Ab) has not been reported in lung transplantation.

**Methods.** Forty-two consecutive lung-transplant patients were included in this cross-sectional study. The presence and specificity of HLA-Ab was determined at the same frequency with transbronchial biopsies. Soluble C4d levels were measured by enzyme-linked immunosorbent assay in all 42 patients. In a subgroup of 32 patients with available timely matched parafin-embedded tissue sections, the vascular C4d deposition was also assessed.

**Results.** The presence of HLA-Ab in 16 patients was associated with biopsy-proven acute rejection (10/16 vs. 3/16, P<0.01) and increased immunosuppression (13/16 vs. 4/16, P<0.005). Pulmonary function was also decreased in patients with HLA-Ab (mean forced expiratory volume in 1 second=49%) when compared with the control group (mean forced expiratory volume in 1 second=66%, P<0.05). Nine patients exhibited specific vascular C4d deposition and in eight of nine (89%) cases HLA-Ab were detected, versus 8 of 23 (35%) in C4d-negative patients (P<0.05). Soluble C4d in BAL was highly (>0.5 µg/mL) elevated in patients with HLA-Ab and vascular C4d and was moderately (0.2 µg/mL) increased in patients with antibodies but C4d-negative. In contrast, only a slight elevation of soluble C4d (<0.1 µg/mL) was detected in patients without HLA-specific antibodies.

**Conclusions.** The association of HLA-specific antibodies with vascular C4d deposition and soluble C4d in BAL, in addition to the reduced pulmonary function, might constitute a diagnostic triad for antibody-mediated rejection in lung transplant patients.

**Keywords:** Lung transplant, Complement C4d, HLA, Antibodies, Rejection.

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We have reported that the development of anti-human leukocyte antigen (HLA) alloantibody (HLA-Ab) in lung transplant (LTX) patients is associated with high-grade and persistent or recurrent acute rejection and lymphocytic bronchiolitis (1, 2). We and others have also described associations between HLA-Ab and chronic lung allograft dysfunction (1–6). Furthermore, antibody detection preceded by at least 1 year the diagnosis of chronic lung allograft dysfunction (1, 4). However, there is still a debate regarding the pathogenic role of HLA-Ab in LTX (7).

By comparison, complement activation in renal allografts as documented by vascular C4d deposition, has become one of the criteria for the diagnosis of antibody-mediated rejection (8–11). Our previous investigation showed an association between circulating HLA-Ab and soluble C4d in broncho-alveolar lavage (BAL) of LTX patients (12). Furthermore, in another cohort, we established an association between vascular C4d in lung allografts and the presence of circulating anti-HLA antibodies (3).

In the present study we concomitantly evaluated (1) vascular C4d deposition in lung allograft, and (2) the levels of soluble C4d in time matched broncho-alveolar lavage fluid, in relationship with (3) circulating HLA-specific antibodies.

**MATERIALS AND METHODS**

Forty-two consecutive patients who received LTX between 2001 and 2003 at the University of Pittsburgh Medical Center and had at least 1 year of follow-up were included in the study. The Institutional Review Board of the University of Pittsburgh Medical Center and Johns Hopkins University approved the protocol and informed consent was obtained from all patients. All patients received antilymphocytic (antithymocyte globulin [rabbit] or alemtuzumab) antibodies before transplantation, followed by maintenance immunosuppression with FK506 and low-dose steroids. We performed a cross-sectional analysis of vascular and soluble C4d, in association with circulating anti-HLA alloantibodies, in LTX recipients.

**Detection of Human Leukocyte Antigen-Specific Antibodies**

A total of 338 serum samples were tested. Blood samples were collected before lung transplantation and after LTX at the time of transbronchial biopsy (TBB) procedure. We...
used commercial enzyme-linked immunosorbent assay (ELISA) kits (LATM, One Lambda; Canoga Park, CA) in accordance with the manufacturer’s instructions to identify IgG anti-class I or class II HLA-specific alloantibodies. All tests were duplicated and the positive cutoff calculated as 20% of average positive serum control and 10% of average positive IgG control. Antibody specificity was further determined by Luminex HLA class I or HLA class II single-antigen beads (Labscreen, One Lambda; Canoga Park, CA).

