Evidence That Humoral Allograft Rejection in Lung Transplant Patients Is Not Histocompatibility Antigen-Related

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We have recently recognized humoral rejection (HR) in lung allograft recipients and its association with acute and chronic graft dysfunction. We have shown that C4d, a stable marker of classic complement activation, is deposited in lung allografts, correlating with clinical rejection and parenchymal injury. The antigenic target may be endothelium in the setting of recurrent acute rejection while varying components of the bronchial wall may be important in chronic graft dysfunction. We sought to establish whether there is a role for antibodies with histocompatibility antigen specificity in the lung humoral allograft phenomenon. Flow cytometric and ELISA assays to assess donor-specific antigens were conducted on sera from 25 lung transplant recipients who had experienced one or more episodes of clinical rejection; in addition, the serum samples were tested for evidence of antiendothelial cell antibody activity. Morphologically, each case had biopsies showing septal capillary injury with significant deposits of immunoreactants with microvascular localization and positive indirect immunofluorescent antiendothelial cell antibody assay. Panel-reactive antibody testing showed absence of MHC Class I/II alloantibodies; ELISA based crossmatch detecting donor-specific MHC Class I/II specific antibodies was negative. HR can occur in the absence of antibodies with HLA specificity; antigenic targets may be of endothelial cell origin.

Key words: Histocompatibility, lung transplantation, rejection

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Introduction

We have recently shown a role for humoral immunity in the propagation of acute and chronic lung graft dysfunction (1). Features of the acute syndromic complex are those of dyspnea, fever, and lung infiltrates, while light microscopically there is evidence of a necrotizing septal capillary injury syndrome accompanied by immunoreactant deposition within the interalveolar septal capillaries (1). In patients fulfilling the criteria for bronchiolitis obliterans syndrome (BOS), the prototypic cause of chronic lung graft dysfunction, bronchial wall immunoreactant deposition with localization to the bronchial epithelium and microvasculature of the bronchial wall has also been recently identified (2). As further evidence of the role of humoral immunity in the pathogenesis of lung allograft dysfunction is the direct correlation of the degree of C4d deposition with the clinical status of the patient and the degree of parenchymal injury (3). C4d is a stable component of classic complement pathway activation and is held to be a marker of humoral allograft rejection (4,5). Recognizing that humoral allograft rejection in the lung appears to be clinically and pathogenetically significant, the question arises as to the nature of the involved antigenic target.

We and others have reported that the de novo elaboration of alloantibodies during the post transplant period correlates with both acute and chronic rejection of the renal allograft (2,3). As antigen-antibody binding is the main basis for C4d deposition in allograft tissue, we sought to explore the possibility that antibodies being induced in lung transplant recipients were on the basis of donor-mismatched major histocompatibility complex (MHC) Class I and/or Class II antigens. There are several studies that suggest an important role for histocompatibility antigen (HLA)-associated antibodies in the evolution of humoral allograft rejection in the kidney and heart (4–6). To test a similar role for HLA related antibodies in the lung allograft population, sera obtained from 25 lung transplant recipients at various times post transplant were tested for panel-reactive antibody (PRA) using a commercially available solid-phase flow cytometry assay. All patients had clinical evidence of rejection and a pathologic evaluation compatible with humoral rejection, the morphologic and immunofluorescent features of which are discussed later. The assay employs purified HLA antigens covalently attached to microspheres.
Antibody binding is detected using flow cytometry: to date the most sensitive method available for alloantibody detection. Furthermore, our earlier studies implicate endothelial-based antigen as the potential antigenic target, and in this regard an indirect immunofluorescent assay to assess for the presence of antiendothelial cell antibodies was also performed using the same patient serum samples.

Materials and Methods

The patient population was defined by 25 patients who had undergone a unilateral lung transplant. All of these patients had at variable times in their post-transplantation course experienced clinical episodes of rejection with biopsy confirmation, whereby the combined light microscopic picture and immunofluorescent findings were most compatible with humoral rejection (1).

Patients were categorized into those manifesting acute rejection, BOS (i.e., chronic graft dysfunction) and/or who were clinically well. Features of acute rejection included dyspnea, cough, fever, and chest roentgenogram showing infiltrates and/or effusions and a decrease in forced expiratory volume (FEV1). The cardinal hallmark of BOS was a significant reduction in their FEV1 compared with their best post transplant baseline values.

