Chronic Humoral Rejection: Identification of Antibody-Mediated Chronic Renal Allograft Rejection by C4d Deposits in Peritubular Capillaries

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Abstract. The pathogenesis of chronic renal allograft rejection (CR) remains obscure. The hypothesis that a subset of CR is mediated by antidonor antibody was tested by determining whether C4d is deposited in peritubular capillaries (PTC) and whether it correlates with circulating antidonor antibodies. All cases (from January 1, 1990, to July 31, 1999) that met histologic criteria for CR and had frozen tissue (28 biopsies, 10 nephrectomies) were included. Controls were renal allograft biopsies with chronic cyclosporine toxicity ($n = 21$) or nonspecific interstitial fibrosis ($n = 10$), and native kidneys with end-stage renal disease ($n = 10$) or chronic interstitial fibrosis ($n = 5$). Frozen sections were stained by two-color immunofluorescence for C4d, type IV collagen and Ulex europaeus agglutinin I. Antidonor HLA antibody was sought by panel-reactive antibody analysis and/or donor cross matching in sera within 7 wk of biopsy. Overall, 23 of 38 CR cases (61%) had PTC staining for C4d, compared with 1 of 46 (2%) of controls ($P < 0.001$). C4d in PTC was localized at the interface of endothelium and basement membrane. Most of the C4d-positive CR tested had antidonor HLA antibody (15 of 17; 88%); none of the C4d-negative CR tested (0 of 8) had antidonor antibody ($P < 0.0002$). The histology of C4d-positive CR was similar to C4d-negative CR, and 1-yr graft survival rates were 62% and 25%, respectively ($P = 0.05$). Since August 1998, five of six C4d-positive CR cases have been treated with mycophenolate mofetil ± tacrolimus with a 100% 1-yr graft survival, *versus* 40% before August 1998 ($P < 0.03$). These data support the hypothesis that a substantial fraction of CR is mediated by antibody (immunologically active). C4d can be used to separate this group of CR from the nonspecific category of chronic allograft nephropathy and may have the potential to guide successful therapeutic intervention.

Chronic rejection (CR), clinically defined as progressive loss of renal function with hypertension and proteinuria more than 3 mo after transplantation, is the leading cause of late allograft dysfunction, with an overall incidence of 25% when all renal allografts, functioning and nonfunctioning at death, are considered (1). CR accounts for renal failure in 50 to 80% of recipients who return to dialysis after transplantation. The morphologic diagnosis can be difficult. The two most definitive features of chronic renal allograft rejection are the arterial intimal thickening with mononuclear cell infiltration and the duplication of the glomerular basement membrane (GBM). However, the arterial lesion preferentially affects the larger vessels, which are not always sampled in needle biopsies. The glomerulopathy can be mild or focal and by itself is not pathognomic of CR because it is also seen in thrombotic microangiopathy and certain chronic immune complex diseases. Interstitial fibrosis and tubular atrophy are similarly nonspecific findings that are compatible with a variety of causes, including past ischemic injury, hypertension, and chronic cyclosporine toxicity (CsAT). The inability to distinguish these conditions by histologic characteristics is reflected in the literature by the nonspecific term *chronic allograft nephropathy*, which is used to encompass the end result of chronic injury resulting from immunologic reaction to donor alloantigens as well as from nonimmunologic mechanisms (2,3). Because effective treatment is likely to be fundamentally different, a test that could reliably distinguish chronic immunologic rejection from other causes of allograft dysfunction is much needed.

We recently reported that C4d in peritubular capillaries (PTC) is a useful diagnostic marker of acute humoral rejection (4), a possibility initially suggested by the work of Feucht and colleagues (5,6). C4d is a fragment of the classical complement pathway component C4, which is activated by antigen-antibody complexes (7). C4 is activated and proteolytically cleaved into C4a and C4b, exposing a reactive and short-lived thiolester group in C4b that binds to nearby molecules covalently. C4b is subsequently inactivated by cleavage into C4c

and C4d, the latter fragment containing the covalent bond to the tissue that thereby can remain at the site of complement activation.