Transbronchial Biopsy Procedures
As per our center policy, to diagnose rejection or infection, surveillance fluoroscopically guided TBB and fiberoptic bronchoscopies were routinely performed 2 weeks after lung transplantation and every 3 months thereafter (13). Additional bronchoscopies were performed when changes in clinical or functional parameters occurred, such as infiltration by chest radiography, oxyhemoglobin desaturation, or significant (>10%) decline in forced expiratory volume in 1 second (FEV1) from baseline. Furthermore, follow-up bronchoscopies were also done 3 to 4 weeks after pulse methylprednisolone treated or cytolytic treated rejection. Both in surveillance, and in indication biopsies, BAL was collected and stored at –80°C for future use (12). A total of 64 BAL samples were tested for the presence of soluble C4d in 42 patients. The values of FEV1 were expressed as percentage of the best airflow of every patient in the first 100 days after lung transplantation. The surveillance spirometry was performed in accordance with American Thoracic Society criteria, whereas the grading of perivascular acute rejection followed the ISHLT published criteria (14, 15). In all TBB included in the present analysis infection was excluded.

Immunohistochemistry for C4d
TBB were selected from the paraffin archives of the University of Pittsburgh Medical Center (n=38 TBB and 196 biopsy fragments) for 32 lung recipients where timely matched serum and BAL samples were available. In patients with circulating HLA-specific antibodies, the biopsy corresponding to initial antibody detection (“sentinel biopsy”) was assessed in 16 cases and compared with 16 temporally matched biopsies from the antibody-negative group (3). There was no significant difference between the postoperative TBB testing day in patients with circulating antibodies (448±190, range 203–734 days) when compared with antibody-negative patients (449±198, range 125–855 days). Another criterion in our cross-sectional study was the availability of BAL fluid for soluble C4d evaluation. Hematoxylin-eosin sections were analyzed for the presence of acute cellular rejection. C4d immunohistochemical stain was performed in formalin fixed, paraffin-embedded tissue sections. As we previously described, positive immunoreactivity was defined as linear, continuous subendothelial deposition identified in capillaries, arterioles and venules (Fig. 1) (3).

The C4d deposition was also assessed in LTX patients with diagnosis of infection (CMV-pneumonitis, Candida and Cryptococcus) and without acute rejection. CMV-pneumonitis was defined by the presence of viral inclusions identified on hematoxylin-eosin or on special stains for CMV accompanied by an inflammatory reaction.

Quantitation of Soluble C4d in Bronchoalveolar Lavage Fluid

BAL samples were collected, centrifuged at 800g for 5 min at room temperature and stored at –80°C until testing. Soluble C4d BAL levels were determined using a commercially available ELISA kit (QUIDEL, San Diego, CA). Low and high controls provided with the kit were tested together with undiluted BAL samples. Testing was conducted in duplicate according to the manufacturer’s instructions.

A VERSAmax tunable microplate reader ( Molecular Devices, Sunnyvale, CA) was used to determine the optical density of all analytes at 405 nm. The results were calculated from a standard curve and reported in micrograms per milliliter. The limit of detection for the C4d assay is 0.001 μg/mL.

Statistical Analysis

For continuous dependent variables we used ANOVA and Mann-Whitney U test. For nominal outcomes we used contingency tables and the chi-square test (P values expressed as two-tailed Fisher’s exact test or Yates correction for small numbers, when appropriate). An alpha level of 5% was considered significant (STATISTICA software from StatSoft) (16).

RESULTS

Anti-Human Leukocyte Antigen Alloantibodies
All the historical and actual lymphocytotoxic cross-matches were negative and donor-specific anti-HLA antibodies were not detected by solid-phase methods (ELISA and Luminex) before transplantation. After lung transplantation, the analysis of 338 serum samples revealed the presence of HLA-specific antibody in 16 patients, whereas 26 patients remained antibody-negative by ELISA and Luminex. Ten patients developed anti-class I HLA-specific antibodies (63%), two patients developed anti-class II (12%), whereas both anti-class I and class II HLA-specific antibodies were found in four (25%) lung recipients. There were no significant differences regarding recipient demographic data between patients with or without antibodies (Table 1). Donor specific anti-HLA antibodies were detected in thirteen of the 16 patients (81%). The specificity distribution was toward class I HLA in nine patients, six against HLA-A and three toward
HLA-B, whereas anti-class II donor-specific antibodies were encountered in seven patients: one HLA-DRB1 and six HLA-DQB1 cases (Table 2).