The two salient features assessed microscopically were the presence or absence of septal capillary necrosis, the morphologic hallmark of humoral allograft rejection and of acute cellular rejection, as defined by perivascular and interstitial collections of lymphocytes (1). The demonstration of septal capillary injury in the absence of discernible infection and reperfusion ischemia injury was considered morphologic evidence of humoral allograft rejection, whereby the extent of necrosis determined the severity of this type of rejection; the light microscopic features of humoral allograft rejection in the lung has been previously published (1). In addition, bronchial wall changes characteristic of BOS were evaluated, specifically in the context of the presence or absence of epithelial thinning, chondrocyte necrosis, bronchial wall fibrosis and vasculitic changes of the bronchial wall (2).

Direct immunofluorescent studies were an important diagnostic adjunctive test carried out on each case to substantiate a role for humoral immunity in bronchial wall rejection, whereby the extent of necrosis determined the severity of this type of rejection; the light microscopic features of humoral allograft rejection in the lung has been previously published (1). In addition, bronchial wall changes characteristic of BOS were evaluated, specifically in the context of the presence or absence of epithelial thinning, chondrocyte necrosis, bronchial wall fibrosis and vasculitic changes of the bronchial wall (2).

Serum samples were available on these patients. Specifically, antibody testing directed against MHC-related antigen of both Class I and Class II subtypes were carried out on these samples. Two methodologies were implemented to determine the presence of HLA-related antibodies. One technique was a flow cytometric assay utilizing a commercially available pool of major histocompatibility antigens of known specificity, while the other was an ELISA test to assess for antibodies directed against donor-specific antigens of MHC Class I and Class II subtypes. Each assay will be described separately. In addition, the serum was also assessed for antiendothelial cell antibody activity through an indirect immunofluorescent assay as described later.

Flow cytometric alloantibody detection

As outlined by Pelletier et al. (17–10), a commercially available pool of microparticle beads coated with various purified MHC antigens of known specificity were used according to manufacturer’s instructions (FlowPRA, OneLambda, Canoga Park, CA). Briefly, 20 μL of recipient sera was incubated with 5 μL of MHC Class I plus 5 μL of MHC Class II microparticle beads for 30 min at room temperature (RT). The beads were washed twice with buffer and centrifuged at 10,000 r.p.m. for 2 min. The beads were resuspended in 100 μL of solution containing FITC-conjugated goat anti-human IgG and incubated for 30 min at RT. The wash step was repeated and the beads were resuspended in 500 μL of wash buffer. Negative control serum using pooled sera from nontransfused males was similarly prepared. Samples were read with the aid of a Beckman Coulter XL2 flow cytometer. The fluorescence profile obtained with negative control sera was used as the baseline fluorescence. MHC Class I and Class II beads were readily distinguishable, as they are fluorescent (excited at 488 nm and maximum emission at 580 nm) and have unique emission spectra. The positive/negative cutoff was empirically determined for each assay by setting a histogram region that excluded 98% of the peak obtained with the negative control serum. The median channel associated with this cut point was recorded for each assay. A test was deemed positive for alloantibody if there a distinct peak was noted or if there was a shift to the right in bead fluorescence of ≥6% to the right of the cutoff point.

Anti-donor MHC Class I/Class II ELISA

For the antidonor MHC Class I/Class II ELISA, a commercially available ELISA for detecting patient IgG antibodies specific to donor-derived MHC Class I and Class II molecules was used according to manufacturer’s instructions (Transplant Monitoring System, GTI, Waukesha, WI). Briefly, donor spleen cells were used as a source of lysate, which was added to microtiter wells precoated with murine monoclonal antibody (capture antibody) with specificity to either MHC Class I antigens or MHC Class II antigens. The plates were washed and patient sera was next added to the microwells. Patient alloantibody binding was detected by the addition of enzyme conjugated antihuman IgG, addition of substrate, and reading at 405 nm on an ELISA reader. Controls included a known negative serum, a known positive serum, and a lysate control measuring the amount of Class I and Class II molecules captured from the lysate. A test was considered positive (presence of alloantibody to donor MHC) if the optical density (OD) of the patient sample was twice the value of the OD for the negative serum. This ratio is referred to as the reactivity index for MHC I or MHC II. All samples were performed in duplicate.