With increasing evidence for the role of antibody in CR in animals (8,9), we sought to determine whether CR in humans might also be associated with antibody-mediated mechanisms, which could be identified by C4d expression in renal allograft tissue samples, and correlated with circulating antidonor HLA antibody, histology, and clinical features.

Materials and Methods
Patients

From January 1, 1990, to July 31, 1999, 649 renal allograft biopsies were performed at the Massachusetts General Hospital, 152 of which were done for evaluation of chronic allograft dysfunction. The histopathologic diagnoses in this group were CR ($n = 30$), chronic cyclosporine toxicity (CsAT; $n = 21$), recurrent ($n = 24$) or de novo ($n = 20$) glomerular disease, and nonspecific tubulointerstitial fibrosis ($n = 57$). Twenty-eight of the 30 cases fulfilled the inclusion criteria for CR upon review and had sufficient frozen tissue available for this study. The histologic criteria for CR were either (1) GBM duplication (chronic allograft glomerulopathy) in the absence of de novo or recurrent glomerulonephritis or (2) arterial intimal fibrosis (chronic allograft arteriopathy) with intimal mononuclear cell infiltration. When sequential biopsies were performed in a patient, the first biopsy that showed CR was included in this study. In addition, 38 allograft nephrectomies were done in this time period, 10 of which met the criteria of CR and were included.

The control group ($n = 46$) included (1) all renal allograft biopsies from the same time period that showed chronic CsAT ($n = 21$), with the typical peripheral nodular medial hyalinosis of arterioles (chronic CsA arteriolopathy); (2) random renal allograft biopsies with nonspecific chronic interstitial fibrosis (TxCIN; $n = 10$); (3) random samples from the same time period of native kidney biopsies with chronic interstitial fibrosis (CIN; $n = 5$), and native nephrectomies with end-stage renal disease (ESRD; $n = 10$) as a result of diabetic glomerulosclerosis, IgA nephropathy, or hypertension.

Clinical data on all renal transplant patients were obtained by chart review and the clinical database. Data gathered included patient demographics (age at biopsy, gender), allograft variables (% cadaveric versus living donor, previous transplantation), and posttransplantation variables (acute rejection rate in the first 6 mo, time from transplantation to allograft sampling, level of creatinine and proteinuria at allograft sampling, and immunosuppressive agents at induction and at time of biopsy). Follow-up of all allograft biopsy cases was complete either to graft failure or to February 2000 in the surviving grafts. Graft failure was defined as return to dialysis or retransplantation. The 1-yr graft survival rate was analyzed for all renal allograft cases and controls.

Light Microscopy

Histologic sections from all transplant patients were reviewed without knowledge of clinical or immunopathologic information and scored for (1) chronic allograft glomerulopathy, defined as global, diffuse, or segmental duplication of the GBM with cellular interposition; (2) chronic allograft arteriopathy with arterial intimal proliferation and hypertrophy, with or without infiltrating mononuclear inflammatory cells; (3) interstitial fibrosis, tubular atrophy; and (4) the presence of interstitial mononuclear inflammatory cells and plasma cells.

Immunofluorescence Microscopy

Cryostat sections of renal tissue cut at 4 μ from all CR cases ($n = 38$) and controls ($n = 46$) were used. C4d was detected with a sensitive three-layer immunofluorescence technique and combined with stains for type IV collagen, as described previously (4).

Frozen sections were incubated in 100 mg/ml avidin D (Vector Laboratories, Burlingame, CA) to block endogenous biotin. Sections were washed, and excess avidin was saturated by adding 10 mg/ml D-biotin, (Sigma Chemical, St. Louis, MO). Monoclonal antibody to C4d (clone 10-11; Biogenesis, Sandown, NH) was applied for 30 min. Sections were washed and incubated sequentially with biotinylated horse anti-mouse IgG (Vector Laboratories), 1:100 dilution, followed by FITC-streptavidin (Biomeda, Foster City, CA), 1:50 dilution, after washing; each step was incubated for 30 min. Afterward, a monoclonal anti-collagen type IV antibody (clone 24.12.8; Silenius, Hawthorn, Australia) followed by a tetramethylrhodamine isothiocyanate (TRITC)-rabbit anti-mouse IgG (DAKO, Carpinteria, CA) were applied, with washing after each step.

