

Complement Activation in Acute Humoral Renal Allograft Rejection: Diagnostic Significance of C4d Deposits in Peritubular Capillaries

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Abstract. The distinction between acute humoral rejection (AHR) and acute cellular rejection (ACR) in renal allografts is therapeutically important, but pathologically difficult. Since AHR is probably mediated by antibodies to the donor endothelium that activate the classical complement pathway, it was hypothesized that peritubular capillary C4d deposition might distinguish this group. Renal biopsies ($n = 16$) from 10 patients with AHR who had acute graft dysfunction, neutrophils in peritubular capillaries, and a concurrent positive cross-match were stained for C4d by immunofluorescence. Control biopsies for comparison showed ACR ($n = 14$), cyclosporin A toxicity ($n = 6$), or no abnormality ($n = 4$). Peribiopsy sera were tested for anti-donor HLA antibody. C4d deposited prominently and diffusely in the peritubular capillaries in all AHR

biopsies (16 of 16). IgM and/or C3 were also present in 19 and 44%, respectively. With two-color immunofluorescence, C4d was localized in basement membranes (type IV collagen⁺) and in the endothelium (*Ulex europaeus* agglutinin-I⁺). In ACR, no more than trace C4d was found in peritubular capillaries ($P < 0.0001$ versus AHR), and no patient had anti-donor HLA antibodies (0 of 8); 27% had neutrophils in peritubular capillaries. One of six biopsies with cyclosporin A toxicity had similar C4d deposits, and circulating anti-donor class I antibody was detected. Grafts with AHR were lost (40%) more often than those with ACR (0%; $P < 0.02$). C4d in peritubular capillary walls distinguishes AHR from ACR, is more specific and sensitive than traditional criteria, and is a potentially valuable adjunct in the diagnosis of graft dysfunction.

Acute humoral rejection (AHR) is a form of renal allograft rejection that typically occurs a few weeks after transplantation and is associated with circulating antibodies to donor HLA class I (1), class II (2,3), or non-MHC (4–6) antigens on endothelium. AHR may coexist with T cell-mediated injury (acute cellular rejection [ACR]), and probably contributes to graft injury more often than is generally appreciated. Indeed, cytotoxic anti-donor class I antibody was found in the circulation at the time of acute rejection in 20 to 25% of patients (1,7). Such antibodies correlate with an increased risk of clinically severe acute rejection and graft loss (1,7).

Identification of AHR in biopsy specimens has been problematic, because no morphologic feature described to date is either pathognomonic or universal. Several are helpful in raising the possibility, however, including neutrophils in peritubular capillaries, fibrinoid necrosis, and glomerular thrombi (1,7–

9). Unfortunately, immunofluorescence for IgG, IgM, C3, or fibrin is not as helpful as one might expect, revealing no statistically significant difference between AHR and ACR (8). At present, the diagnosis of AHR is made by identification of suspicious morphologic features on biopsy with subsequent serologic confirmation of circulating anti-donor antibodies.

The prognosis of AHR is uniformly worse than ACR (1,5–8). However, those that recover from the acute episode of AHR have a similar long-term outcome (8), suggesting that the pathogenetic humoral response can be transient if treated effectively. Indeed, plasmapheresis combined with mycophenolate mofetil and tacrolimus can be successful in reversing AHR and restoring graft function (10). These important therapeutic implications of AHR have stimulated our efforts to develop more sensitive and specific diagnostic criteria.

Materials and Methods

Patients

The study group consists of all renal biopsies evaluated from January 1994 to August 1998 (16 biopsies) at the Massachusetts General Hospital that met the following inclusion criteria: (1) acute graft dysfunction; (2) prominent neutrophils in cortical peritubular capillaries or fibrinoid necrosis of arteries; and (3) a positive anti-donor T and/or B cell cross-match in a concurrent serum. These criteria are generally accepted to favor AHR (11). All but one had a negative pretransplant cross-match (patient 4 was weakly positive, retrospectively). All except two received a cadaveric kidney; two were

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living related donors (daughter, mother) (Table 1). Maintenance immunosuppression included cyclosporin A (CsA), prednisone, and azathioprine. Six patients were treated for AHR with plasmapheresis, and/or mycophenolate mofetil and tacrolimus, as described (10).