Indirect immunofluorescence assay

Acetone-fixed cytocentrifuge preparations of human pulmonary microvascular endothelial cells (HPMVEC) and of human umbilical vein endothelial cells (HUVEC), as well as commercially prepared slides of human epithelial cells (HEP2, Inova, San Diego, CA) were incubated with each serum sample as previously described (1). Antibody binding was detected with fluoresceinated goat antihuman IgG (1:100 in PBS; Caltag, Burlingame, CA). Fluorescent antibody complexes were visualized using an Olympus BX51 microscope, and images were recorded with a Paxit digital camera. A negative control was run with each assay using normal pooled human serum. The methods have been previously described (1).

Human pulmonary microvascular endothelial cells (BioWhittaker, Inc., Walkersville, MD) were propagated in endothelial cell basal medium-2 (EBM-2, BioWhittaker) supplemented with the EGM-2-MV bullet kit [fetal bovine serum (FBS), hydrocortisone, human fibroblast growth factor-β, vascular endothelial growth factor, insulin-like growth factor, ascorbic acid, human epidermal growth factor, gentamycin; BioWhittaker]. Human umbilical vein endothelial cells (HUVEC) were isolated from vessels and propagated in endothelial cell growth medium (ECGM) consisting of M-199 (Gibco,
Grand Island, NY) supplemented with 20% FBS (U.S. Bio-Technologies Inc., Parker Ford, PA), 50 μg/mL of bovine brain extract (prepared according to Maciag et al. [11]), 12 U/mL of sodium heparin (Sigma Chemical Company, St. Louis, MO), and 20 mM HEPES buffer. All HUVEC growing surfaces were pretreated with human fibronectin (25 μg/mL, Upstate Biotechnology, Inc., Lake Placid, NY). The HPMECs and HUVECs were maintained at 37 °C in a humidified 5% CO2/95% air atmosphere, passed weekly by brief trypsin digestion at a ratio of 1:4, and used in experiments at passage 5–8.

Results

Clinical features (see Tables 1 and 2)
All 25 patients had experienced one or more episodes of acute rejection clinically according to the criteria outlined, which in 10 of the cases was temporally associated with the dates when their panel reactive antibodies were run. In the remaining cases, the rejection episodes occurred within weeks to months from when their panel reactive antibodies were determined. The same serum samples were also sent for quantitative antibody measurement against cytomegalovirus, to refute a potential role for anergy as a basis for the anti-HLA negativity.

Pathology (see Table 1)
Of the 25 patients, all 27 biopsies showed morphologic evidence of humoral allograft rejection as defined by the presence of septal capillary necrosis accompanied by significant deposits of intra-alveolar fibrin (Figures 1 and 2). All biopsies showed variable deposition of complement and immunoglobulin. Specifically, C4d deposition was observed in 23 of the biopsies, while C1q was present in 18 of the biopsies and C5b-9 in 25 of the biopsies (Figures 3 and 4). Those four without C4d deposition had no evidence of clinical rejection although there were other immunoreactants deposited within the lung parenchyma. The degree of parenchymal necrosis determined the grade of humoral allograft rejection, which is indicated in Table 1. In 11 biopsies there were concomitant features of acute cellular rejection.

Pre-transplant alloantibody status
As shown in Table 1, PRA testing demonstrated an absence of both MHC Class I and MHC Class II circulating alloantibodies in this patient population during the pre transplant period, excluding patient 8.

Post transplant alloantibody status
As shown in Table 1, PRA testing demonstrated a remarkable absence of both MHC Class I and MHC Class II circulating alloantibodies in this patient population at all of the time points tested during the post transplant period. PRA values were less than the 6% value our program employs as an indicator of sensitization in all cases with one exception. No MHC Class II specificity could be assigned when the one sample which demonstrated Class II sensitization (PRA = 12%) was further tested for MHC Class II specificity.

