C3d and the Septal Microvasculature as a Predictor of Chronic Lung Allograft Dysfunction

Cynthia M. Magro, Abbas E. Abbas, Kay Seilstad, Amy L. Pope-Harman, Tibor Nadasdy and Patrick Ross, Jr.

ABSTRACT: Studies have shown a potential role for humoral rejection in the evolution of lung graft dysfunction, apparently based on antibodies without human leukocyte antigen specificity. The correlation between extent of C4d deposition with clinical status further illustrates the importance of humoral immunity. Our study examines the potential value of C3d as a further diagnostic adjunct. C3d deposition was examined in lung allograft specimens using frozen tissue indirect immunofluorescence (IIF) and avidin biotin immunohistochemical applied to paraffin embedded tissue. Intermediate/extensive amounts of C3d using IIF and immunohistochemical (IH) methodologies correlated with chronic graft dysfunction; IIF C3d deposition was associated with septal and bronchial wall fibrosis (p < 0.0001). Weak/absent amounts of IIF and IH C3d correlated with clinical stability (p < 0.0001). Higher levels of C3d by IH were more sensitive than by IIF as a bronchiolitis obliterans syndrome determinant. C3d and C4d deposition using immunofluorescence and IH were correlated (p < 0.00001). C3d deposition appears prognostically significant. Higher tissue expression of C3d mark chronic graft dysfunction/persistent graft failure following transplantation. The close correlation between C3d and C4d lends credence to the role of humoral allograft rejection as a pulmonary graft dysfunction contributing factor. C3d by IH manifests higher sensitivity but similar specificity compared to C3d by IIF. Human Immunology 67, 274 –283 (2006). © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: Lung transplantation; C3d deposition; chronic graft dysfunction

ABBREVIATIONS
- BAL bronchoalveolar lavage
- BMZ basement membrane zone
- BOS bronchiolitis obliterans syndrome
- HLA human leukocyte antigen
- Ig immunoglobulin
- HPF high power field

INTRODUCTION
We have shown in earlier studies that humoral immunity directed at endothelium of the septal microvasculature and bronchial wall, bronchial epithelium, and cartilage may contribute to both acute and chronic allograft dysfunctions [1, 2]. We have also recognized that the antibodies that we have implicated in the lung humoral allograft phenomenon are more analogous to autoantibodies as opposed to alloantibodies [3]. The basis for this proposal is one of absence of human leukocyte antigen (HLA) antibodies despite reactivity of the patient’s serum to generic lung epithelium and endothelium.

In a previously published article, we demonstrated that clinical status could be closely correlated with C4d deposition, a stable and covalently bound component of classic complement activation and held to be a marker of humoral allograft rejection. In those patients with recurrent acute rejection, higher levels of C4d in the lung allograft tissue were observed during times of rejection while lower levels were seen during periods of clinical quiescence [4]. In a very recent article by Miller and coworkers, the authors discovered that C4d concentrations in bronchoalveolar lavage (BAL) fluids correlated with anti-HLA antibodies although not with clinical status per se [5].

More recently, it has been suggested that C3d, another stable component of complement activation pro-
duced through activation of either the classic or alternative pathways, may potentially constitute a further prognostic adjunct in the setting of solid-organ transplantation [6]. The purported mechanisms by which C3d may contribute to graft failure, however, have not been fully elucidated nor has its role as a diagnostic adjunct received significant attention in the transplantation literature.

The purpose of our study was to explore the value of C3d as a morphologic predictor of lung graft dysfunction and to determine its pathogenetic significance as a factor contributing to long-term graft failure. We examined two methods of C3d deposition, one in the context of indirect immunofluorescent (IIF) testing using frozen tissue and the other employing an avidin biotin technique applied to formalin-fixed paraffin embedded tissue, the latter representing a novel application of C3d.

MATERIALS AND METHODS
A correlation was sought between C3d deposition and clinical status as well as between C3d and light microscopic findings, specifically in regards to (1) extent of terminal lung parenchymal necrosis (as a morphologic hallmark of humoral allograft rejection), (2) presence of acute cellular rejection defined by perivascular and interstitial lymphocytic infiltrates, and (3) subsegmental bronchial wall and septal fibroplasia indicative of chronic graft dysfunction [2,7]. The study was presented to and approved by the institutional review board of The Ohio State University Medical Center.