Cases from three other groups were compared. These cases were included if they met the diagnostic criteria for either: (1) acute cellular rejection (14 patients) according to the Cooperative Clinical Trials in Transplantation criteria (six type I, seven type II, and one type III) (12); (2) acute CsA toxicity (six patients, including two cases with thrombotic microangiopathy) (11); or (3) had no diagnostic abnormality (four patients, biopsies taken at the time of transplantation). Cases were screened sequentially from the same time period as the AHR cases, starting with the most recent samples; cases with sufficient frozen tissue available for study were used. Biopsies were analyzed by light, immunofluorescence, and electron microscopy, as described (13).

Detection of Anti-Donor Antibodies

Donor T and B cells were isolated with immunomagnetic beads at transplantation and stored at -70°C (10). Serial serum samples were collected prospectively. Cytotoxic cross-matches were performed using patient sera taken within 1 d of the biopsy. Both anti-human globulin-enhanced T cell and standard complement-dependent cytotoxic B cell assays were used (10). Panel-reactive antibodies were determined with a local frozen cell panel. OKT3 was removed from

patient sera with immunomagnetic beads coated with anti-mouse IgG (15). Flow cytometric cross-matches were done on the patient on antithymocyte globulin (patient 9).

Immunofluorescence Microscopy

Biopsy sections were stained with a three-step immunofluorescence technique developed in our laboratory. Four-micrometer frozen sections were incubated in 100 $\mu\text{g}/\text{ml}$ avidin D (Vector Laboratories, Burlingame, CA) to block endogenous biotin. Sections were washed and excess avidin was bound by adding 10 $\mu\text{g}/\text{ml}$ d-biotin (Sigma Chemical Co., St. Louis, MO). Monoclonal antibody to C4d (clone 10-11; Biogenesis, Sandown, NH) was applied for 30 min. Sections were washed and incubated sequentially first with biotinylated horse anti-mouse IgG (1:100) (Vector Laboratories) and after washing then with FITC-streptavidin (1:50) (Biomedex, Foster City, CA) each for 30 min. Endothelial cells were detected with biotinylated *Ulex europaeus* agglutinin-I (Ulex lectin, Vector Laboratories), which binds to α -L-fucose (in blood group substance O) (14).

For simultaneous detection of vascular basement membranes and C4d, monoclonal anti-type IV collagen (Silenus, Hawthorne, Australia) and tetramethylrhodamine isothiocyanate-rabbit anti-mouse IgG (Dako, Carpinteria, CA) were added to the above C4d protocol. For simultaneous C4d and endothelial staining, C4d was detected with tetramethylrhodamine isothiocyanate-rabbit anti-mouse IgG and Ulex lectin was detected with FITC-streptavidin.

Table 1. Acute humoral rejection in patents with renal allografts^a

Patient	Donor Source	Circulating Antibodies to Donor		Biopsy No. ^b	PTC PMN	PTC IgM	PTC C3	PTC C4d	Graft Survival ^b	Creatinine (mg/dl) at 1 yr
		T Cells	B Cells							
1	CD	1:4	>1:8	1 (5)	2+	—	—	2 to 3+	Failed (8)	
2	CD	>1:8	>1:8	1 (8)	3+	+	—	4+	Survived	1.6
3	CD, 3	ND	ND	2 (17)	2+	—	—	1 to 2+	Failed (180)	
4 ^c	CD, 2	>1:8	>1:8	3 (33)	2+	—	—	2+	Failed (23)	
		1:32	Negative	1 (6)	2+	—	+	3+		
5	Daughter	1:16	ND	2 (12)	2+	—	+/-	3+	Survived	
		1:32	>1:8	1 (7)	2+	—	—	4+		
6	CD	1:16	ND	2 (34)	3+	—	—	4+	Survived	1.8
		>1:8	1:4	2 (26)	2+	—	—	4+		
7	CD	1:128	1:4	1 (14)	2+	—	+	4+	Survived	
		>1:512	1:64	2 (21)	3+	—	—	4+		
8	CD, 3	Negative	>1:8	1 (7)	1 to 2+	—	—	4+	Survived	1.3
9 ^d	CD, 3	Positive ^e	Positive ^e	1 (3)	3+	+	+	4+	Failed (10)	
10	Mother	Negative	1:256	1 (6)	3+	+	+	4+	Survived ^f	
		1:2	1:128	2 (17)	3+	—	+	3+		
		Weak	1:16	3 (24)	2 to 3+	—	+	2 to 3+		

^a PTC, peritubular capillaries; PMN, polymorphonuclear leukocytes; ND, not determined; CD, cadaveric kidney. The number after CD indicates the number of renal allografts the patient has received.