Post transplant donor-specific ELISA
In light of the virtual absence of detectable circulating alloantibodies in the lung transplant recipients during the post transplant period, it was decided to further examine selected sera with an ELISA-based crossmatch. This assay has recently been made commercially available (Transplant Monitoring System, GTI, Waukesha, WI) and utilizes a lysate prepared from donor lymphoid cells. ELISA trys capture soluble MHC Class I or MHC Class II antigens in plastic microwells which can be used to detect donor-specific MHC Class I- and MHC Class II-specific antibodies using routine enzyme-conjugated ELISA technology. Donor lysate material was available for seven lung transplant recipients. Patient sera was compared with a known negative serum control included in each assay, resulting in a ratio of the OD of the patient serum/optical density of the negative control serum. This ratio, called a MHC I or MHC II reactivity index, was considered positive if it was greater than 2.0. As seen in Table 2, all of the post transplant sera tested resulted in a negative test for the presence of donor-specific MHC Class I and MHC Class II alloantibodies.

Assessment of ability to form antibody not histocompatibility related
Despite the absence of detectable histocompatibility-related antibodies, the IgG antibody titers to cytomegalovirus were very high, excluding three patients in which the test was negative. The samples tested were those used to assess PRAs.

Indirect immunofluorescent assay
Twenty-two of the 25 samples demonstrated significant granular nuclear staining of the pulmonary microvascular endothelium (Figures 5 and 6); concomitant cytoplasmic staining was seen in the sample from patient 8. Of the three negative cases, one was clinically well while the other two cases represented pretransplant specimens.

Discussion
We have recently described a post transplant reaction pattern characterized by a necrotizing pauci-inflammatory capillary injury syndrome, accompanied by significant localization of an immunoreactant to the septal capillaries and bronchial wall, suggestive therefore of humoral immunity (1). In this earlier study, the specificity of the reaction had been suggested by the absence and or substantive diminution of staining in either pretransplant serum and or postpheresis specimens. With respect to this current study, all of the patients had one or more episodes of pulmonary humoral allograft rejection according to criteria previously outlined by us (1). Furthermore, the presence of complement and immunoglobulin deposition in biopsy material was further corroborative evidence of the role of humoral immunity.
Table 1: Correlation of panel reactive status with clinical and pathological features of rejection

<table>
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<th>Tx date</th>
<th>Clinical status</th>
<th>Biopsy date</th>
<th>Humoral rejection</th>
<th>Acute cellular rejection</th>
<th>Immunofluorescence</th>
<th>% Reactive antibody</th>
<th>CMV Ab results</th>
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<td>neg</td>
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<td>pos</td>
<td>+ + ND + - - - - -</td>
<td>11/22/02</td>
</tr>
</tbody>
</table>

CMV = cytomegalovirus; Ab = antibody; - = negative; BOS = bronchiolitis obliterans syndrome; pos = positive; CL = class; PRA = panel reactive antibody; NR = not rejecting; SC = septal capillary; R = rejecting; Tx = transplant; ND = not done; NA = not available; + = positive; neg = negative.
immunity in lesional propagation, and more specifically, the presence of C4d and of C1q within the lung interstitial was indicative of antibody-driven classic complement activation.

There is some precedent in the human literature regarding humoral allograft immunity as a deleterious factor contributing to lung graft failure, especially in the context of chronic rejection. For instance, increased post transplantation ventilator days, decreased survival and/or a higher incidence of BOOP were observed in patients with PRAs (8,9). Many studies have closely linked HLA antibodies to humoral allograft rejection (7,10). The presence of lymphocytotoxic antibodies in recipient serum before transplantation is considered a contraindication to solid organ transplantation, because of the high incidence of graft failure, the basis being one of accelerated humoral allograft rejection (11–14). To identify patients who are at risk of reacting adversely to transplanted donor tissue, the transplant candidates are tested preoperatively from a donor panel encompassing all possible HLA antigens. The potential antibodies that could be formed fall under the designation of PRAs. Studies have shown the importance of humoral immune reaction to these donor-specific HLA-related antigens (14). Specifically, the presence of pretransplantation PRA is associated with an adverse post-transplantation outcome compared with those patients in whom the PRA test is low or negative (15,16). Among the adverse post transplant events are acute cellular rejection, decreased long-term graft survival, increased mortality and accelerated coronary heart disease (17–19). One study found that preformed anti IgG antibodies directed against MHC Class II antigens were a major risk factor for early and more frequent cellular rejection episodes (20).

However, despite the donor and recipient mismatch, we found no evidence of PRAs for Class I and Class II antigens either before or subsequent to transplantation. As shown in Table 1, PRA testing demonstrated a remarkable absence of both MHC Class I and MHC Class II circulating alloantibodies in this patient population at all of the time points tested during the post transplant period. PRA values were less than the 6% value that our program employs as an indicator of sensitization in all cases with one exception. No MHC Class II specificity could be assigned when the one sample that demonstrated Class II sensitization (PRA = 12%) was further tested for MHC Class II specificity.