### Soluble C4d in Broncho-Alveolar Lavage Fluid

The levels of soluble C4d in broncho-alveolar lavage fluid ranged from 0.01 to 3.23 μg/mL, with an average of 0.25±0.18 μg/mL. Higher soluble C4d levels (mean=0.28 μg/mL) were observed in the 16 patients with circulating HLA-Ab, as compared with the 26 antibody-negative patients (mean=0.11 μg/mL, P<0.005, Fig. 2).

Elevated soluble C4d in BAL were also encountered in four patients with a diagnosis of infection (Table 2). In three

### TABLE 1. Demographic characteristics in lung transplant patients with or without circulating anti-HLA alloantibodies

<table>
<thead>
<tr>
<th>Lung recipient</th>
<th>HLA antibodies (N=16)</th>
<th>No HLA antibodies (N=26)</th>
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<td>449±198 d</td>
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<td>Female</td>
<td>11/16 (69%)</td>
<td>14/26 (54%)</td>
<td>NS</td>
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<tr>
<td>Double lung</td>
<td>14/16 (88%)</td>
<td>17/26 (65%)</td>
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<td>50.8±11.8 yr</td>
<td>54.4±13.5 yr</td>
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</table>

* Postoperative day for trans-bronchial biopsy and antibody screening.
NS, not significant.

### TABLE 2. The distribution of vascular C4d, soluble C4d, infection, acute rejection, steroid-resistant rejection, and pulmonary function in patients with circulating anti-HLA antibodies versus control

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<th>Pts</th>
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<th>HLA-Ab*</th>
<th>DSAb</th>
<th>ACRc</th>
<th>ACR±1 Bx*d</th>
<th>Sol. C4d (μg/mL)e</th>
<th>FEV1 (%)</th>
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* Circulating anti-HLA alloantibodies.
b Donor-specific HLA antibody.
c Biopsy-proven acute rejection at the same time with vascular C4d staining and soluble C4d evaluation.
d Biopsy-proven acute rejection in the previous or the next biopsy following vascular C4d staining and soluble C4d evaluation.
e ELISA-detected soluble C4d in broncho-alveolar lavage fluid.
f Continuous, linear, subendothelial C4d deposition in lung allograft.
POD, postoperative day; LBB, lymphocytic bronchiolitis.
patients no circulating antibodies were detected, whereas one LTX recipient had both HLA-specific antibodies and infection. The infection complications were CMV-pneumonitis (soluble C4d/H11005 $3.23$ g/mL), *Candida albicans* (soluble C4d/H9262 $1.00$ g/mL), *Cryptococcus neoformans* (soluble C4d/H11005 $0.62$ g/mL) and pneumonia (soluble C4d/H11005 $0.44$ g/mL).

When these four cases of infection were excluded from the analysis, we observed that LTX recipients with circulating HLA-Ab still exhibited significantly higher levels of soluble C4d levels as compared with the antibody-negative group ($0.26$ g/mL, $n=15$ vs. $0.088$ g/mL, $n=23$, $P<0.001$).

**Anti-Human Leukocyte Antigen Alloantibodies and Vascular C4d**

In 32 cases, out of 42 LTX patients, we had available timely matched paraffin biopsy blocks for vascular C4d staining evaluation (Table 2). Sixteen patients in this group exhibited significantly higher levels of soluble C4d levels as compared with the antibody-negative group ($0.26$ µg/mL, $n=15$ vs. $0.088$ µg/mL, $n=23$, $P<0.001$).

