

C4d Deposition in Cardiac Allografts Correlates With Alloantibody

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Background: The presence of C4d along the peritubular capillaries in kidney allografts correlates with the presence of anti-donor serum alloantibodies. We applied C4d staining to cardiac allograft and non-allograft biopsies to determine if C4d staining in heart allografts correlates with anti-donor serum alloantibodies.

Methods: We stained for C4d all available frozen tissue biopsies from cardiac transplant recipients between 1997 and 2002, including autopsies. Two hundred twenty-one tissue samples from 124 patients were analyzed. Included in both groups were a variety of International Society for Heart and Lung Transplantation (ISHLT) grades of rejection plus post-implant cardiac ischemic injury (PIMD), and biopsies from patients who had received OKT3. Patients were matched by age, gender and interval after transplantation. Forty-four additional controls were included from patients biopsied for non-transplant-related cardiac disease.

Results: C4d staining of the myocardial capillaries correlated well with the presence of anti-donor alloantibodies. Twenty-one of 25 biopsies from patients with anti-donor alloantibodies showed C4d staining (84%), whereas only 7 of 60 without anti-donor alloantibodies stained for C4d. C4d staining did not correlate with ischemia or OKT3 therapy. Only 4 of 44 non-transplant biopsies stained for C4d (9%). An example of the clinical utility of C4d staining in patient care is presented.

Conclusions: C4d staining of the capillaries in cardiac allografts correlates well with anti-donor serum alloantibodies, is a useful assay to verify alloantibody deposition, and can be used to establish one of the criteria for antibody-mediated cardiac rejections. *J Heart Lung Transplant* 2005;24:1202-10. Copyright © 2005 by the International Society for Heart and Lung Transplantation.

Patients with heart allografts receive right ventricular biopsies to monitor for episodes of allograft rejection, and their pathologic diagnoses assist with patient management.¹ Complicating their interpretation are inflammatory infiltrates, post-transplant ischemic injury and Quilty lesions that may mimic rejection histologically.^{2,3} In addition, the clinical care of patients with biopsies showing moderate-grade rejection (International Society for Heart and Lung Transplantation [ISHLT] Grade 2) is unclear because treatment protocols vary due to uncertainty about the effect of treatment on clinical outcome.⁴⁻⁶

Also complicating both the interpretation of allograft

biopsies and patient treatment is that, on occasion, alloantibodies can deposit in vivo in allografts and cause a spectrum of pathologic injury, from acute humoral rejection to possible chronic rejection.⁷⁻¹² In some cases, the presence of alloantibody may have no immediately detectable pathologic or clinical consequences, i.e., accommodation.¹³ In heart allografts, the presence of anti-donor-specific alloantibodies seems to prejudice cardiac allograft survival, both short and long term.¹⁴⁻¹⁸

Presently there is no reliable histologic marker to verify cardiac allograft antibody deposition that can identify and monitor those patients at risk for antibody-mediated rejection. To detect histologically antibody deposition in cardiac allografts, investigators have used indirect immunofluorescence of tissue immunoglobulins (IgM and IgG) and complement (C3, C1q).^{15,19} These studies readily identify immunoglobulins and complement deposition in cardiac capillaries, arterioles and sometimes myocytes. This methodology seems to show a poor correlation between fluorescence and the actual presence of anti-donor-specific antibodies,^{15,19} especially as fluorescence is commonly observed in biopsies from patients without antibodies.²⁰ This is similar to the experience in kidney biopsies, in which staining of IgG and C3 correlates poorly with anti-donor antibodies.²¹

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Dramatic improvement in our ability to diagnose humoral rejections in kidney allografts is provided by C4d staining of the peritubular capillaries in renal allografts.^{7,9,10,22,23} C4d, an inactive split product of C3 convertase and complement activation, covalently binds to proteins near sites of complement activation after Factor I inactivation of the convertase. Thus, C4d deposition indicates recent complement activation through the classical pathway. Indirect immunofluorescence of C4d on kidney allograft biopsies shows a strong correlation with the presence of anti-donor-specific antibodies,^{7,22,24} including ABO.²⁵ C4d staining, by itself and alone, predicts poorer graft survival in renal allografts^{12,26-28} and in heart allografts.²⁹ C4d peritubular capillary staining also identifies a sub-set of renal allograft patients with chronic rejection, most of whom have anti-donor antibodies.^{10,11} To determine whether deposition of alloantibody within cardiac allografts is detectable by C4d staining and to investigate the utility of C4d staining in cardiac allograft rejection, we correlated C4d staining in endomyocardial allograft biopsies with anti-donor-specific alloantibodies.