Dual staining for C4d and Ulex europaeus agglutinin I (UEA; an endothelial marker) was used to determine the relationship of the C4d with the vascular endothelium using biotinylated UEA (Ulex Lectin, Vector Laboratories) which binds to α L-fucose (10). C4d was detected with unlabeled monoclonal anti-C4d and TRITC-rabbit anti-mouse IgG; biotinylated UEA was detected with FITC-streptavidin as above. C4d/UEA combined stains were done in nine C4d-positive CR, three C4d-negative CR, and two chronic CsAT cases. Combined staining for UEA and collagen type IV was done to outline the architecture of renal structures in all cases and controls. Biotinylated UEA was detected by FITC streptavidin and anti-type IV collagen was detected with TRITC-rabbit anti-mouse IgG as above.

Routine direct immunofluorescence studies were also carried out with FITC-labeled polyclonal antisera to human IgG, IgM, IgA, C3, albumin, and fibrin/fibrinogen (11).

All slides were reviewed using an Olympus BX60 (Tokyo, Japan) vertical illumination fluorescence microscope and graded by pattern and intensity of staining, without knowledge of the clinical or morphologic diagnosis.

Circulating Antidonor Antibodies

Circulating antidonor HLA class I and HLA class II antibodies were detected using either local frozen cell panels (panel-reactive antibody) or cytotoxic donor cross matching (where available) using recipient sera taken in the peribiopsy period, i.e., within 7 wk of allograft sampling. Cytotoxic donor crossmatches were performed using patient sera against donor mononuclear cells that had been stored at $-70°C$. T and B cells were isolated at the time of the cross matching using immunomagnetic beads. Both anti-human globulin enhanced T-cell and standard complement-dependent cytotoxicity B-cell assays were used (4,12,13).

Statistical Analyses

Statistical comparison between the groups was done using the Fisher exact test and t tests, where applicable.

Results
Controls

By immunofluorescence microscopy, no C4d was detectable along the PTC in any of the 15 native kidneys with ESRD or CIN, in the 10 renal allografts with nonspecific interstitial fibrosis (TxCIN), or in 20 of 21 cases of CsAT (Figure 1A,

Table 1). One case, originally diagnosed as chronic CsAT, had prominent PTC C4d, similar to that found in the CR cases described below. Unfortunately, no peribiopsy serum from this patient had been saved, so testing for antidonor antibodies was not possible. C4d was detected in most controls in the glomerular mesangium and sometimes in a linear GBM pattern, as described previously in normal kidneys (4,14). Focal deposits of C4d were seen in the arterioles and arterial intima. The tubular basement membranes (TBM) did not show staining for C4d in the renal allograft controls ($n = 31$) or native kidney biopsies ($n = 5$). The native nephrectomies, however, showed focal C4d staining along the TBM in 70% of the cases.

Routine immunofluorescence microscopy studies for immunoglobulins (IgG, IgA, IgM), C3, and albumin were negative in PTC of all of the renal allograft controls. In the chronic CsAT group, 8 of 21 cases showed nonspecific, focal fibrin staining in PTC. None of the 10 chronic interstitial fibrosis cases showed staining for fibrin in PTC. Nondiagnostic staining for IgM, C3, and some IgG were noted along the GBM or in the mesangium.

Chronic Renal Allograft Rejection

In contrast to the controls, prominent C4d deposits in PTC were detected by immunofluorescence in 23 of 38 cases (61%) that had been diagnosed as CR (Table 1). When biopsies and nephrectomies were considered separately, 57% (16 of 28) of the biopsies and 70% (7 of 10) of the nephrectomies were C4d positive ($P = $ NS). C4d staining was present diffusely in all or almost all cross sections of PTC with a bright, broad linear pattern (Figure 1B). C4d staining of the PTC was seen in both the cortex and the medulla (when both were included in the sample), with brighter and more diffuse staining in the cortex.