Selection Criteria
Since June 4, 2004, we have been adding C3d to the lung immunofluorescent profile. Before that, the standard direct immunofluorescent profile comprised immunoglobulin (Ig)G, DAKO Cytomation, Carpinteria, California, IgM, DAKO Cytomation, Carpinteria, California, IgA, DAKO Cytomation, Carpinteria, California, C1q, DAKO Cytomation, Carpinteria, California, C3, DAKO Cytomation, Carpinteria, California, C4d, Quidel, San Diego, California, and C5b-9, DAKO Cytomation, Carpinteria, California. Between June 4, 2004, and March 15, 2005, we performed C3d on 49 transplant biopsies from a patient population of 23 unilateral lung allograft recipients and 1 bilateral lung graft recipient. All patients had received primary cadaveric nonliving donor lung transplants and all procedures were performed at Ohio State University Medical Center. The indications for biopsy were in the context of three distinct clinical scenarios: (1) routine surveillance, (2) acute rejection, or (3) chronic graft dysfunction compatible with bronchiolitis obliterans syndrome (BOS). The reason for biopsy was indicated in all cases.

In addition to the prospective direct immunofluorescent studies for C3d conducted during the designated period, we also applied retrospectively an immunohistochemical technique to assess for C3d deposition in the corresponding paraffin-embedded, formalin-fixed biopsy samples. To further verify the potential significance of C3d via this immunohistochemical technique, we performed paraffin immunohistochemical C3d on an additional 6 patients who did not have concomitant C3d performed on their frozen tissue. These samples antedated 2004 and frozen tissue for further immunofluorescent analysis in regard to C3d was not available.

A correlation was also sought between C3d and C4d both with respect to the immunofluorescent frozen tissue analysis and the paraffin-embedded immunohistochemical results.

C3d Indirect Immunofluorescent Assay
The tissue was cut at 2–4 μm in thickness and placed on Fisher/Superfrost Plus slides; they were allowed to air dry for 30 minutes, followed by a rinse in phosphate-buffered saline (PBS). A total of 150 μl Avidin D (100 g/ml in PBS/1% BSA) was applied as a preblock for 20 minutes followed by a PBS rinse. Subsequently, 150 μl (10 g/ml in PBS) d-biotin preblock for 20 minutes was applied to the slide followed again by a PBS rinse. The monoclonal antibody to C3d DAKO Cytomation, Carpinteria, California (clone 10-11) at a dilution of 1:100 (10 g/ml) was applied for 30 minutes followed by a PBS wash for 2–3 minutes. An anti-mouse immunoglobulin G (H & L) diluted to a concentration of 1:100 was applied for 30 minutes followed again by a PBS wash. FITC-streptavidin at a concentration of 1:50 was then applied for 30 minutes followed by a 2–3 minute PBS wash.

Grading Fluorescent C3d on Frozen Tissue
We used the same criteria that were applied in an earlier study that assessed C4d deposition in lung allograft tissue [4]. The deposition of C3d was assessed with respect to distribution of staining within the tissue (i.e., interalveolar septal versus subsegmental bronchial wall) and extent. The extent was related to the percent of lung parenchyma manifesting staining. In this regard, there were three categories of staining based on the percent of the lung parenchyma showing any C3d deposition regardless of intensity. Thirty percent or less was defined as low; between 30% and 50%, intermediate; and greater than 50%, high. Oil examination was required as weak fluorescent intensity is not apparent under lower powers of magnification. The quality of the granules could be coarse or fine. Bright homogeneous staining was discounted because in most instances it reflected background staining, primarily of elastic fibers.
C3d and C4d Immunohistochemical Staining Procedure on Paraffin-Embedded Tissue
Formalin-fixed, paraffin-embedded tissue was cut at 4 microns in thickness and placed on Fisher/Superfrost Plus slides. The slides were then placed in a 60 °C oven for 1 h, cooled, deparaffinized, and then rehydrated in the usual manner using sequential immersion in xylene, followed by graded percent ethanol solutions, and finally water. All slides were quenched for 5 minutes in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase activity. Antigen retrieval was not needed and was not used for these two antibodies. Slides were placed on a Dako Autostainer immunostaining system designed for use with immunohistochemistry. Before addition of primary antibody, slides were blocked for nonspecific staining using a serum-free protein for 15 minutes at room temperature (Dako code number X0909). Primary antibodies (C3d/C4d rabbit polyclonal antibodies, Dako catalog #A0063) were diluted 1:500 and incubated for 30 minutes. The detection system used was a labeled polymer system, Envision Plus Dual Link (Dako code number K4061). Staining was visualized with DAB chromogen (Dako code number K3468). The slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions and cover slipped.