MATERIALS AND METHODS

Cases

We assayed all available frozen tissue biopsies from cardiac transplant recipients between 1997 and 2002. Two hundred twenty-one tissue samples from 124 patients were analyzed. Also included were autopsy tissue samples from patients who had died after cardiac transplantation. Included in both groups were biopsies with a variety of ISHLT grades of rejection plus post-implant cardiac ischemic injury (PIMI), and biopsies from patients who had received OKT3. Patients were matched for age, gender and interval after transplantation, a factor known to affect the probability of rejection.³ Forty-four additional controls were included from patients biopsied for non-transplant-related cardiac disease, including 18 patients with idiopathic dilated cardiomyopathy, 11 with hypertrophic cardiomyopathy, 1 with hemochromatosis, 3 with coronary artery disease, 7 with cardiac amyloid, 3 with myocarditis and 1 lupus patient with cardiac leukocytoclastic vasculitis. Five infarcts from non-transplant patients were also stained. Because C4d staining can disappear a few weeks after removal of antibody, and antibody has a half-life of about 1 month, we required that the serum sample for antibody analysis and the tissue sample be within 1 month of each other. We identified 9 patients with alloantibodies. These 9 patients with circulating alloantibodies form a heterogeneous group, both pathologically and clinically. One was a pediatric patient, ABO-incompatible, who died from chronic rejection on Day 106. Another patient died of acute humoral rejection 5 days after transplantation due to sensitization while

awaiting transplantation. Three patients died of acute cellular rejection with prominent endothelialitis. Two patients were clinically unsuspected and had ISHLT Grade 0 rejection. Two patients had survived episodes of acute cellular rejection (ISHLT Grades 3a and 4) with alloantibodies after intensive therapy.

C4d Staining by Immunofluorescence

Biopsy sections were stained using a 3-step immunofluorescence technique developed in our laboratory. Four-micrometer frozen sections were incubated in 100 µg/ml avidin D (Vector Laboratories, Burlingame, CA) to block endogenous biotin. Sections were washed and excess avidin was bound by adding 10 µg/ml D-biotin (Sigma Chemical Co., St. Louis, MO). Monoclonal antibody to C4d (Clone 10-11; Biogenesis, Sandown, NH) was applied for 30 minutes at a 1:100 dilution. Sections were washed and incubated sequentially, first with biotinylated horse anti-mouse IgG (1:100) (Vector Laboratories) and then, after washing, with fluorescein isothiocyanate (FITC)-streptavidin (1:50) (Biomedex, Foster City, CA), each for 30 minutes.⁷

C4d Staining by Polyclonal Anti-Sera

Four-micron thickness sections on Superfrost Plus slides were baked at 60°C for 30 minutes, deparaffinized and re-hydrated. For antigen retrieval, slides were heated in a decloaking chamber for 3 minutes in Antigen Decloaker Solution (Biocare Medical), cooled and rinsed in phosphate-buffered saline (PBS). Slides were blocked with normal goat serum at 1:50 dilution for 20 minutes at room temperature. For tissues rich in endogenous biotin, 2 drops of avidin D in 100 µg/ml PBS was added to each slide for 20 minutes. Polyclonal anti-C4d¹¹ was added at 1:50 in Renaissance Background Reduction Solution (Biocare Medical) and incubated at 4°C overnight. After PBS washing, Universal Link (Biocare Medical) was added for 20 minutes. After washing in PBS, streptavidin-horseradish peroxidase (Biocare Medical) was added and incubated for 20 minutes. Slides were rinsed in distilled water and Romulin AEC Chromogen (Biocare Medical) was added and the slides incubated for 2.5 to 5 minutes. The slides were rinsed in distilled water and stained and counterstained, dehydrated in alcohol and xylene, and coverslipped with permanent mounting media.