^b The number in parentheses indicates days posttransplantation.

^c Patient was presensitized at time of transplantation.

^d This patient's original disease was hereditary nephritis. His clinical course was complicated by the presence of anti-glomerular basement membrane antibodies in the kidney.

^e Determined by flow cytometry.

^f Graft has survived the first 6 wk. Patients 5 through 10 were treated with a rescue protocol combining tacrolimus with mycophenolate and/or plasma exchange (10).

Statistical Analyses

Sections were examined in an Olympus BX60 vertical illumination fluorescence microscope and scored for C4d staining by two observers without knowledge of the clinical or pathologic diagnoses. Comparison of groups was done with the Fisher exact test. A *P* value <0.05 was considered significant.

Results

Acute Humoral Rejection

The clinical and pathologic features are summarized in Table 1. Eight of 10 patients had circulating IgG antibody specific for donor T cells (titers 1:4 to >1:512); the two other patients had IgG reactivity only to donor B cells (titers >1:1 and >1:256) (Table 1). Seven patients had donor-specific reactivity to both T and B cells. Cytotoxic assays against HLA-typed panels of T or B cells demonstrated reactivity against donor HLA specificities. The 1-yr graft survival was 60%.

All biopsies had prominent neutrophils in peritubular capillaries (Figure 1), occasionally accompanied by scattered intravascular fibrin. Less frequently, glomerular capillaries had neutrophils, endothelial cell swelling/loss, or fibrin deposits. Fibrinoid arterial necrosis was found in two cases. In addition, features of ACR were present in 10 of 16 biopsies (62%), including interstitial mononuclear infiltrates (56%), tubulitis (50%), and endarteritis (endothelialitis, 31%). Scattered interstitial eosinophils were observed in 38%. Peritubular capillaries had detectable deposits of IgM in 3 of 16 biopsies, C3 in 7 of 16, and IgG in 0 of 16 biopsies (Table 1). Sites of arterial fibrinoid necrosis showed IgM and C3 deposition.

Intense staining for C4d was detected in a widespread, uniform distribution in the peritubular capillaries in all AHR biopsies (Figure 2A, Table 1). At low power, the smaller oval and elongated ring-like fluorescence profiles of peritubular capillaries were readily evident between the larger, negative tubular cross sections. The capillary staining was crisp, linear, and continuous, but also had a finely granular pattern at high power, which extended into the lumen from the more linear deposits. The medullary vessels generally had less intense C4d deposition compared with the cortex. Arteries with fibrinoid

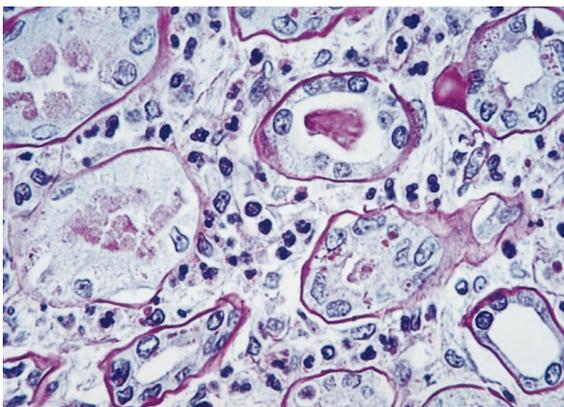


Figure 1. Light micrograph of a renal allograft biopsy with acute humoral rejection, showing accumulation of neutrophils in peritubular capillaries. Periodic acid-Schiff stain. Magnification, $\times 256$.