Grading C3d and C4d on Formalin-Fixed Paraffin-Embedded Tissue
There were three categories of deposition which were recognized: low, intermediate, and high based on the number of positive staining vessels in five 400× fields. The numbers were arbitrarily chosen. A low category was assigned in cases showing positivity amid <10 vessels per 5 high power field (HPF), an intermediate category in those cases showing positive staining of 10–15 vessels per 5 HPF, and a high category in those cases manifesting positive staining of >15 vessels per 5 HPF. An attempt was made to correlate the extent of staining with evidence of chronic graft dysfunction, both morphologically and clinically.

Statistical Analysis
The statistical analysis was conducted using Fisher’s exact tests and Spearman rank correlation coefficients. Significance level was set at α = 0.05.

RESULTS
Clinical Features
A total of 49 biopsies were procured from 24 patients for direct immunofluorescent studies; these patients were 13 to 2178 days posttransplantation (Table 1). Of these, 25 biopsies were part of routine surveillance; the patients were clinically assessed as being well by the attending clinician with neither features of acute rejection nor chronic graft dysfunction according to information provided on the requisition. There were two biopsies prompted by acute dyspnea clinically held to represent acute rejection. Eighteen biopsies were obtained from patients with chronic graft dysfunction who at the time of the biopsy fulfilled clinical criteria for BOS based on a significant decrement in forced expiratory volume in 1 s from their best baseline value. Finally, there was an additional category represented by four biopsies from 2 patients who did not improve significantly after transplantation. These patients were categorized as “persistent graft failure following transplantation.” These patients had a protracted hospital stay and remained on ventilator support for long periods of time and the one patient in whom pulmonary function testing could be performed had never achieved an adequate baseline value to allow any determination of a meaningful BOS score.

In regard to the immunosuppressive protocol, it was individualized according to the clinical scenario and hence was quite variable. The individual regimens are listed in Table 2. Most patients were on multiple immunosuppressive agents representing combinations of two or more of prednisone, cyclosporine, sirolimus, tacrolimus, and mycophenolate.

The panel-reactive antibodies were determined before transplantation in all patients. Only two of the patients were found to be sensitized (Patient 12 and Patient 6). In the case of Patient 6, 15% of the tested sera showed reactivity with DQ2 specificity, whereas the donor was DQ2 negative; however, the patient was crossmatched negative. In the case of Patient 12, although the panel reactive antibodies were very high, the crossmatches were confirmed to be negative using T- and B-cell flow cytometry. The remainder of the cases had negative panel-reactive antibodies. There was no panel-reactive antibody testing after transplantation. Although C3d and C4d were measured in tissue samples, there was no determination of complement levels in the serum.

IIF C3d Levels and Clinical Status
Intermediate and high levels of IIF C3d were associated with BOS/chronic graft dysfunction or persistent graft failure (p < 0.0001) with a sensitivity of 66.67% (Figures 1–3). Also, the false-positive rate was low, with only 1/17 cases (5.88%) of those with high or intermediate levels of C3d not having BOS/chronic graft dysfunction. Conversely, absent or low amounts correlated with a state of clinical stability whereby the patients’ biopsies were done for routine surveillance or were procured at a point in their clinical course when they had not developed BOS (p < 0.0001). The false-negative rate was high, whereby 26.67% of patients with BOS/PGF had low or no C3d in
### TABLE 1  C3d and C4d expression on biopsy of lung allograft dysfunction patients