Alloantibodies

Circulating donor-specific antibodies were assayed in the departmental tissue typing laboratory by using T- and B-cell cytotoxicity assays and/or flow cytometry.^{9,10,23} Pre-transplant donor-specific antibodies were tested in all patients by anti-human globulin cytotoxicity assays. Circulating anti-donor antibodies are not sought routinely after cardiac transplantation but were assayed in patients with

cardiac dysfunction that was thought to be rejection-related (biopsy-proven or not).

Statistical Analysis

Paired data sets were analyzed using chi-square analysis (SPSS Software).

RESULTS

Correlation of C4d Staining and the Presence of Serum Alloantibodies

We correlated C4d fluorescence on frozen allograft cardiac tissue with serum anti-donor antibody using 85 tissue samples (biopsies and autopsies) from 38 patients. We were able to identify 9 patients who had both antibodies and concurrent tissue samples ($N = 25$). For patients with alloantibodies, we stained 2 or 3 tissue samples per patient between 21 and 100 days post-transplant. We also compiled an additional set of 60 paired data sets from 29 patients without serum alloantibodies, also matched for age and gender, in which C4d staining of tissue and alloantibody could be compared. Two frozen biopsies at different timepoints post-transplant were assayed per patient and matched for interval after transplantation with the intervals of the antibody-positive group. C4d staining was found lining the walls of capillaries, on the endothelium of arterioles, in intravascular plasma, and occasionally on ischemic myocardium. Only capillary C4d staining correlated with presence of serum alloantibody (Figure 1). Of 25 tissue samples paired with positive antibodies, 21 showed capillary C4d staining (84%) (Table 1). Four biopsies from 4 alloantibody-positive patients were negative for C4d staining. However, the negative C4d

staining observed on 2 biopsies from 2 patients with alloantibody turned C4d-positive on their next biopsies. In the renal literature it is well accepted that C4d staining in a minority of cases may precede or follow the detection of alloantibody. Fifty-three of 60 samples without antibody were negative for C4d (88%). The association of C4d capillary staining in allografted hearts and alloantibodies was statistically significant ($p < 0.001$). Arteriolar positive staining was identified in biopsies from patients with and without alloantibodies ($p = 0.2$). In 4 cases, myocytes clearly stained and correlated with areas of ischemic injury. In 65 cardiac allograft biopsies, which could not be correlated with alloantibody, 7 of 65 were positive for C4d and 58 were negative.

C4d Staining in Patients Receiving OKT3

Potentially, C4d deposition could be related to therapy with OKT3, which could cause complement activation and possible C4d in vivo deposition.^{30,31} To test this hypothesis, we correlated the biopsies of 18 patients who had received OKT3. Biopsies were taken between 7 and 41 days after transplantation. Eight of 18 of these patients had alloantibodies, and 10 of 18 did not. Seven of 8 patients with alloantibody were C4d-positive, but 0 of 10 without alloantibody showed C4d staining (Table 2). This association was statistically significant ($p < 0.001$). To confirm this correlation pathologically, we stained the allografted heart and native kidney for C4d from a patient, who had anti-donor alloantibodies and died from acute humoral rejection and who also had received OKT3 as part of therapy. In Figure 2, the capillaries in the allografted heart stained for C4d (Figure 2A). This is similar