To identify vessels and to identify the site of C4d deposition, we double stained sections with anti-C4d and either antibody to type IV collagen (which binds to basement membranes) or UEA (which binds to endothelial cells). In sections that were double stained for C4d and type IV collagen, the C4d was localized primarily on the luminal side of the type IV collagen (Figure 2A). In contrast, in sections that were double stained for UEA and C4d, the C4d was distributed largely contiguous and external to the UEA staining of endothelium (Figure 2B). These results taken together localize C4d primarily at the interface between the endothelium and the outer basement membrane of PTC. UEA staining generally was reduced in the C4d-positive CR cases (Figure 2B) compared with the C4d-negative CR cases or other renal transplant controls. Double staining for type IV collagen and UEA also showed a qualitative reduction in intensity and number of PTC staining for UEA in the majority (80%) of C4d-positive CR cases. In contrast, the majority of C4d-negative CR cases (80%) and the controls (79%) overall had widespread, bright UEA staining along the endothelium of PTC. C4d staining was also noted along the TBM in some cases from both CR groups, *i.e.*, CR cases that were positive for C4d in PTC and those that were negative for C4d in PTC; however, no statistically significant difference was identified.

By routine immunofluorescence microscopy, no specific or distinct reactivity for immunoglobulins (IgG, IgA, IgM), C3, fibrin, or albumin were identified in the PTC of the C4d-positive or C4d-negative CR cases. In the C4d-positive CR group, one case showed focal IgM, two showed fibrin, and three showed C3 deposition in PTC. The remaining C4d-positive CR cases did not show staining for Ig, C3, fibrin, or albumin in PTC. In the C4d-negative CR group, one case had focal IgA and two showed fibrin staining in the PTC; the remaining cases were negative for Ig, C3, albumin, and fibrin. Direct immunofluorescence findings in glomeruli are not discussed in detail because there were no diagnostic findings. Nonspecific staining for IgM, C3, and some IgG were noted along the GBM or in the mesangium.

By light microscopy (Table 2), no routine histologic feature distinguished the C4d-positive CR cases from the C4d-negative CR cases: global GBM duplication (61 *versus* 50%; Figure 3A), arterial intimal fibrosis (91 *versus* 80%), arterial intimal mononuclear cell infiltration (48 *versus* 40%; Figure 3B), moderate to severe interstitial mononuclear cell infiltrate (57 *versus* 40%), presence of plasma cells (43 *versus* 33%), degree of interstitial fibrosis (69 *versus* 55% of cortex), and tubular

Table 1. C4d deposition in chronic rejection and controls[a]

	N	C4d−	C4d+	*P* Value[b]
Chronic rejection	38	15	23 (61%)	
Controls				
renal allografts				
CsAT	21	20	1 (5%)	0.00001
TxCIN	10	10	0	0.0005
native kidneys				
ESRD	10	10	0	0.0005
CIN	5	5	0	0.016
all controls	46	45	1 (2%)	<0.0001