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Days post transplant (paraffin)</th>
<th>C4d on paraffin</th>
<th>C3d on paraffin</th>
<th>C3d IIF</th>
<th>C4d IIF</th>
<th>HR/CR by biopsy</th>
<th>Pathologic BOS (BW/F)</th>
<th>Reason for biopsy</th>
<th>BOS score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69 high</td>
<td>nd</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>965</td>
<td>nd</td>
<td>negative</td>
<td>low</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>1042</td>
<td>nd</td>
<td>moderate</td>
<td>high</td>
<td>AHR/CR</td>
<td>SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1065</td>
<td>nd</td>
<td>moderate</td>
<td>negative</td>
<td>BWF</td>
<td>BO</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>58 high</td>
<td>UR</td>
<td>negative</td>
<td>ACR</td>
<td>S</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
</tr>
<tr>
<td>378</td>
<td>moderate</td>
<td>BMZ training only</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>946</td>
<td>high</td>
<td>moderate</td>
<td>AHR</td>
<td>BW/SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>976</td>
<td>nd</td>
<td>UR</td>
<td>high</td>
<td>BWF/SF</td>
<td>BOS</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>134 low</td>
<td>Negative</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>415 low</td>
<td>UR</td>
<td>negative</td>
<td>negative</td>
<td>BWF</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>456</td>
<td>high</td>
<td>Negative</td>
<td>negative</td>
<td>negative</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>657</td>
<td>high</td>
<td>moderate</td>
<td>moderate</td>
<td>AHR/CR</td>
<td>SF/BWF</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>108 prominent BW</td>
<td>High</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>319</td>
<td>low</td>
<td>UR</td>
<td>low</td>
<td>AHR</td>
<td>SF</td>
<td>BOS</td>
<td>S</td>
<td>0-p</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>61 high</td>
<td>High</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>nd</td>
<td>High</td>
<td>high</td>
<td>AHR</td>
<td>S</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>19 low</td>
<td>Negative</td>
<td>low</td>
<td>Negative</td>
<td>S</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>low</td>
<td>Negative</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>317 low</td>
<td>Negative</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>464</td>
<td>nd</td>
<td>Nd</td>
<td>moderate</td>
<td>ACR</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1465 nd</td>
<td>High</td>
<td>low</td>
<td>negative</td>
<td>BWF</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1360 nd</td>
<td>Nd</td>
<td>BMZ only</td>
<td>negative</td>
<td>BWF</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>36 low</td>
<td>High</td>
<td>negative</td>
<td>AHR/ACR</td>
<td>S</td>
<td>negative</td>
<td>BWF/SF</td>
<td>0-p</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>low</td>
<td>Low</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>moderate</td>
<td>High</td>
<td>BMZ only</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1467 high</td>
<td>High</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>186 moderate</td>
<td>High</td>
<td>nd</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>nd</td>
<td>Nd</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>379 high</td>
<td>High</td>
<td>negative</td>
<td>low</td>
<td>negative</td>
<td>S</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>641</td>
<td>nd</td>
<td>Nd</td>
<td>negative</td>
<td>negative</td>
<td>BW/SF</td>
<td>Decrease in PFTs</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>103 moderate</td>
<td>UR</td>
<td>low</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>ur</td>
<td>UR</td>
<td>low</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1601 low</td>
<td>Negative</td>
<td>low</td>
<td>negative</td>
<td>ACR</td>
<td>Negative</td>
<td>BOS</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2178 high</td>
<td>High</td>
<td>high</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>330 nd</td>
<td>High</td>
<td>negative</td>
<td>high</td>
<td>AHR</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>379</td>
<td>low</td>
<td>High</td>
<td>negative</td>
<td>negative</td>
<td>ACR</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>414</td>
<td>high</td>
<td>High</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>557 ur</td>
<td>Ur</td>
<td>moderate</td>
<td>negative</td>
<td>SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>15 nd</td>
<td>High</td>
<td>high</td>
<td>AHR</td>
<td>Negative</td>
<td>PGD</td>
<td>ur</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>nd</td>
<td>Ur</td>
<td>high</td>
<td>ur</td>
<td>PGD</td>
<td>ur</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>nd</td>
<td>Negative</td>
<td>high</td>
<td>low</td>
<td>HR</td>
<td>SF</td>
<td>PGD</td>
<td>ur</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>79 nd</td>
<td>High</td>
<td>nd</td>
<td>negative</td>
<td>HR</td>
<td>Negative</td>
<td>BOS</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>521</td>
<td>nd</td>
<td>Nd</td>
<td>high</td>
<td>negative</td>
<td>Negative</td>
<td>BOS</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>802</td>
<td>nd</td>
<td>High</td>
<td>high</td>
<td>HR</td>
<td>SF/BW</td>
<td>BOS</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>241 moderate</td>
<td>Low</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>370</td>
<td>low</td>
<td>Low</td>
<td>negative</td>
<td>HR</td>