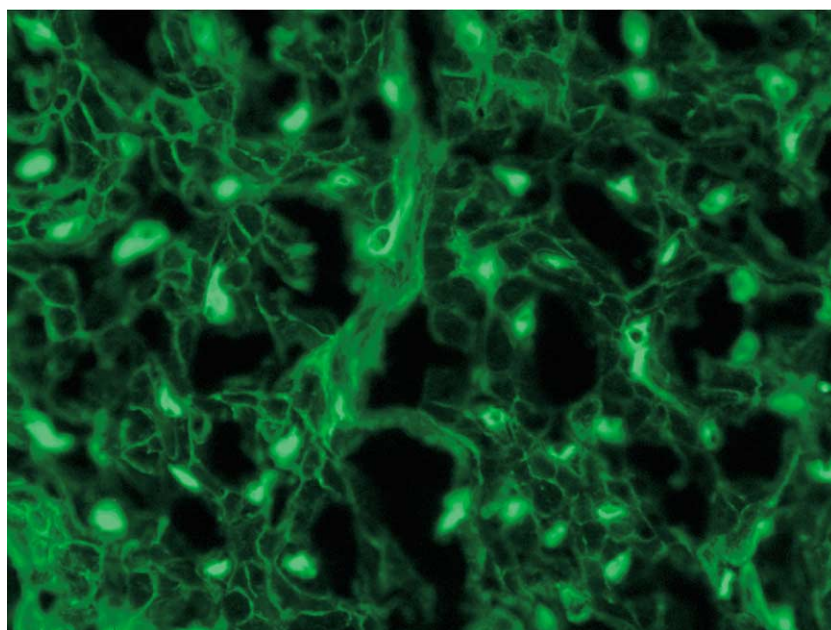


Figure 1. Capillary staining for C4d (original magnification $\times 400$).

Table 1. Correlation of Capillary C4d and Serum Alloantibody

	Serum antibody		Total
	Negative	Positive	
C4d staining			
Negative	53	4	57
Positive	7	21	28
Total	60	25	85

$p < 0.001$.

to the pattern of peritubular capillary C4d staining in an allografted kidney from a case of acute humoral rejection of the kidney (positive control, Figure 2B). In contrast, a biopsy with ISHLT Grade 3a rejection, from a patient who did not have alloantibodies, lacked capillary C4d staining (Figure 2C). There was no C4d staining in the native kidney from the patient with acute humoral cardiac rejection (Figure 2D). These data suggest that cardiac capillary C4d staining is associated with anti-donor antibodies deposited in the cardiac allograft and is not attributable to OKT3 therapy or generalized C4d deposition.

C4d Staining and Post-Transplant Ischemic Injury

Since ischemic myocardial tissue can stain for complement,³²⁻³⁴ biopsies from patients with or without antibodies, but with diagnoses of post-transplant ischemic injury, were stained for C4d. Of 33 cases in which the biopsies showed post-transplant myocardial ischemia, 5 of 7 biopsies from patients with serum alloantibody were C4d-positive. However, 25 of 26 (96%) without alloantibody did not stain (Table 3). This correlation was statistically significant ($p < 0.001$). In the absence of alloantibody, capillaries are unlikely to stain diffusely for C4d in heart allograft biopsies with diagnoses of post-transplant ischemia.

C4d Staining in Frozen and Paraffin-Embedded Tissue

C4d staining was initially performed on frozen sections and has now been extended to include formalin-fixed and paraffin-embedded sections of kidney.¹² To validate C4d staining on formalin-fixed and paraffin-embedded tissue, we analyzed those specimens for which both frozen and paraffin tissue were available. Of 38 specimens available for review, 13 positive for C4d on frozen sections were also positive on paraffin (Figure 3). Of 25 that were negative on frozen sections, only 1 was positive on paraffin. This pattern of staining of the 4 samples in Figure 2A-D were the same when C4d staining of corresponding formalin-fixed and paraffin-embedded sections were used.