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**Anti-Human Leukocyte Antigen Alloantibodies**

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A positive vascular C4d deposition was diagnosed in 9 of 32 LTX recipients tested (28%, Table 2). In 89% (8/9) of those cases we also found circulating HLA-specific antibodies. In contrast, only 35% (8/23) of C4d-negative cases exhibited circulating HLA-Ab ($P<0.05$). Compared with the presence of circulating anti-HLA antibodies, specific vascular C4d deposition was characterized by a sensitivity of 50% and a specificity of 93%.

In addition, there was a significant association between vascular C4d deposition and the level of soluble C4d in BAL: a mean of 0.76 µg/mL in C4d-positive patients versus a mean of 0.21 µg/mL in C4d-negative cases ($P<0.001$). When infection cases were excluded, the association between vascular C4d deposition and soluble C4d in BAL continued to be significant: a mean of 0.46 µg/mL in C4d-positive patients compared with a mean of 0.14 µg/mL in C4d-negative LTX recipients ($P<0.005$) (Fig. 3).

The levels of soluble C4d in BAL exhibited the following stepwise pattern: (1) The highest levels were observed in patients who exhibited both circulating antibodies and positive vascular C4d deposition (mean=0.5 µg/mL), followed by (2) patients with HLA-specific antibodies but no vascular C4d (mean=0.2 µg/mL), and the least in (3) patients without circulating antibodies (mean=0.1 µg/mL) (Table 2).

In the infection-free cohort, patients with circulating HLA-Ab and vascular C4d deposition had soluble C4d levels in BAL more than 0.3 µg/mL in five of seven cases, compared with only one of seven in antibody-positive and C4d-negative group ($P<0.05$) and none out of 14 antibody-negative patients ($P=0.001$).

**Anti-Human Leukocyte Antigen Alloantibodies are Associated With Decreased Pulmonary Function and Increased Immunosuppression**

In this cross-sectional analysis, both the histopathological findings and the pulmonary functional tests were considered at the same time points with the assessment of circulating HLA-specific antibodies, vascular C4d deposition and solu-
ble C4d in the broncho-alveolar lavage fluid (Table 2). Furthermore, one previous and one consecutive biopsy after the vascular C4d staining were also counted. Ten of the 16 patients with circulating HLA-Ab encountered biopsy-proven acute rejection, when compared with only three lung recipients in the antibody-negative group (P<0.01). When all biopsies were considered, 13 of 16 patients with circulating HLA-Ab were diagnosed with biopsy-proven acute cellular rejection, compared with only 5 of 16 in antibody-negative group (P<0.005). Furthermore, the pulmonary function was also decreased in patients with circulating antibodies (mean FEV₁=49%) when compared with patients without antibodies (mean FEV₁=66%, P<0.05) (Table 2).

Augmented immunosuppression was necessary in 13 of 16 patients with circulating HLA-Ab, compared with only 4 of 16 in the antibody-negative group (P<0.005, Table 2).

**DISCUSSION**

In lung transplantation biopsy-proven acute cellular rejection is considered the most important risk factor for chronic allograft dysfunction, whereas the role of the humoral arm of alloimmune response remains to be fully defined (17). We have shown that anti-HLA antibodies correlated with high-grade and refractory forms of acute rejection in lung allografts, and with increased use of steroids (2). In addition, we and others have found significant associations between circulating HLA-specific antibodies and lung allograft dysfunction (1, 4–6, 13, 18).

Several studies highlighted the risk-factor role of anti-class I and anti-class II HLA-Ab in lung transplantation (19–21). In the present cohort, and in previous studies, both anti-class I and anti-class II HLA-specific antibodies were associated with a worse outcome in LTX patients (1–3, 22, 23).

In renal and cardiac transplantation, vascular C4d deposition is a reliable marker of antibody-mediated rejection (8–11, 24–26). The C4d fragment that is generated through the classical, antibody-dependent pathway of complement cascade has the diagnostic advantage of remaining covalently bound to the endothelial cell membrane for days or weeks (24, 27). In a rat model of cardiac allografts C4d was determined to clear within 5 days (28).

Vascular C4d and C3d deposition correlated with heart allograft dysfunction and graft loss (29). Furthermore, in many renal allograft studies vascular C4d deposition was an important diagnostic element for antibody-mediated rejection, in addition to circulating antibodies and clinical phenotype (30). The diagnosis of humoral or mixed forms of rejection is important because antibody-mediated rejection might benefit from specific therapeutic strategies (31, 32).