<td>SF/SF</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>458</td>
<td>moderate</td>
<td>High</td>
<td>low</td>
<td>negative</td>
<td>BW/SF</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>1199 low</td>
<td>Negative</td>
<td>low</td>
<td>negative</td>
<td>BWF</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>70 moderate</td>
<td>Negative</td>
<td>negative</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>195</td>
<td>moderate</td>
<td>Low</td>
<td>low</td>
<td>low</td>
<td>AHR</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>410 low</td>
<td>Low</td>
<td>nd</td>
<td>negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>39 low</td>
<td>Low</td>
<td>nd</td>
<td>high</td>
<td>HR/ACR</td>
<td>SF</td>
<td>S</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>38 low</td>
<td>High</td>
<td>nd</td>
<td>moderate</td>
<td>HR</td>
<td>SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>high</td>
<td>High</td>
<td>nd</td>
<td>high</td>
<td>HR/ACR</td>
<td>SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>269</td>
<td>nd</td>
<td>High</td>
<td>BMZ only</td>
<td>negative</td>
<td>BWF</td>
<td>BOS</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>221 low</td>
<td>Negative</td>
<td>nd</td>
<td>High</td>
<td>HR</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
</tr>
<tr>
<td>536</td>
<td>low</td>
<td>Negative</td>
<td>nd</td>
<td>Negative</td>
<td>HR</td>
<td>Negative</td>
<td>S</td>
<td>0-p</td>
<td></td>
</tr>
<tr>
<td>476</td>
<td>low</td>
<td>Negative</td>
<td>nd</td>
<td>Negative</td>
<td>Negative</td>
<td>S</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>385 moderate</td>
<td>Nd</td>
<td>Moderate</td>
<td>HR</td>
<td>SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>495</td>
<td>high</td>
<td>Nd</td>
<td>Moderate</td>
<td>negative</td>
<td>SF</td>
<td>BOS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>495 low</td>
<td>Nd</td>
<td>Low</td>
<td>negative</td>
<td>SF</td>
<td>S</td>
<td>0-p</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** 0-p = potential BOS stage; ACR = acute cellular rejection; AHR = acute humoral allograft rejection; BMZ = basement membrane zone; BOS = bronchiolitis obliterans syndrome; BW = bronchial wall; BWF = bronchial wall fibrosis; CR = cellular rejection; DIF = direct immunofluorescence; HR = humoral rejection; PGD = persistent graft dysfunction following transplantation; PFT = pulmonary function tests; S = surveillance; SF = septal fibrosis; UR = unsatisfactory result.
<table>
<thead>
<tr>
<th>Patient #</th>
<th>Days post transplant</th>
<th>C4d on paraffin</th>
<th>C3d on paraffin</th>
<th>Immunosuppressive Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>high</td>
<td>High</td>
<td>Cyc, pred, mm</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>Moderate</td>
<td>Ur</td>
<td>Cyc, pred, mm, siro</td>
</tr>
<tr>
<td>3</td>
<td>134</td>
<td>Low</td>
<td>Negative</td>
<td>Cyc, pred</td>
</tr>
<tr>
<td>4</td>
<td>413</td>
<td>Low</td>
<td>High</td>
<td>Cyc, pred, mm</td>
</tr>
<tr>
<td>5</td>
<td>108</td>
<td>High</td>
<td>High</td>
<td>Cyc, pred, mm</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>high</td>
<td>High</td>
<td>Cyc, pred, mm, siro</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>low</td>
<td>Negative</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>8</td>
<td>317</td>
<td>low</td>
<td>Negative</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>9</td>
<td>1465</td>
<td>Nd</td>
<td>Low</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>10</td>
<td>1360</td>
<td>Nd</td>
<td>Low</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>11</td>
<td>56–1</td>
<td>low</td>
<td>High</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>12</td>
<td>1467</td>
<td>high</td>
<td>High</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>13</td>
<td>186</td>
<td>moderate</td>
<td>High</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>14</td>
<td>1138</td>
<td>Nd</td>
<td>High</td>
<td>Pred, pred, sir</td>
</tr>
<tr>
<td>15</td>
<td>103</td>
<td>moderate</td>
<td>UR</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>16</td>
<td>1601</td>
<td>low</td>
<td>Negative</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>17</td>
<td>2178</td>
<td>high</td>
<td>High</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>18</td>
<td>330</td>
<td>Nd</td>
<td>High</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>19</td>
<td>557</td>
<td>UR</td>
<td>UR</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>20</td>
<td>13</td>
<td>nd</td>
<td>High</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>21</td>
<td>79</td>
<td>nd</td>
<td>High</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>22</td>
<td>241</td>
<td>moderate</td>
<td>Low</td>
<td>Pred, pred, sir</td>
</tr>
<tr>
<td>23</td>
<td>1199</td>
<td>low</td>
<td>Negative</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>24</td>
<td>195</td>
<td>moderate</td>
<td>Low</td>
<td>Pred, pred, tac</td>
</tr>
<tr>
<td>25</td>
<td>410</td>
<td>low</td>
<td>Low</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>26</td>
<td>39</td>
<td>low</td>
<td>Low</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>27</td>
<td>38</td>
<td>low</td>
<td>High</td>
<td>Pred, pred, mm</td>
</tr>
<tr>
<td>28</td>
<td>221</td>
<td>low</td>
<td>Low</td>
<td>Pred, pred, mm</td>
</tr>
<tr>
<td>29</td>
<td>389</td>
<td>moderate</td>
<td>Nd</td>
<td>Pred, tac</td>
</tr>
<tr>
<td>30</td>
<td>495</td>
<td>low</td>
<td>Low</td>
<td>Pred, pred, sir</td>
</tr>
</tbody>
</table>