Additional Controls

To evaluate additional controls, we performed C4d staining on 44 non-transplant patients who received cardiac biopsies for various diseases, including cardiac

amyloid, dilated cardiomyopathy, hypertrophic cardiomyopathy, coronary artery disease, hemochromatosis, myocarditis and cardiac leukocytoclastic vasculitis, in a lupus patient. Using the criterion of diffuse capillary staining, only 4 were positive (9%) (all dilated cardiomyopathy cases). The 3 patients with myocarditis were negative, including 1 who received a transplant and has remained alloantibody and C4d-negative. Some additional staining patterns of uncertain significance were observed, including plasma staining and arteriolar endothelial staining. Amyloid in 6 of 7 patients with cardiac amyloid stained for C4d. Four of 44 (9%) non-transplant biopsies were C4d-positive on paraffin-embedded formalin-fixed tissue. These are presumed to represent false-positive results, but the presence of anti-cardiac autoantibodies was not excluded (nor sought after) in these patients. Capillary-specific staining was not identified in autopsied hearts with infarcts ($N = 5$).

Chronic Allograft Rejection

In chronic renal allograft rejection, a significant percentage (34% to 61%) showed C4d staining of their biopsies,¹¹ correlating mostly with the presence of serum alloantibody.¹⁰ Chronic rejection in heart allograft manifested as vasculopathy may also be associated with alloantibodies.³⁵ To test the hypothesis that chronic cardiac allograft vasculopathy, like chronic allograft nephropathy, may be associated with C4d staining, we evaluated 33 samples from 33 patients without chronic cardiac vasculopathy (264 to 4,828 days after transplantation) and 43 samples from 25 patients with chronic cardiac vasculopathy (106 to 5,277 days after transplantation). Chronic cardiac vasculopathy (CCV) is defined as luminal narrowing of coronary arteries detected by intravascular ultrasound or intimal hyperplasia of coronary arteries at autopsy.

Table 2. C4d Staining and Serum Alloantibody in Patients Receiving OKT3

Serum alloantibody	OKT3 therapy		Total
	No	Yes	
No			
C4d staining			
No	45	10	55
Yes	1	0	1
Total	46	10	56
Yes			
C4d staining			
No	1	1	2
Yes	9	7	16
Total	10	8	18
Total	56	18	74

$p < 0.001$.