[a] CsAT, chronic cyclosporine toxicity; TxCIN, renal transplant chronic interstitial fibrosis; ESRD, end-stage renal disease; CIN, chronic interstitial fibrosis.
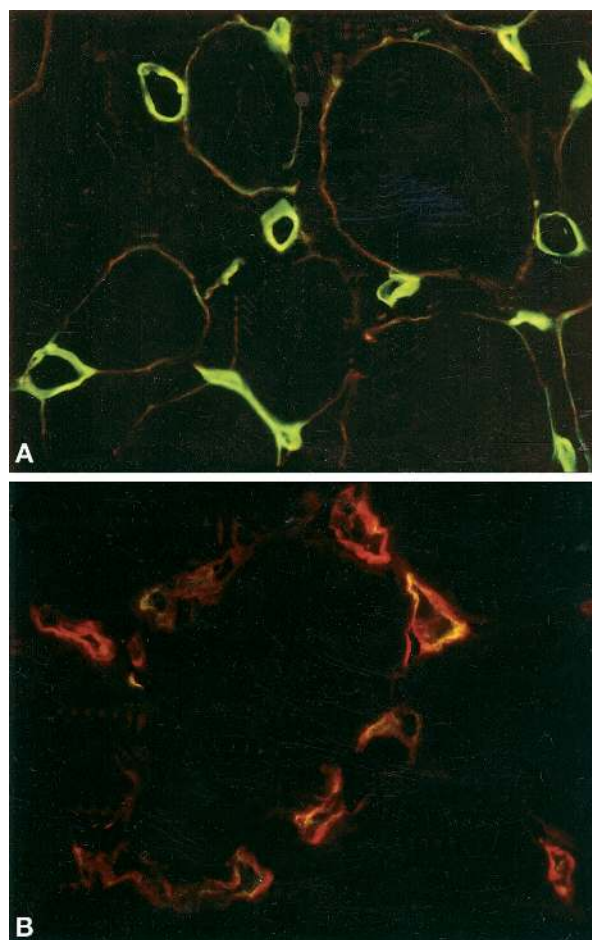[b] *Versus* chronic rejection group.
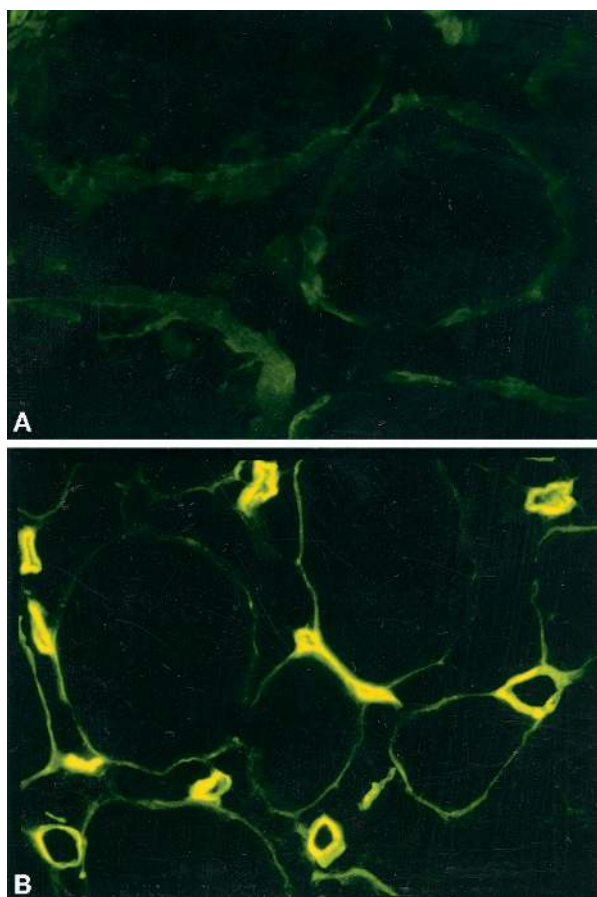
Figure 1. Single-color immunofluorescence microscopy, FITC-streptavidin–conjugated monoclonal antibody to C4d (A) Renal allograft biopsy from a control case, with chronic cyclosporine toxicity: the faint outline of tubular basement membranes can be seen. Staining for C4d is absent in peritubular capillaries (PTC). (B) Chronic humoral rejection: renal allograft biopsy from a case of chronic rejection (CR). Bright and diffuse staining for C4d is present in PTC that are scattered between the tubules. Magnification, ×300.