**Abbreviations:** cyc = cyclosporine; mm = mycophenolate mofetil; pred = prednisone; siro = sirolimus; tac = tacrolimus; UR = unsatisfactory result.
their biopsies. There was a statistical concordance between fluorescent C3d and C4d deposition ($p < 0.0019$).

**C3d Immunohistochemical Staining and Clinical Status**

Negative C3d and/or low amounts of C3d ($<10$ vessels in 5HPF) predicted patients without BOS (specificity of 77.8%), whereas intermediate and higher values of C3d (in excess of 10 vessels per HPF) predicted BOS (sensitivity of 70%) (Figures 5–8).

In addition to vascular staining, biopsies from patients with BOS showed C3d deposition within the bronchial epithelial basement membrane zone (BMZ) alone and within chondrocytes of bronchial wall (Figure 9). C3d deposition could be seen in biopsies before the development of BOS. All patients with higher values of C3d within the septae or C3d localized to the bronchial wall eventually developed BOS (Figures 5 and 9). There was also a correlation between C3d and C4d on paraffin embedded tissue ($p = 0.0021$).

**Correlation Between Paraffin-Embedded C3d and IIF C3d**

There was a correlation between paraffin C3d and C3d by IIF ($p = 0.088$) with a sensitivity of 80% and specificity of 52.38%. The negative predictive value of low or absent C3d by paraffin-embedded immunohistochemical analysis with low or absent C3d by IIF was 84.62%.

**FIGURE 1** Patient 17 in this series was 2168 days post-transplantation with a bronchiolitis obliterans syndrome score of 2 at the time of the biopsy. The biopsy shows prominent deposition of immunoglobulin G in a granular array within the septae corroborative of chronic persistent humoral allograft rejection.

**FIGURE 2** The biopsy from Patient 17 illustrated in Figure 1 shows significant deposition of C3d, involving greater than 50% of the sampled lung parenchyma.

**FIGURE 3** Patient 20 from this series had biopsies that showed prominent C3d within the septae as early as Day 13 after transplantation. She has had persistent graft dysfunction since transplantation.

**FIGURE 4** Patient 10 was 1360 days post-transplantation and had bronchiolitis obliterans syndrome. There was prominent granular localization of C3d within the BMZ of the subsegmental bronchus, analogous to a positive lupus band test.
whereas the positive predictive value of intermediate and high levels of C3d by paraffin with high levels of C3d by DIF was only 44.44%.

**Correlation Between C3d Levels With Days After Transplantation**

There was no correlation between number of days posttransplantation and C3d levels ($r = 0.231, p = 0.35$).