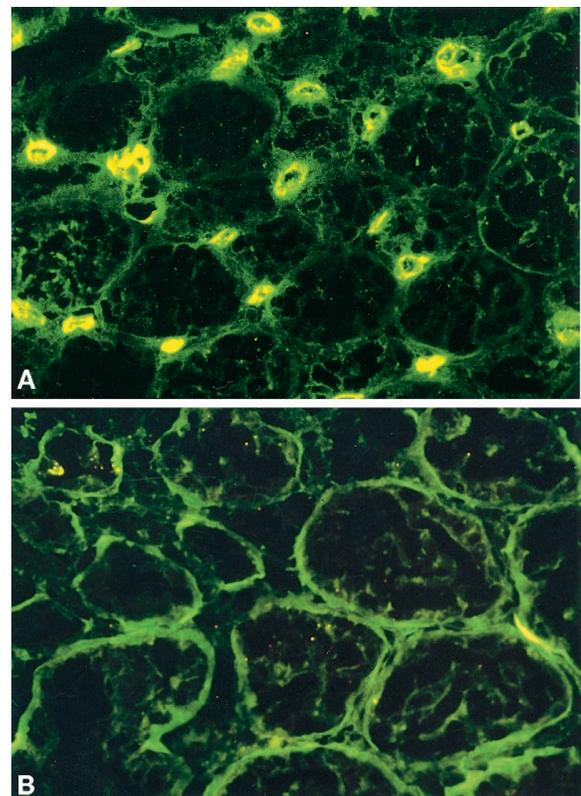


Figure 2. Immunofluorescence micrograph of renal allograft biopsies stained for C4d (see Materials and Methods). (A) Acute humoral rejection, with diffuse bright staining for C4d in the peritubular capillaries. (B) Acute cellular rejection, which has C4d in tubular basement membranes, but no detectable deposition of C4d in peritubular capillaries. Magnification, $\times 200$.

necrosis had C4d deposits in the media. Other sites of C4d deposits were not unique for AHR (see below), including broad deposits in the glomerular mesangium, bright linear glomerular basement membrane (GBM) deposits, and focal deposits in arterioles and arterial intima. Tubular basement membranes (TBM) were generally negative.

Using dual stains, C4d fluorescence codistributed with anti-type IV collagen in peritubular capillaries and was sometimes also evident on the luminal side of the basement membrane (Figure 3, A and B). Staining of endothelium with the lectin Ulex was variable, and showed a focal loss in peritubular capillaries and large vessels in those cases in which endothelial injury was detected at the light microscopic level. In dual stains, C4d fluorescence overlapped that of Ulex, but was also present external to the Ulex lectin and in occasional peritubular capillaries that were Ulex lectin-negative (Figure 3, C and D).

Acute Cellular Rejection

ACR biopsies had negative (11 of 14) or trace, focal (3 of 14) staining of C4d in peritubular capillaries by immunofluorescence, including the four that had intracapillary neutrophils (Figure 2B, Table 2) (*P* < 0.0001; AHR *versus* ACR). As in normal kidneys (see below), the glomeruli had mesangial C4d and sometimes prominent linear GBM deposits, arterioles often had bright C4d, and arteries also had focal intimal C4d.

