C4d Staining of Pulmonary Allograft Biopsies: An Immunoperoxidase Study

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Background: The role of antibody-mediated/humoral rejection in lung allografts is not fully elucidated. In other organ systems, deposition of a specific complement product, C4d, is a sensitive and specific marker for humoral rejection. C4d can be evaluated in tissue biopsies by immunofluorescence or light microscopic immunohistochemical staining techniques. Using immunohistochemical staining techniques we sought to determine whether there was any specific staining pattern for C4d in lung allograft biopsies with or without the diagnosis of acute or chronic cellular or humoral rejection.

Methods: A total of 68 lung transplant biopsies, performed at UCLA Medical Center from January 2002 to August 2004, were collected and the paraffin blocks were re-cut and stained for C4d by an immunoperoxidase technique. The cases were separated by the presence or absence of features of acute and/or chronic rejection based on the International Society for Heart and Lung Transplantation working formulation for the classification of pulmonary allograft rejection, revised 1995. The pattern of staining for C4d was then systematically examined.

Results: Positive staining in a variable, focal non-specific pattern was observed. There was no consistent staining pattern within the different diagnostic groups.

Conclusions: C4d staining of paraffin-embedded lung allograft biopsies, using currently available techniques, does not identify acute or chronic cellular or humoral rejection in lung allograft tissue.

Lung transplantation is an effective treatment for end-stage chronic lung disease. However, long-term survival is poor, <50% at 5 years. Risk factors for 1-year mortality include: surgical complications; non-cytomegalovirus (non-CMV) infection; graft failure; cardiac failure; and acute cellular rejection. Risk factors that impact 5-year survival are primarily chronic graft failure, manifested by bronchiolitis obliterans syndrome (BOS), and cytomegalovirus (CMV) and non-CMV infections. In lung transplantation, there are few data regarding the impact of antibody-mediated rejection on short- and long-term survival.

On the other hand, in heart and kidney allografts, it is recognized that in some patients acute and chronic rejection may be related primarily to antibody-mediated humoral rejection. The introduction of morphologic markers for humoral rejection has made accurate diagnosis easier, and has improved the specificity and results of treatment. One such marker in the heart and kidney is complement deposition in the capillaries. In both heart and kidney allograft biopsies there is a strong correlation of C4d deposition in capillaries with active humoral rejection. The development of an immunoperoxidase (IP) staining method for C4d now allows demonstration of complement activation in paraffin-embedded tissues.

In heart and kidney transplant patients, humoral rejection is treated differently from cellular rejection. The treatment options available for humoral rejection are now diverse and effective based on the clinical scenario. Chemotherapeutics include mycophenolate mofetil and tacrolimus, sometimes used prophylactically to reduce the incidence of humoral rejection. Plasmapheresis is also used if the rejection episode is clinically severe. Therefore, it would be very useful to determine if there is a biologically significant humoral component involved in lung allograft rejection.

C4d is a component of the classical complement pathway. The initial activation of the classical pathway involves binding of C1 activators, usually Fc domains of immunoglobulins, which in turn results in the confor-
mational activation of C1q, which in turn proteolytically activates C2 and C4. Activation of C4 results in cleavage into C4a and C4b. C4d is an inactive fragment of C4b but maintains a reactive thioester group that forms a strong covalent bond with nearby structures. In complement activation associated with antibody-mediated humoral rejection, the nearby structure is the vascular intima. Therefore, strong and diffuse staining of capillaries in the heart and of peri-tubular capillaries in the kidney with immunofluorescence (IF)
or IP techniques is a marker of acute humoral rejection (Figure 1a and 1b).