The ELISA based crossmatch assay gave a negative test for the presence of donor-specific MHC Class I and MHC Class II alloantibodies (Figure 7). A relative state of anergy would be one explanation, because of iatrogenic hypogammaglobulinemia; however, all of these patients, excluding three, had detectable antibodies directed at viral antigen as revealed by positive antibody titers to cytomegalovirus. Another potential mechanism would be one of circulating alloantibody adsorption onto the allograft lung tissue. The tissue fixed/bound antibody would then clearly not be available for serologic detection. Supportive of this potential mechanism are two studies in which eluates of renal allografted tissue contained immunoglobulin and fractions

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**Table 2: Detection of donor MHC-specific alloantibody by ELISA**

<table>
<thead>
<tr>
<th>Transplant date</th>
<th>Sera date</th>
<th>Donor MHC I reactivity index</th>
<th>Donor MHC II reactivity index</th>
<th>Anti-MHC I Ab</th>
<th>Anti-MHC II Ab</th>
<th>Antibody to third-party antigen</th>
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<td>neg</td>
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</table>

MHC reactivity index is positive if > 2.

Ab = antibody; neg = negative; pos = positive; Tx = transplant; ND = not done; EBV = Epstein-Barr virus; CMV = cytomegalovirus; OD = optical density.

*At the same time that the patient’s sera was run, a known MHC reactive serum was also run as a positive control. The MHC I reactivity indices for the positive control sera ranged from 6.0 to 9.4 with a mean index of 7.8, and the MHC II reactivity indices for the positive control sera ranged from 4.0 to 11.5 with a mean index of 9.5. The reactivity index is the amount of antibody in the serum that can bind to donor-derived MHC Class I or Class II molecules; it is a ratio of the OD of the patient serum/OD of the negative control serum.
Humoral Allograft Rejection is Not HLA Related

Figures 1 and 2: Fibrinoid alteration of the septae accompanied by extensive intra-alveolar fibrin deposition, the defining morphologic hallmark of humoral allograft rejection.

of complement (21,22). We were unable to find additional human studies to support this hypothesis as a potential mechanism.

There is some precedent in the literature that humoral allograft rejection and C4d deposition may not always be correlated with PRAs (23,24). Hence, alternative conclusions to explain the C4d deposition would be either in the context of antigenic targets that are not histocompatibility based vs. other mechanisms unrelated to antibody deposition by which classic complement cascade sequence activation can occur. Regarding the former, the microvascular endothelial bed has been held to be an important antigenic target of humoral rejection in the heart and kidney; not surprisingly therefore, antiendothelial cell antibodies have been inversely correlated with long-term graft failure (25–30). Until recently, the intrapulmonary septal capillary network was not commonly recognized as a potential antigenic source contributing to human lung graft dysfunction. In our earlier study, we proffered the hypothesis that the septal capillary endothelium is the antigenic target based on the direct localization of C1q and IgG immunoreactivity to the nucleus and or cytoplasm of endothelial cells, defining the prototypic immunofluorescent profile of an antiendothelial cell Gell and Comb’s type II immune reaction (1). Also, indirect immunofluorescent testing has shown the presence of antiendothelial cell antibodies that are reactive to nondonor related umbilical and pulmonary microvascular endothelial cells (1).

In this current study, there was considerable data to support an endothelial-based antigenic target. Firstly, the dominant pattern of injury was one of septal capillary injury. Secondly, the presence of immunoglobulin and components of classic complement cascade activation within the septal microvasculature implicates a direct role for humoral immunity in the propagation of the microvascular injury. Thirdly, there was localization of immunoreactivity to the

Figures 3 and 4: Granular deposition of C1q within the capillary endothelium corroborative of antibody activity directed against endothelium (Figure 3). A similar deposition pattern is seen with C5b-9 (Figure 4). The patient had clinical evidence of rejection and as well as a morphologic reaction pattern compatible with humoral rejection.
Figures 5 and 6: Indirect immunofluorescent assay to assess for antiendothelial cell antibodies. The sample shows prominent granular nuclear staining of endothelium compatible with a positive reaction; the serum sample was obtained during a rejection episode.