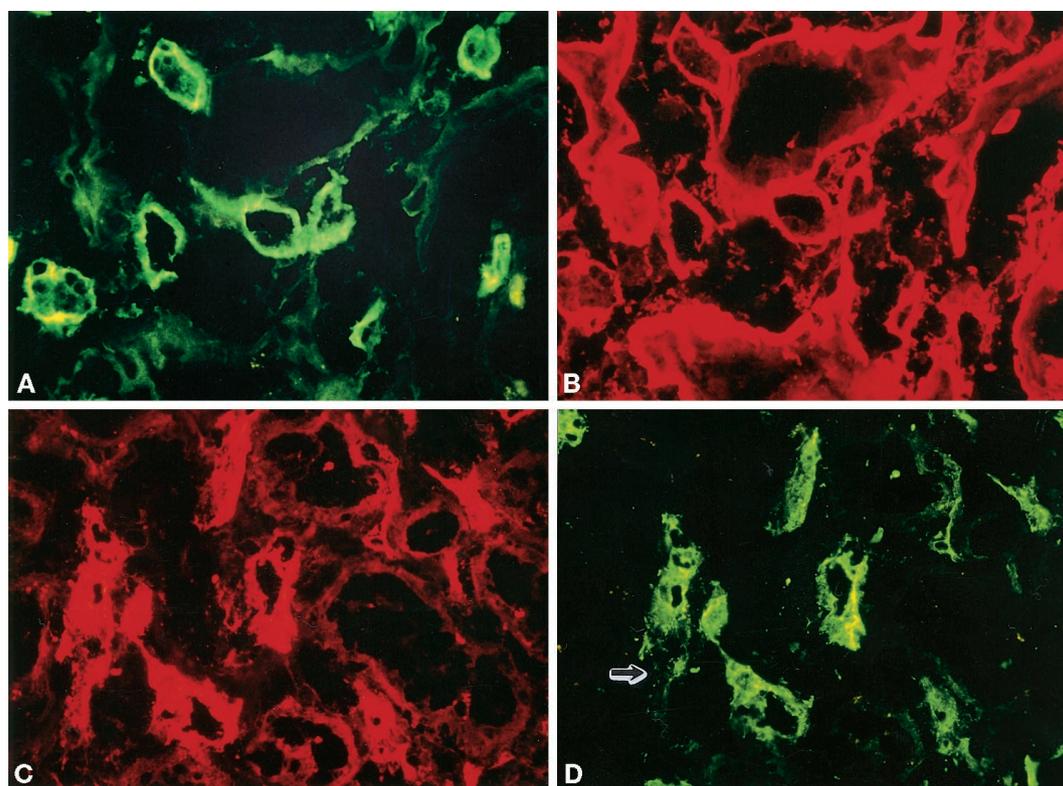


Figure 3. Double immunofluorescence stains of renal allograft biopsies with acute humoral rejection. In the same field, the green filter (A) shows C4d and the red filter (B) type IV collagen codistributed in the peritubular capillaries. The tubular basement membrane stains uniformly for type IV collagen but only focally for C4d. In another pair of fields, the red filter (C) shows C4d in the same distribution as the endothelial marker, Ulex lectin (D, green filter). Portions of vessels show C4d deposits without Ulex lectin reactivity (arrow), suggesting that the C4d remains after loss or injury of the endothelium. Magnification, $\times 200$.

Table 2. Other categories of allograft biopsies examined^a

Diagnosis	Biopsies Examined	No. with Antibodies	No. with PTC	No. with PTC C4d
Acute cellular rejection	14	0 of 8	4 of 14	0 of 14
CsA toxicity	6	1 of 6	3 of 6	1 of 6 ^b
NDAR/donor biopsy	4		0 of 4	0 of 4

^a CsA, cyclosporin A; NDAR, no diagnostic abnormality recognized. Other abbreviations as in Table 1.

^b In one of the six cases with morphologic features of CsA toxicity and without ptc pmn, the ptc stained positively for C4d. Retrospective testing of this patient's serum revealed circulating anti-class I antibodies.

TBM sometimes had focally intense linear or segmental C4d. Ulex lectin binding was variably diminished in peritubular capillaries compared with normal kidneys. The reduction was patchy and more prominent in arterial vessels than in peritubular capillaries. Because of the variable intensity and distribution of staining with Ulex lectin, this reagent was not useful in distinguishing humoral from acute cellular rejection. No graft loss occurred in the ACR group ($P < 0.02$ versus AHR).

CsA Toxicity

The six biopsies on which a diagnosis of CsA toxicity was made had a range of changes that included focal acute tubular injury (3 of 6), focal swelling of glomerular endothelial cells, thrombotic microangiopathy (2 of 6), and/or neutrophils in

peritubular capillaries (3 of 6). C4d staining in peritubular capillaries was positive in one case, which had no evidence of AHR or thrombotic microangiopathy. Retrospective examination of this patient's serum revealed anti-donor antibodies (Table 2). A biopsy 2 wk later showed neutrophils in peritubular capillaries, consistent with AHR, and was positive for C4d. In those with thrombotic microangiopathy, intense arterial intimal and medial deposits of C4d were present. In other cases, the overall C4d distribution was similar to that in normal kidneys (see below) as was the Ulex lectin staining pattern.

Normal Kidneys

No C4d was found in peritubular capillaries. C4d was detected in the glomerular mesangium and in a linear GBM

pattern, as described previously (16). Focal deposits of C4d were in the TBM, arterioles, and arterial intima. Ulex lectin stained the endothelium of all glomerular and peritubular capillaries and some arteries/arterioles.