One previous study using IF techniques has examined C4d deposition in lung biopsies. The study showed deposition of C4d in septal capillaries correlating with septal capillary necrosis and C4d deposition in bronchial walls in BOS. However, septal capillary necrosis is not currently a part of the International Society for Heart and Lung Transplantation (ISHLT) classification of lung allograft rejection, and similar data have not been reported from other centers. Additional studies are needed to determine if C4d is a useful marker in lung transplant biopsies. One difficulty with evaluation of transbronchial biopsies is the relative paucity of diagnostic tissue. The ISHLT recommends that at least 5 pieces of alveolated lung parenchyma containing bronchioles and >100 air sacs are needed to adequately grade rejection. If BOS is a consideration, then even more pieces may be necessary, as small airways tend to disappear and are therefore difficult to find in this entity. A surgical lung biopsy would supply ample tissue for all types of diagnostic modalities and has been proposed for patients who require mechanical ventilation. This is clearly too invasive in most clinical scenarios but could be useful in the very ill for whom a definite and fast diagnosis is imperative. A recent study by Hopkins et al has suggested that sampling 10 to 12 pieces of tissue provides a very high diagnostic yield with a very low procedure complication rate. In any event, with diagnostic tissue being at such a premium, it may be difficult to know what and how much tissue should be frozen for IF studies.

In this study we applied an IP stain for C4d that can be used to study fixed, paraffin-embedded tissues. We routinely use this method to evaluate cardiac allograft biopsies. Our goal was to see if C4d deposition occurs in lung allograft tissues in acute and chronic rejection.

METHODS

A total of 68 lung transplant biopsies from 63 patients performed at UCLA Medical Center, from January 2002 to August 2004, were collected and reviewed. The age range of the patients was 27 to 70 years (mean 57.1 years). The age of the allografts ranged from 1 day to approximately 15 years (mean 16.0 months). Twenty patients had been diagnosed with acute rejection: minimal/A1 (6 cases); mild/A2 (7 cases); or moderate/A3 (7 cases). Twenty-eight patients had bronchial/bronchiolar inflammation; 11 had evidence of BOS, consistent with chronic rejection; and 5 had diffuse alveolar damage (DAD). Twenty-one patients showed no evidence of rejection or significant inflammation.

Paraffin blocks were re-cut and stained for C4d by an IP technique, as reported recently. Four-micron-thick sections were cut, re-hydrated, and steamed in citrate buffer (10 mmol/liter, pH 6.0) for 25 minutes. The sections were then cooled for 10 minutes and washed in running water 5 times. Endogenous peroxidase was blocked by using 3% H2O2 in methanol for 15 minutes, followed by washing in TBS (50 mmol/liter, pH 7.6) with 0.1% Tween-20. To block non-specific background staining, the tissue was incubated with 10% normal goat serum (NGS) for 30 minutes. The slides were then incubated with 1:50 rabbit anti-human C4d antibody (C4dpAB; American Laboratory Products Co., Windham, NH) in 3% NGS overnight at 4°C, followed by washing in TBS (3 × 5 minutes). Secondary antibody (biotinylated goat anti-rabbit IgG; 1:200) was applied for 40 minutes, followed by washing in TBS (3 × 5 minutes). Horseradish peroxidase–avidin (1:1,000) was applied for 30 minutes, followed by washing in TBS (3 × 5 minutes). DAB was applied for 10 minutes followed by further washing in running water. The slides were then counterstained with dilute aqueous hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped.

The biopsies were separated by the presence or absence of features of acute and/or chronic rejection based on the ISHLT working formulation for the classification of pulmonary allograft rejection, revised 1995. Presence or absence of DAD and BOS was also noted. The pattern of staining for C4d was then systematically examined. The structures examined included: peri-bronchial and alveolar capillaries; alveolar septae; and endothelial lining of arteries and arterioles. Any and all structures that stained were analyzed and recorded. A “specific” staining pattern was defined as diffuse capillary endothelial cell staining as observed in antibody-mediated rejection in heart and kidney transplant biopsies. All other staining patterns were considered “non-specific.”

Six non-transplant lung biopsies were examined as negative controls. Four cases had constrictive bronchiolitis, similar to the pattern seen in chronic rejection. Two cases had DAD, similar to the ischemia–reperfusion injury seen in early post-transplant biopsies. Positive and negative controls for IP C4d staining were performed on heart allograft biopsies with a known diagnosis of humoral rejection (Figure 1b and c).