Figures 7: Donor MHC I and II reactivity indices of each patient compared with their respective positive controls.

septal endothelium and, finally, there was antiendothelial cell antibody seroreactivity. With respect to the latter, in our hands, this test demonstrated prominent nuclear staining of acetone-fixed endothelial cell culture lines. The positive staining was in the context of patients with either acute rejection episodes and or BOS. The specificity of the reaction was suggested by the absence and or substantive diminution of staining in either pretransplant serum and or postpharesis specimens.

The fact that none of the patients had antibodies to major histocompatibility antigen is in itself corroborative of an anti-endothelial cell-specific alloantigen system, as other work has been published that suggests this system’s alloantigenicity is not related to major histocompatibility antigens. In the context of the allografted endothelium, it falls under the designation of the vascular endothelium system, which is also shared by peripheral blood monocytes (31). In these prior reports involving renal allografts, in the absence of discernible anti-HLA antibodies against the donor determined at transplant and during rejection, antibodies with endothelial cell antibody avidity could be detected (32,33). Hence these authors have concluded that non-HLA antigens expressed by endothelial cells may have an important role in the rejection process in patients who are ABO and HLA compatible. The mechanisms by which these non-HLA endothelial cell-based antibodies mediate damage include the induction of various adhesion molecules such as ICAM-1, VCAM-1, ELAM-1 by these antibodies (34), complement-mediated lysis (35) and or antibody-dependent cellular cytotoxicity (36).

With respect to other potential mechanisms of complement cascade sequence activation independent of antibody deposition, in the context of the lung allograft recipient, infection and a reperfusion ischemia injury syndrome would be the main contenders. There are three complement systems: the classic pathway, the alternate pathway, and the mannose binding lectin pathway (37). The alternate pathway is triggered with microbial antigen and ischemia. However, it does not involve C1q, C4, or its stable counterpart C4d (38,39). The classic complement pathway is activated primarily through antigen-antibody binding. Nevertheless there are some recent studies that suggest a potential role for this pathway in regards to the reperfusion ischemia injury. Specifically, administration of inhibitors to C1q has resulted in a significant reduction in the reperfusion ischemia injury, indicative of classic complement activation. One potential mechanism of classic complement activation independent of antibody antigen interaction is the mannose lectin binding pathway (40). The mannose lectin binding pathway is activated by mannose binding lectin interacting with its ligand. Studies have shown lectin complement activation after myocardial ischemia reperfusion, where blockade of the lectin pathway protects from reperfusion ischemia injury. In this setting therefore, it has been hypothesized that the deposition of C4d in early cardiac biopsies reflects reperfusion ischemia injury rather than being indicative of true humoral immunity (41). The mechanism by which reperfusion ischemia activates the complement system has been recently elucidated. It has now been established that intermediate
keratin filaments can activate the lectin pathway whereby endothelial cells subjected to oxidative stress demonstrate enhanced cytokeratin expression, hence defining the potential link between reperfusion ischemia injury and endothelial cell activation (42). For the activation of complement to occur, the mannose binding ligand must be associated with serine proteases. The result is activation of C4 and C2, generating the C3 convertase: C4bC2b. The C4/C2 cleaving activity of the MBL complex is in essence shared with the C1 complex of the classical pathway of complement activation (43–45). In most of the cases presented herein, the biopsies were obtained within a few months post transplantation, which makes reperfusion ischemia injury a less likely contender of complement activation. In addition there are other aspects of the direct and indirect immunofluorescent profile supportive of humoral immunity all of which have already been alluded to. Among these features are the deposition of IgG within the interalveolar septae, the cellular decoration of endogenous lung constituents with complement and immunoglobulin and the positive indirect antiendothelial cell results.

In summary then, we have found evidence of humoral allograft rejection based on the morphologic pattern of vascular injury and the localization of classic complement components to the septal microvasculature in the absence of HLA-associated antibodies. Endothelium is likely an important if not the critical target, and it is very possible that the antigenic targets are those that fall under the general rubric of the ‘vascular endothelial cell system’ (31–33). We are continuing to conduct improved studies and to more precisely characterize the endothelium as the probable antigenic-target operative in the lung humoral allograft rejection phenomenon. This work will be the basis of a forthcoming paper.

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References