Figure 2. Two-color immunofluorescence microscopy in C4d-positive chronic humoral rejection. (A) renal allograft biopsy double stained for C4d (FITC, green) and type IV collagen (tetramethylrhodamine isothiocyanate [TRITC], red). The red immunofluorescence reactivity for type IV collagen outlines the tubular and peritubular capillary basement membranes. Bright green immunofluorescence staining for C4d is present along the PTC and internal to type IV collagen staining. (B) Renal allograft biopsy double stained for C4d (TRITC, red) and Ulex europaeus agglutinin I (UEA; FITC, green), an endothelial marker. Bright red staining for C4d is present along the PTC, external to the green staining for UEA. The UEA staining is significantly reduced in intensity or absent in many PTC, compatible with endothelial injury. Magnification, ×300.

atrophy (53 *versus* 48% of cortex). Each feature was more severe in the C4d-positive group, but these trends did not achieve statistical significance. The cases of chronic CsAT and nonspecific interstitial fibrosis had an absence, as expected, of the features that defined CR, either widespread GBM duplication *or* arterial intimal fibrosis with mononuclear cells.

The PTC in CR were identified easily as dilated structures in the interstitial space by light microscopy. Neutrophils were not prominent in the lumens of PTC, as they often are in acute humoral rejection (4,15), and any other features associated with acute humoral rejection were not detected (fibrinoid arterial necrosis, glomerulitis, thrombi). The PTC basement membranes were often thickened and laminated on periodic acid-Schiff stains (Figure 4), and occasional empty basement membrane lined spaces were present without obvious endothelial cells, correlating with loss of Ulex staining.

Circulating Antidonor HLA Antibodies

C4d deposits in PTC correlated with circulating antidonor HLA antibody (Table 3). Of those tested in the peribiopsy

period, 88% (15 of 17) of C4d-positive CR cases had circulating antidonor HLA antibody, whereas none of the C4d-negative CR cases had antidonor HLA antibody (0 of 8; $P = 0.0002$). Of the remaining two (12%) C4d-positive CR cases that were tested for antidonor antibodies, the cross match was negative in one; in the other, whereas increased levels of circulating antibody were identified, the specificity to donor HLA could not be determined because of a high panel-reactive antibody.

Patient Demographics, Clinical Features, and Follow-Up Data

There were no differences in patient demographics when all CR cases were compared with the renal allograft controls. To

Table 2. Pathologic features of chronic rejection cases with or without C4d deposits and allografts with chronic CsAT or nonspecific interstitial fibrosis

Feature		Chronic Rejection		Control Allografts	
		C4d$^+$	C4d$^-$	CsAT	TxCIN
	N	23	15	21	10
GBM^a duplication (%)^b					
global		61	50	0	0
segmental		39	50	–	–
none		–	–	100	100
Arterial intimal fibrosis (%)^b		91	80	57	25
Endarteritis (%)^b		48	40	0	0
Interstitial mononuclear infiltrate (%)^b					
moderate to severe		57	40	14	10
none or sparse		43	60	86	90
with plasma cells		43	33	19	10
Interstitial fibrosis (mean % cortex)		69 ± 23	55 ± 29	32 ± 16	37 ± 26
Tubular atrophy (mean % cortex)		53 ± 30	48 ± 28	27 ± 12	23 ± 15

^{a} GBM, glomerular basement membrane.
^{b} Percentage of cases with.

compare specific clinical features and outcome data (Table 4), we analyzed posttransplantation variables in all biopsied cases. The proportion of cases that had received previous transplants was higher in the C4d-negative CR group (50%) compared with C4d-positive CR cases (19%) or chronic CsAT cases (5%) and TxCIN cases (0%). The induction immunosuppression and therapy at biopsy were not significantly different between the CR and control groups and included prednisone plus azathioprine or CsA, or a combination of all three agents (data not shown). The acute rejection incidence at 6 mo after transplantation was 63% in C4d-positive CR, 66% in C4d-negative CR, 52% in the chronic CsAT group, and 30% in the TxCIN group. One case from the C4d-positive CR group had a previously documented episode of acute humoral rejection 22 d after transplantation. The mean time from transplantation to allograft biopsy was similar in the four groups. The mean creatinine and proteinuria at biopsy were higher in the C4d-positive and C4d-negative CR cases compared with the controls.