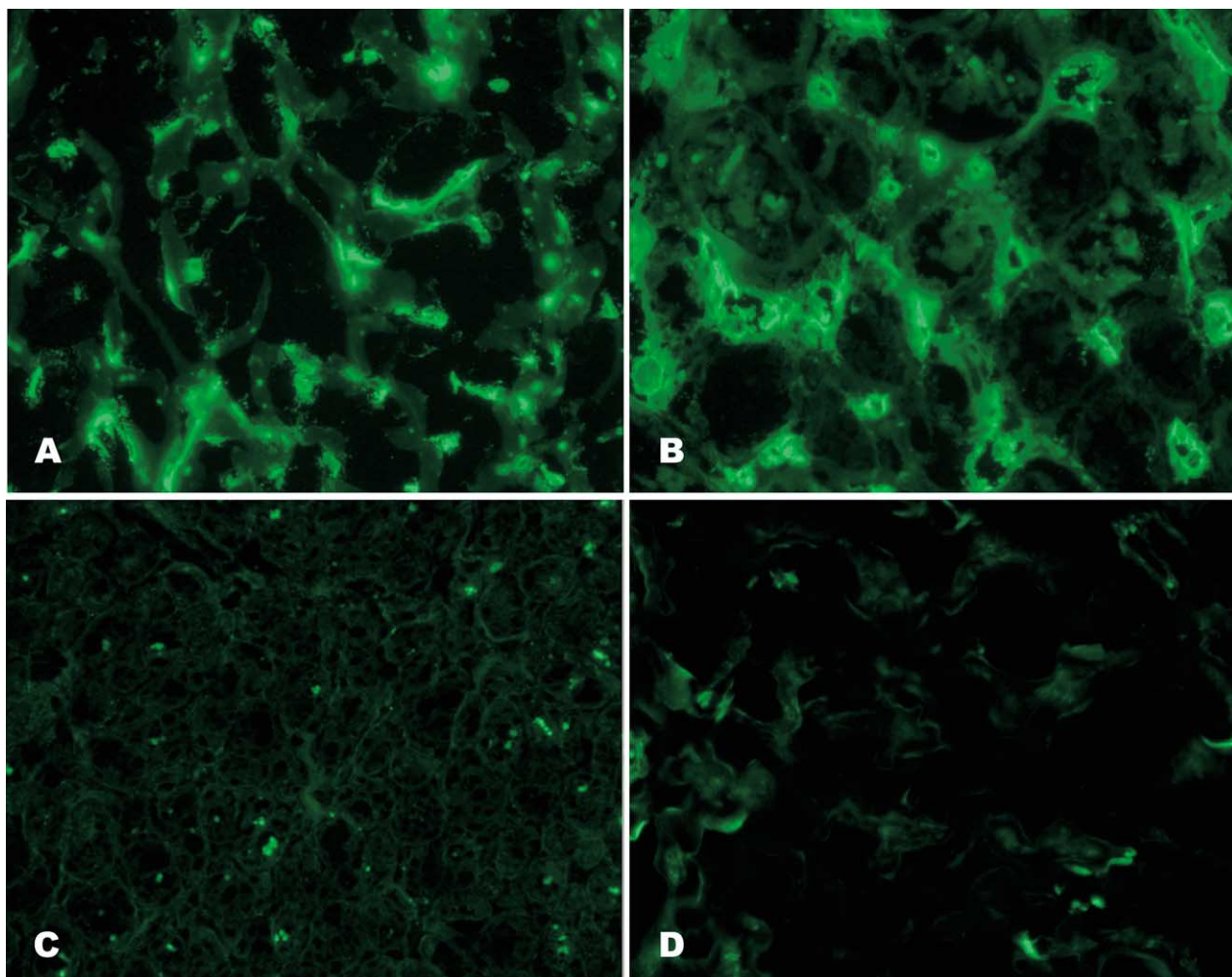


Figure 2. (A) Capillary staining in the cardiac allograft from a patient who died of acute humoral rejection (original magnification $\times 200$). (B) Peritubular capillary staining in a kidney allograft with acute humoral rejection (original magnification $\times 200$). (C) Absence of capillary staining in a cardiac allograft biopsy from a patient with ISHLT Grade 3a rejection. (D) Absence of staining for C4d in the peritubular capillaries of a native kidney from the same patient who died of acute humoral cardiac allograft rejection.

Seven of 33 samples without CCV were positive for C4d (21%), whereas 8 of 43 samples with CCV were positive for C4d (23%). This correlation is not statistically significant ($p = 0.9$). We lack sufficient serum alloantibody data for additional correlations.

Clinical Utility of C4d Staining

The prospective utility of C4d staining is illustrated in a 49-year-old man who received a heart transplant for ischemic cardiomyopathy. For over 1.5 years he was clinically stable on prednisone, imuran and cyclosporine. At Day 605 post-transplant, on routine follow-up, he was clinically stable. His biopsy was Grade 0 with 3 Quilty B lesions. His trough cyclosporine level was 120 mg/ml, slightly low for this patient, who may have been partially non-compliant. During the next 3 weeks, he became progressively more symptomatic and presented on Day 629 with severe orthopnea and an inability to

walk 2 to 3 feet. Urgent right-heart catheterization showed a right atrial pressure of 29 mm Hg and a wedge pressure of 32 mm Hg, with output of 4.2 liters/minute. Troponins were normal. Echocardiogram showed an ejection fraction of 25% (from 66% previously). Right ventricular endocardial biopsy showed moderate ISHLT Grade 2 rejection. The frozen C4d assay showed diffuse capillary fluorescence, consistent with in vivo deposition of alloantibody. Levels on the paraffin block identified an additional focus of lymphocytic infiltrate with myocyte necrosis. The diagnosis was changed to ISHLT Grade 3a rejection. A retrospective panel-reactive antibody (PRA) was positive at 60% and showed anti-donor-specific titers of Class I (1/8) and Class II (1/256) alloantibodies. The patient was treated for 10 days with OKT3, 5 days of plasmapheresis, and then an infusion of rituximab (anti-CD20) on Day 680.³⁶ On Day 690, his biopsy showed an ISHLT Grade 0 rejection, but re-