Anti-HLA antibody screening and flow cross-matching with donor T and B lymphocytes was performed as previously described in many but not all patients.

RESULTS

Infrequent positive staining in a variable pattern was observed (Table 1). The staining patterns included: focal alveolar capillary endothelium; epithelial cells lining the alveoli; internal elastic lamina of arteries and elastic fibers throughout bronchial walls; endothelium of arteries and post-capillary venules; macrophage aggregates; and peri-bronchial capillaries.
Three of 20 acute rejection cases (15.0%) showed staining of elastic fibers; 2 of 11 cases of BOS (18.2%) showed staining of a post-capillary venule and the internal elastic lamina of some arteries in 1 case (Figure 1d-f); and 2 of 5 cases of DAD showed focal strong alveolar wall capillary staining (40%) (Figure 1g) and 3 cases (60.0%) showed staining of hyaline membranes (Figure 1h). Two of 20 cases of acute rejection (10.0%), 4 of 5 cases of DAD (80.0%) and 11 of 33 cases with no rejection, DAD or BOS (33.3%) showed varying degrees of non-specific staining. Seven of 28 cases featuring bronchial/bronchiolar inflammation (25.0%) showed non-specific background staining of alveoli and macrophages. Overall, 17 of the 68 cases (25.0%) showed some form of C4d staining.

The non-transplant control cases showed similar staining patterns. One of 2 cases of DAD (50.0%) and 2 of 4 cases of constrictive bronchiolitis (50.0%) showed arterial and venous endothelial staining; and 1 of 4 cases of constrictive bronchiolitis (25.0%) showed non-specific background staining of alveoli and macrophages.

The staining patterns did not show any consistency within the different diagnostic groups. Convincing endothelial cell staining for C4d was seen in both transplant and non-transplant arterial and venous endothelium.

The only episodes of alveolar capillary staining occurred in 2 cases of DAD immediately post-transplant. Both cases had negative HLA antibody cross-matches by flow analysis performed at the time of biopsy and did not have laboratory evidence of donor-specific antibodies.

DISCUSSION

The development of a reliable marker for humoral rejection in renal and cardiac allografts has resulted in a better understanding of mechanisms of rejection, and better treatment. C4d staining of peri-tubular capillaries in renal transplant biopsies has been associated with acute humoral rejection with a sensitivity and specificity of 95% and 96%, respectively. Other less sensitive and somewhat less specific histologic features of acute humoral rejection in the kidney allograft include neutrophils in peri-tubular capillaries, edema, and fibrinoid necrosis of arteriolar walls in severe cases. Evaluation of heart biopsies for C4d deposition can be performed reliably with either IF or IP techniques. Histologic features of acute humoral rejection in heart transplant biopsies include interstitial edema, capillary endothelial cell swelling and accumulation of macrophages within capillaries, and, in severe cases, neutrophilic infiltration and hemorrhage. Intercapillary accumulation of CD68+ monocytes in the absence of an interstitial T-cell infiltrate is virtually diagnostic of humoral rejection.

It is reasonable to suggest that there may be a humoral component associated with lung allograft rejection. The ISHLT lung allograft rejection criteria includes 4 categories: (A) acute (with or without Category B); (B) airway inflammation; (C) chronic airway; and (D) chronic vascular forms of rejection. Airway inflammation refers to the infiltration of the lamina propria by mononuclear cells and is predictive of future BOS, the diagnostic feature of chronic airway rejection. The etiology of BOS is complex and undoubtedly multifactorial. It has been hypothesized that both immune-related and non-immune-related factors are involved. There are certainly well-established risk factors including, but not limited to: prior episodes of acute rejection; cytomegalovirus infection; histoincompatibility (HLA mismatch); other infections (Pneumocystis carinii pneumonia, bacterial pneumonia, etc.); airway ischemia; and lymphocytic bronchitis/bronchiolitis. Airway ischemia can be secondary to non-immune injury by disruption of the bronchial blood supply after the transplant procedure.