**C3d Levels and Morphologic Features of Chronic Graft Dysfunction**

From a morphologic perspective, intermediate and high levels of C3d were associated with bronchial wall or septal fibrosis ($p < 0.0016$), which are morphologic hallmarks of chronic graft dysfunction (Figures 10 and 11). In addition, the specific localization of C3d within the bronchial epithelial basement membrane zone was only seen in patients with BOS (Figure 4). There was, however, no correlation between the extent of C3d and the presence of absence of acute cellular rejection or acute humoral rejection, although there was a trend to suggest that higher levels of C3d deposition were associated with humoral rejection.

**FIGURE 5** Patient 11 was Day 99 posttransplantation and had developed a progressive decrement in his pulmonary function test. Even though at the time of the biopsy, he did not have diagnostic features at least from a physiologic perspective of bronchiolitis obliterans syndrome (BOS), he had a progressive decline in his forced expiratory volume and eventually fulfilled criteria for BOS. There is prominent deposition of C3d within the capillaries of the septal microvasculature.

**FIGURE 6** Patient 1 had a relatively rapid progressive course of deterioration eventuating in severe bronchiolitis obliterans syndrome. This biopsy is Day 90 after transplantation. There is prominent homogeneous and granular staining within the microvasculature of the septae.

**FIGURE 7** Patient 21 is 800 days posttransplantation and developed bronchiolitis obliterans syndrome quite rapidly. The biopsy shows prominent deposition of C3d within the septal microvasculature.

**FIGURE 8** This patient was clinically stable and continues to do very well. There is no significant staining in the microvasculature for C3d.
Predictive Value of C3d by Either Methodology as a Predictor of BOS

C3d as an independent risk factor predicting BOS was associated with a sensitivity of 70% and specificity of 77.8%, with an overall positive predictive rate 91.3% and negative predictive value of 43.75%.

DISCUSSION

We have demonstrated a correlation between the deposition of C3d within the septal microvasculature and the clinical status using two independent methodologies of staining. Biopsies that were devoid of C3d or showed a lesser extent of C3d within the lung parenchyma were observed most frequently in patients who were clinically well. In contrast, higher degree of tissue expression of C3d was a marker of chronic graft dysfunction/BOS or primary graft failure after transplantation. It is also apparent that the immunohistochemical C3d technique was superior to the IIF methodology because of the high false-negative rate associated with the latter. Specifically, a negative C3d by IIF did not guarantee clinical stability or a clinical course uncomplicated by BOS. Almost all of the discordant C3d IIF and immunohistochemical C3d cases were in the context of patients who were clinically unwell and in whom there were high levels of C3d by immunohistochemistry while fluorescent C3d was negative. The reason why immunohistochemical C3d is more sensitive than IIF reflects the nature of the detection system. Specifically, it is widely accepted that the detection system employed for IH testing is much more sensitive compared with the fluorescent technique.

We propose that the postulated pathogenetic basis by which higher levels of in situ deposition of C3d are associated with chronic graft dysfunction/BOS is two-fold. Its deposition with the septae reflects in situ activation of the complement cascade sequence with C3d representing a covalently bound stable component of complement activation. Until recently, chronic lung graft dysfunction was held to represent a progressive scarring process of the conducting airways. However, in a recently published study that assessed the role of ultrastructure as a diagnostic adjunct in lung allograft biopsies, chronic septal vasculopathic changes as deter-
mined ultrastructurally were important predictors of chronic graft dysfunction [7]. The described ultrastructural vascular changes are characteristic for the end sequelae of humorally mediated microvascular injury, being observed in a heterogeneous group of disorders having a commonality of pathogenesis, namely one of antiendothelial cell antibodies. Among the diversified spectrum of diseases are scleroderma, dermatomyositis, or chronic allograft rejection (i.e., chronic transplant capillaropathy) [7].

The fact that our in situ C3d results could not be correlated with HLA status emphasizes the nature of the implicated antigenic target (i.e., endothelium). It has been demonstrated in prior allograft studies that the endothelial-based epitopes evoking antibody formation are likely not HLA related [8–10]. With respect to our own work, we have established a direct correlation between the in situ assessment of C4d with clinical status despite the absence of HLA antibodies [3,4]. In the study by Miller and coworkers, the authors established a correlative relationship between the extent of C4d in bronchiolar lavage fluid and the presence of HLA antibodies albeit C4d BAL levels could not predict clinical status [5]. Because C3d and C4d remain covalently bound to tissue, it cannot be assumed that C4d and C3d within BAL fluid accurately reflects intraparenchymal levels of C4d and C3d. The results of Miller and coworkers are similar to renal allograft studies that have failed to correlate complement levels in fluid samples such as blood and urine with clinical features of rejection [11–13].