Table 3. C4d Staining vs Post-Transplantation Ischemic Injury

Serum antibody	Ischemic injury		Total
	No	Yes	
No			
C4d staining			
No	47	25	72
Yes	1	1	2
Total	48	26	74
Yes			
C4d staining			
No	2	2	4
Yes	19	5	24
Total	21	7	28
Total	69	33	102

$p < 0.001$.

mained positive for C4d. He received plasmapheresis again with a decline in antibody titers. He is now clinically stable on tacrolimus, mycophenolate mofetil, prednisone and anti-cytomegalovirus prophylaxis with persistence of some anti-donor alloantibodies. Retrospective C4d on frozen tissue (Day 13 post-transplant) and paraffin-embedded tissue (Days 13, 264, 305, 361 and 444 post-transplant) were all negative. The patient's PRA was negative before transplantation. However, biopsies on Days 605, 629, 643 and 676 were positive for C4d (Figure 4). The most recent biopsy on Day 676 showed capillary C4d staining consistent with persistent anti-donor alloantibodies (Figure 4). Another recent report also highlighted the utility of C4d staining in cardiac transplant humoral rejection.³⁷

DISCUSSION

Of the 254 heart transplant patients evaluated, we identified only 9 with alloantibodies. The exact incidence in our population is unknown because alloantibody screening is not routinely performed on all cardiac transplant patients post-transplantation, so a few antibody-positive, but clinically stable patients may have been missed. The true incidence is likely to be low because transplanted patients usually lack sensitization and alloantibodies before transplantation. Nevertheless, from this patient population we have been able to test enough tissue samples to show that C4d capillary staining of cardiac biopsies correlates well, although not perfectly, with the presence of serum anti-donor alloantibodies. The sensitivity is 84% and the specificity is 89%. This correlation is similar to that identified with renal biopsies.^{7-10,24} Other patterns of cardiac staining, including plasma, arteriolar endothelial, ischemic myocardial and amyloid, were also identified, but their significance is unclear and do not correlate with the presence of alloantibodies. The interpretation of these data may be limited by the difficulty of accruing sufficient numbers of cardiac allografted patients with alloantibodies.

Diagnostic criteria for the pathologic diagnosis of cardiac humoral rejection, either acute or chronic, have not been established.³⁸ Few studies correlate the presence or absence of anti-donor alloantibodies with pathologic findings. Criteria differ among centers.^{15,39-42} Nevertheless, reliable identification of those patients with alloantibodies and cardiac allograft tissue injury is vital to: (1) establish a diagnosis of acute

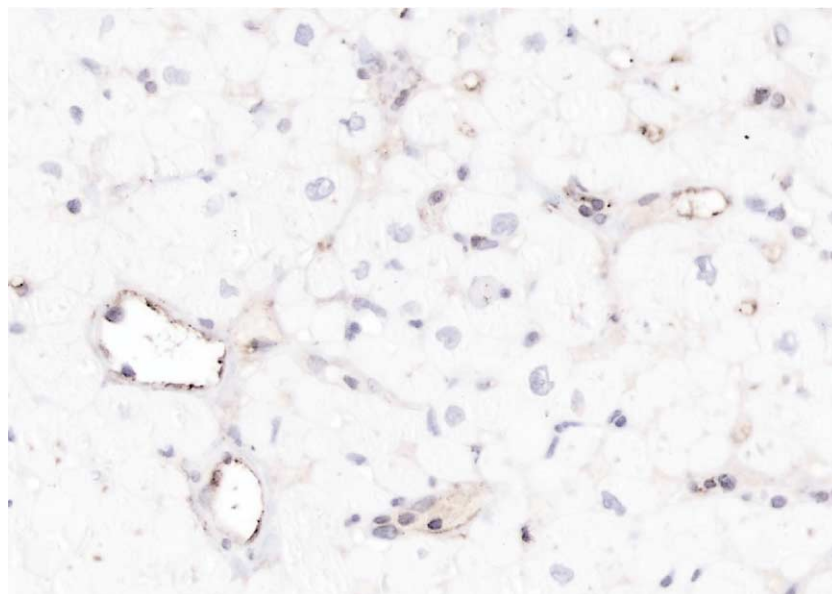


Figure 3. Capillary staining for C4d using formalin-fixed paraffin-embedded tissue from a patient with acute cardiac acute humoral rejection.