A recent study by Luckraz et al demonstrated that microvascular changes in small airways predispose to BOS in lung transplant patients. In their study, the
investigators measured presence of large blood vessels (circumference \(>0.20\) mm) around small airways in 2 groups of patients. The first group consisted of 5 patients who died within 24 hours of transplant, and the second group consisted of 11 patients who had survived at least 1,000 days but died with BOS, confirmed by post-mortem examination. The first group showed the presence of large vessels 95% of the time. The second group demonstrated large vessels around small airways from 11% to 21% of the time. This staining pattern may be a component of BOS, or could represent microvascular injury leading to BOS. Thus, although there is evidence of microvascular changes in BOS, whether or not antibodies or complement cause the injury and play a role contributing to the development of BOS has not been elucidated.

It is tempting to speculate that microvascular injury in lung allografts could be antibody-mediated. When evaluating our study results we took care to examine the tissue capillaries, as these vessels are the sites of C4d deposition in heart and kidney allografts with humoral rejection. In these organs arterial and venous staining is non-specific. The only incidence of convincing and detectable capillary endothelial cell staining involved 2 cases of DAD that did not have demonstrable donor-specific antibodies in the serum at the time of biopsy. When Liebow and Carrington first described diffuse alveolar damage they indicated that damage included the full wall thickness, including the endothelium. It is possible that the ischemia/reperfusion injury associated with DAD could cause non-significant staining in the capillaries. Complement deposition associated with ischemic injury in heart transplant biopsies has been reported. In any event, the staining did not appear to be associated with humoral rejection in either of our patients. Of note is the relatively focal nature of the staining. In both the heart and the kidney transplant biopsies, the staining must be diffuse to be considered positive.

In a recent study by Magro et al., deposition of C4d in lung allograft biopsies was investigated using immunofluorescent techniques. Their study showed C4d deposition in all cases of acute rejection with staining in septal capillaries associated with parenchymal injury and septal necrosis. In patients with BOS, C4d and C1q deposits were seen in the bronchial wall. This finding was not noted in cases of acute rejection. This study and a further study by the same group also found no donor-specific MHC Class I/II antibodies associated with these patients. Therefore, it was postulated that humoral rejection targets endothelial cell antigens regardless of HLA mismatch. Indeed, studies regarding the significance of histocompatibility loci have presented mixed conclusions. Further studies may be necessary to prove the link between C4d staining in the bronchial walls by IF and clinically significant humoral rejection.

Several recent studies have introduced the concept of complement-independent humoral rejection (CIHR). The proposed mechanisms include: crosslinking of HLA Class I molecules that transduce intracellular signals, resulting in increased survival and proliferation of endothelial and smooth muscle cells; antibody binding of MICA, a non-classic MHC Class I molecule, that can activate endothelial cells and cause thrombosis; and binding of anti-phospholipid antibodies to endothelial membranes, resulting in transcription of pro-inflammatory genes by induction of nuclear factor-κB.

Thus, the absence of C4d staining in our patients does not rule out the possibility that an antibody-mediated rejection, by a complement-independent mechanism, may be occurring. Finding components of the HLA crosslinked signaling pathway in lung biopsies in patients with donor-specific HLA antibodies at the time of biopsy, in the absence of demonstrable C4d staining, would be evidence of the presence of CIHR in the lung and requires further study. Another possibility is that humoral rejection in the lung is complement-dependent, but C4d is not a sensitive marker, as it is in the heart and kidney.

Unfortunately, in our study, we could not demonstrate C4d deposition in the microvasculature in lung biopsies of patients with acute or chronic rejection. If our results are representative, then C4d staining of paraffin-embedded lung allograft biopsies will not be a useful tool in identifying antibody-mediated rejection in lung allograft tissue, if indeed antibody-mediated rejection is a clinically relevant event.

The authors thank Longsheng Hong for histopathologic studies.

REFERENCES