Discussion

This study demonstrates a potentially useful and reliable pathologic marker of acute antibody-mediated rejection, which has hitherto been difficult to diagnose. All 10 cases of AHR had bright, extensive staining for C4d in cortical capillaries, in marked contrast to acute cellular rejection, CsA toxicity, or other controls. The one exception (CsA toxicity) had anti-donor antibodies in retrospect and a later biopsy consistent with AHR. Thus, even in a biopsy in which the usual features of AHR are not observed, the presence of C4d appears to be a sensitive indicator of anti-donor antibodies. Other sites of C4d deposition in the kidney (glomeruli, arterioles, arteries, and tubules) were not unique for AHR. The present study, although retrospective, included all patients with biopsies that met our strict inclusion criteria for AHR over an extended period of time, without regard to the C4d stain.

Our results are consistent with previous studies that associated peritubular C4d with more severe “cell-mediated” rejection and pretransplant sensitization (17). Circulating, panel-reactive antibodies were detected in most of these patients before transplantation, including HLA-DR antibody (18). However, in contrast to the present study, no correlation with the morphologic features of humoral rejection or with concurrent anti-donor antibodies was reported.

All patients with AHR in this series had donor-reactive antibodies to HLA class I or class II antigens, alone or in combination. C4d will presumably also be deposited in cases of AHR due to non-HLA antibodies (4–6), and therefore has potential value as an antigen-independent test for humoral rejection. For example, in conventional hyperacute rejection (*e.g.*, ABO blood group incompatibility), we found a similar striking pattern of C4d in peritubular capillaries (unpublished observations).

The C4d stain compares favorably with other diagnostic criteria of AHR. Previously, no single pathologic feature consistently distinguished those patients with circulating antibodies (8). The lesions that favored AHR over ACR were a higher frequency of neutrophils in peritubular capillaries (46% *versus* 5%), fibrinoid necrosis (25% *versus* 5%), glomerulitis (45% *versus* 10%), thrombi (46% *versus* 15%), and infarction (38% *versus* 0%). Of these criteria, we found peritubular neutrophils to be the most useful, and these were present in all of our prototypical AHR cases. However, neutrophils can be observed in peritubular capillaries in ACR and CsA toxicity, even to a quite prominent degree, with no associated circulating antibody. Fibrinoid necrosis of arteries is also commonly regarded as an indicator of humoral rejection, and was present in two of our AHR cases. However, fibrinoid necrosis can occur without demonstrable circulating anti-HLA antibody, as we observed in one current ACR case; in this case, C4d was in affected arteries but not in peritubular capillaries, perhaps due to anti-

bodies to non-HLA antigens expressed in arteries but not capillaries.

Of considerable disappointment to immunopathologists, deposition of Ig and C3 are not sensitive indicators of AHR (8), an observation confirmed by the present study. In fact, many centers do not advocate staining cryostat sections of transplant biopsies for Ig and complement. Although this might argue against a pathogenetic role for antibody or complement, it is more likely that the lack of detectable Ig is due to modulation from the surface of activated endothelium or loss of the target cell itself. Indeed, loss or injury of endothelium was apparent by the patchy Ulex lectin stain in AHR (while the basement membrane type IV collagen remained). However, a similar loss of Ulex lectin reactivity occurred in ACR. These data support the hypothesis that the peritubular capillary endothelium is a prime target in ACR (19,20). We have also tested a large panel of monoclonal antibodies to endothelial antigens in pursuit of a marker of injury that would discriminate between ACR and AHR, but have so far been unsuccessful (*e.g.*, CD31, CD34, CD62, eNOS, CD41; unpublished data). Of all the markers we tested, only C4d in peritubular capillaries showed specificity for AHR. Other sites of C4d deposition in the kidney (glomeruli, arterioles, arteries, and tubules) were not unique for AHR.