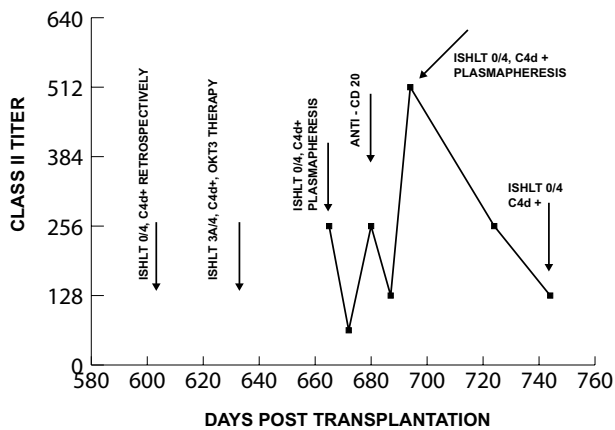


Figure 4. Anti-Class II titer and therapy after cardiac transplantation.

humoral rejection^{38,43}; (2) identify mixed rejections in which the presence of alloantibodies may exacerbate cellular rejections^{44,45}; and (3) determine if chronic rejection (or a sub-set, thereof) is mediated in part by alloantibodies that may contribute to shortening graft survival in chronic rejection.^{14,19}

In our small group of antibody/C4d-positive patients, the presence of alloantibody correlated with C4d capillary staining but did not identify a homogeneous group, either clinically or pathologically. Our findings are similar to those of Behr et al,²⁹ who showed positive C4d staining in a pathologically heterogeneous group that included biopsies both with and without inflammation. In our study 1 patient died of chronic rejection associated with C4d staining and anti-B ABO alloantibody. This patient had no histologic evidence of acute cellular (lymphocytic infiltrate) or humoral rejection (neutrophils, endothelial injury or intravascular macrophages). Two patients were identified with alloantibodies and C4d staining, who were clinically stable and with ISHLT Grade 0 rejection. Their biopsies had no histologic evidence of acute cellular or humoral rejection. These 2 patients with alloantibody and C4d staining, but without acute tissue injury, might represent examples of accommodation.¹³ Six patients suffered severe clinical injury (4 of the 6 died), and met the criteria (alloantibody, C4d deposition, pathologic tissue injury and clinical compromise) for antibody-mediated rejection.^{38,43} One of these 6 patients had acute humoral rejection with capillary injury, intravascular macrophages and patchy myocytonecrosis in the absence of cellular rejection. The other 5 patients had mixed rejection with acute cellular rejection (destructive multi-focal lymphocytic infiltrates and endothelialitis) and histologic evidence of antibody-mediated injury (intravascular macrophages and endothelial injury, with focal capillary neutrophils). Positive capillary C4d staining is not the sole criterion, but is among 4 criteria used to diagnose antibody-mediated rejection.^{38,43}

C4d tissue allograft staining may occur in the absence of detectable serum alloantibodies. The anti-HLA titer could be low or undetectable because the alloantibodies are absorbed entirely into the allograft. Alternatively, the alloantibodies are non-HLA and not detectable by routine tissue-typing techniques.⁴⁶⁻⁴⁸ In some cases, C4d deposition might occur by complement fixation through mechanisms of innate immunity.⁴⁹ C4d deposition without tissue injury—that is, accommodation—might occur if complement-regulatory proteins terminate or regulate complement fixation so that chemotactic factors and the lytic components of the complement cascade are not present to cause tissue injury.^{34,49} If this were the case, C4d, but not C3d, might be deposited.^{50,51} The presence of both C4d and C3d capillary staining may detect more severe pathologic injury with more serious clinical compromise.^{50,52}

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