In situ deposition of C3d may contribute to chronic graft dysfunction in a second way. In the recent study by Palmer and coworkers, the authors demonstrated that patients with an attenuated innate immune system from a mutant Toll-like receptor IV had less severe rejection episodes and a lesser likelihood of developing higher grades of BOS in contradistinction to those patients with no inherent known genetic defect in this particular receptor [14]. It is also interesting that mice defective in the Toll-like receptor IV gene have lower levels of serum C3 and of de novo synthesis of C3. Perhaps the extent of C3d deposition in tissue may ultimately be influenced by the functional expression of this Toll receptor [15]. The mechanism by which innate immunity is augmented by C3d reflects the dependency of proliferation of CD5+ B-1 cells on C3d. CD5+ B-1 cells, the source of natural antibody, will only undergo antigen driven expansion after the binding of antigen-coated C3d with the receptor CR 2 (CD21) found on B cells and antigen-presenting dendritic cells [16, 17]. Studies have shown the importance of C3d in augmenting the humoral response by virtue of enhanced titers of antibody formation when vaccines are conjugated with C3d [18].

The cited literature precedent in regard to C3d as a predictor of graft dysfunction is very limited and, in fact, we are not aware of any prior studies in the field of lung transplantation. In the article by Kuypers and coworkers, the authors explored a potential role of C3d in the evaluation of kidney biopsies [6]. In brief, the authors found that C3d deposition in renal allograft biopsies was correlated with poor outcome. Specifically C3d-positive cases were more likely to be associated with early graft failure and resultant retransplantation and multiple prior episodes of acute rejection compared with C3d-negative cases. In another study, extensive endomyocardial deposition of C3d and C4d correlated with ischemic myocardial injury and also predicted cases in which there were subsequent episodes of acute rejection [19]. In a third study, the authors were able to correlate the deposition of C3d with early severe impairment of renal allograft function [20].

Our study has certain limitations that must be addressed. We have an intermediate-sized transplant program and therefore the number of patients examined is therefore relatively small. Also, the groups of patients for whom the biopsies were performed were not equally represented. There were a disproportionate number of cases that were represented by either the surveillance category or the BOS category. The category of acute decrement in lung function was underrepresented. Undoubtedly these small numbers accounted for the inability to make any specific correlation between C3d and acute decrement in lung function. We have previously shown that C4d levels correlate with acute rejection clinically [4]. In this study, the patients who underwent surveillance biopsy were on average at a lesser number of days posttransplantation compared with those with BOS. Because we could not establish a definitive correlation between acute graft dysfunction and C3d, we cannot rule out timing as a determinant in C3d levels. Specifically, it is possible that C3d deposition in tissue may be influenced by the time lapsed after transplantation, although we were unable validate this possibility with a statistically relevant correlation. Finally, as alluded to, the discrepant IIF C3d and paraffin embedded C3d results are entirely expected because of the nature of the detection systems, a point that has already been discussed. However, unlike C4d which has been assessed using both techniques (i.e., IIF and immunohistochemistry), there are no studies looking at IIF C3d in tissue; however, the enhanced sensitivity using immunohistochemical over IIF is entirely expected.

Despite these limitations, this pilot study does have value based on the demonstration of C3d in lung allograft tissue for the first time, the utilization of a hitherto undescribed methodology for the detection of C3d on paraffin embedded tissue, and a statistical validation.
between high tissue expression of C3d in lung tissue and graft dysfunction. C3d, like C4d, may define another immunologic marker of value in prognosticating lung allograft biopsies.

ACKNOWLEDGMENTS
The authors would like to thank Susie Jones who was instrumental in the development of the C3d and C4d immunohistochemical protocols and who performed all the assays, Cheri Bott and Gyongyi Nadasdy for providing excellent technical assistance in the C3d immunofluorescent assay, Johanna Baran and Aimee Sisinger for editorial assistance, Pat Adams from the Ohio State University Tissue Typing Laboratory for providing information regarding clinical protocols, and Mary Chaeng from the Department of Medical Biostatistics, University of Manitoba, Canada, for her outstanding and comprehensive statistical analysis.

REFERENCES