C4 is the second most abundant complement component after C3. After antibody binds to antigen, C4 is proteolytically cleaved by activated C1 into C4a and C4b. The cleavage exposes the reactive and short-lived thiol ester group in C4b that binds covalently to nearby molecules containing amino or hydroxyl groups, such as proteins and carbohydrates (21,22). Bound C4b is proteolytically inactivated into C4d, a 44.5-kD peptide that contains the thiol ester site and remains covalently bound at the same site. Thus, if C4d is bound to a structural protein, it is potentially a durable marker of local complement activation by the classical pathway. How long C4d may persist was not established by these studies.

C4d was present in all peritubular capillaries, even those that lacked endothelial reactivity with Ulex lectin, suggesting that not all of the C4d is bound to endothelium. Indeed, the staining for C4d extended outside the fuzzy ring of Ulex binding. When these were stained for basement membrane collagen, the C4d was codistributed with type IV collagen, in broad bands around the vessels. Thus C4d appears to be bound to the “bystander” capillary basement membrane. This location would fit with the known ability of C4 to cross-link to nearby proteins at the site of complement activation. The covalent linkage of C4d to structural proteins may explain the mechanism by which C4d remains after alloantibody disappears, since antibody binds to cell surface antigens that can be lost by modulation, shedding, or cell death.

Why peritubular capillaries are the major target of circulating HLA antibodies, sometimes in the absence of obvious glomerular inflammation, is puzzling, since all blood in the peritubular capillaries goes first through glomeruli, which express HLA class I and II antigens. We hypothesize that peritubular capillaries may have less anti-complement protective pathways than glomeruli. Four major cell surface inhibitors of complement activation are abundant in normal human glomer-

uli: decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and CR1 (CD35), which inactivate C3 and/or C5 convertases of the classical and alternative pathways and protectin (CD59), which inhibits the formation of the membrane attack complex (23–25). It is probably important that glomeruli be protected from the spontaneous “ticking over” of C4 and C3 that exposes their reactive thiol ester (21) and from harmful immune complexes. The normal accumulation of C4d in the glomerulus can be taken as evidence for this process. In contrast, only CD59 is prominent in normal cortical peritubular capillaries by immunofluorescence (23,26,27). Thus, at the peritubular capillary surface, classical pathway activation of C4 is relatively unopposed. The critical importance of these regulators of complement activation in resistance to humoral rejection has been proved by the prevention of pig xenograft hyperacute rejection, using organs from pigs transgenic for human CD55 and CD59 (28).

In studies such as this, often one learns the most from the exceptions. The case of CsA toxicity with C4d had no evidence of AHR by light microscopy, but when a concurrent serum was checked, anti-donor class I antibody was detected. This patient was probably having an episode of humoral rejection that did not become clinically or pathologically evident until 2 wk later. Thus, even in a biopsy in which the usual features of AHR are not observed, the presence of C4d appears to be a sensitive indicator of anti-donor antibodies. We have seen another case of ACR superimposed on chronic rejection 5 yr after transplantation that had bright C4d deposits, indistinguishable from those in AHR (unpublished observations). Whether a component of AHR was present was undetermined, since no serum was available; however, the rejection was unusually severe and the graft was lost. We have subsequently reported that the correlation with C4d positivity and circulating antibody also applies to chronic rejection, arguing that a subset of these cases are humorally mediated (29).

We initiated a prospective study in August 1998, staining every renal transplant biopsy regardless of histologic findings for C4d. The results, although preliminary, support the findings reported here. Five of the 29 samples studied to date had widespread, intense C4d deposition in peritubular capillaries and four of five of those with C4d had circulating anti-donor class I and/or class II antibodies. The one exception was on antithymocyte globulin at the time of testing and responded to plasmapheresis. In contrast, none of the six patients tested to date who were negative for C4d had anti-donor antibodies.

AHR typically presents as severe rejection, often resistant to steroid and anti-lymphocyte antibody therapy (1). Graft loss is frequent, ranging from 29 to 75%, compared with 4% for ACR (1,5,9,18). Our data are quite consistent with these reports: 40% of grafts with AHR were lost to rejection, compared with 0% among those with ACR. The differences in prognosis, and presumably the optimal treatment, have encouraged the development of definitive diagnostic criteria. We conclude from the data presented here that assessment of C4d in peritubular capillaries is a useful adjunct in the diagnosis of AHR in renal transplant biopsies and thereby may help guide clinical decisions and stratify clinical trials.

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