In an experimental lung transplantation model, it has also been shown that membrane attack complex leads to vascular destruction and that the local production of complement proteins is important. Complement production was demonstrated at the mRNA and protein levels in lung allografts (33).

In human lung transplantation, significant correlation between anti-endothelial antibodies and vascular C4d deposition was shown by one group, whereas another group concluded that C4d staining of paraffin-embedded lung allograft biopsies did not identify acute or chronic humoral rejection (34, 35). In contrast, we have demonstrated that circulating anti-HLA alloantibodies were associated with vascular C4d deposition in lung allografts (3). The prevalence of C4d deposition was only 5% in protocol biopsies, although significantly higher in indiction biopsies (25%) or in patients with circulating antibodies (33%) (3). Similar differences between the prevalence of positive C4d staining in protocol and indication biopsies were reported in large cohorts of cardiac or renal transplant patients (24, 25, 36). Furthermore, we observed that almost 90% of patients with vascular C4d also exhibited circulating HLA-specific antibodies, similar to the renal transplant reports (Table 2) (8, 10, 11).

Broncho-alveolar lavage fluid might offer a unique window into the lung and the screening of BAL fluid for soluble factors may enhance our understanding of pathogenic processes occurring in the allograft. We have reported on a correlation between increased (>0.1 μg/mL) soluble C4d in broncho-alveolar lavage and circulating HLA-specific antibodies, and on an index case with antibody-mediated rejection that responded to specific therapeutic intervention (12).

In the present study we concomitantly addressed the relationship among the circulating HLA-specific antibody screening, vascular C4d deposition and soluble C4d in broncho-alveolar lavage fluid. We established a significant association among all three components, which individually might be suggestive of antibody-mediated rejection, but the additive effect was more powerful, as shown by the stepwise increment of soluble C4d levels in BAL of lung allografts. The highest levels of soluble C4d were detected in patients with circulating antibodies and vascular C4d deposition; intermediate levels were encountered in patients with circulating antibody or vascular C4d, whereas the lowest levels of soluble C4d in BAL fluid were detected in antibody-negative lung recipients.

Diagnosis of antibody-mediated rejection is complex and requires several pathological criteria (30). One of the hallmarks of humoral rejection is the presence of subendothelial C4d in the allograft, which denotes complement activation (Fig. 1) (10, 30, 35, 36). However, the patchy nature of trans-bronchial biopsies in lung allograft may lead to false-negative results. Soluble C4d in BAL was also increased in the presence of infection, which might be valuable clinical information, but decreased the specificity of association with circulating anti-HLA antibodies. In addition, humoral rejection may occur in the presence of non-HLA antibodies or in the absence of circulating antibodies.

Despite all these caveats, the evidence of vascular complement activation in allograft or soluble C4d in BAL in the absence of infection was significantly associated with circulating anti-HLA antibodies and lung allograft dysfunction (Table 2). Furthermore, the higher prevalence of steroid-resistant rejection in patients with HLA-specific antibodies could be a hallmark of the mixed (cellular and humoral) pattern of these rejections.

In summary, the majority of specific vascular C4d deposition was encountered in patients with HLA-specific antibodies. Soluble C4d in broncho-alveolar lavage fluid was highly (>0.5 μg/mL) elevated in patients with anti-HLA antibodies and vascular C4d, moderately (0.2 μg/mL) rose in patients with antibodies but no C4d deposition, and only slightly elevated in patients without HLA-specific antibodies. However, infections were also associated with high soluble
C4d levels in broncho-alveolar lavage fluid. Although both the diagnosis of infections and rejections are clinically important, soluble C4d alone might not be sufficient for the diagnosis of antibody-mediated rejection. That is why the presence of circulating donor-specific alloantibody and vascular C4d deposition will increase the probability of antibody-mediated rejection, especially in patients with allograft dysfunction or refractory rejection. Furthermore, these patients might benefit from antibody-specific therapeutic strategies.

REFERENCES