The 1-yr clinical follow-up data after biopsy of all renal allograft patients is shown in Table 4. The 1-yr postbiopsy graft survival rate was worse in the CR group compared with the transplant controls (46 *versus* 90%; $P = 0.0002$). Within the CR group, the overall 1-yr postbiopsy graft survival rate was better in the C4d-positive CR cases than in the C4d-negative CR cases (62 *versus* 25%; $P = 0.05$). This may be explained in part by the institution of a new therapeutic protocol for the C4d-positive group beginning in August 1998. At that time, C4d staining was implemented as a prospective test, and all subsequent patients, whose biopsies showed CR and C4d, were switched to tacrolimus and mycophenolate. When the survival of pre-August 1998 cases are examined separately, the 1-yr survival is the same in the C4d-positive and C4d-negative CR groups (40 *versus* 25%; $P = NS$). In contrast, the six patients since August 1998, who had C4d-positive CR and
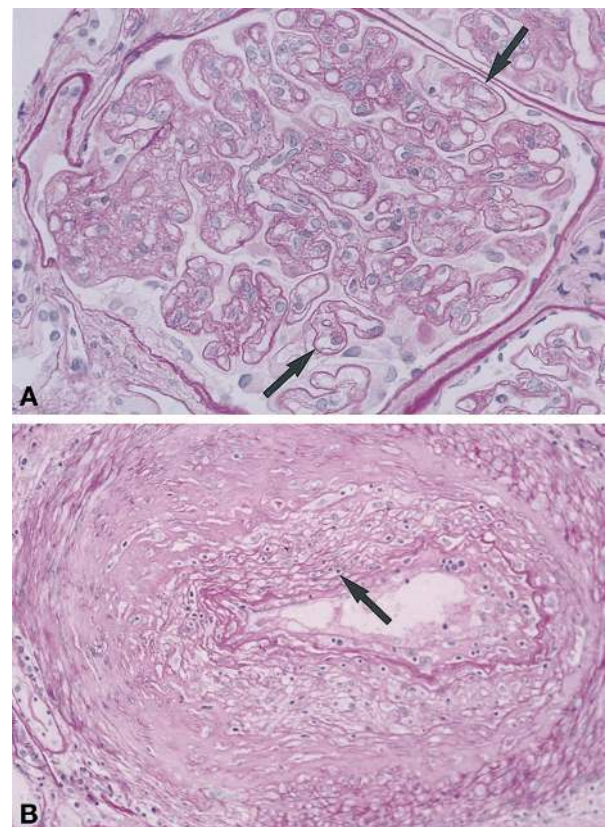
Figure 3. Light microscopic pathology of chronic rejection (CR). (A) Chronic allograft glomerulopathy, widespread duplication of the glomerular basement membrane with cellular interposition (arrow). The mesangium is expanded. (B) Chronic allograft arteriopathy, severe intimal fibroplasia with scattered mononuclear inflammatory cells (arrow). Magnifications: ×300 in A; ×150 in B (periodic acid-Schiff [PAS] stain).
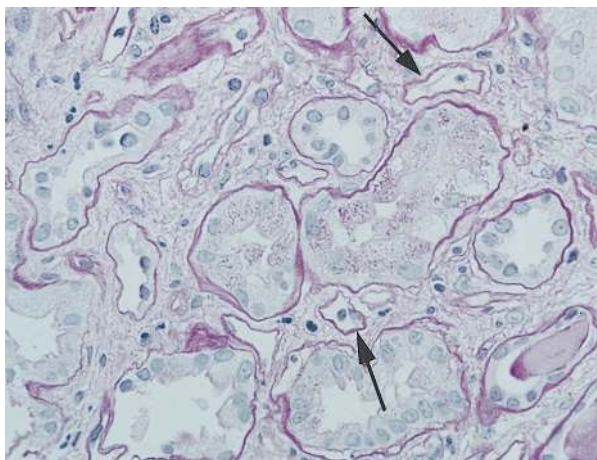
Figure 4. PTC in a case of C4d-positive chronic humoral rejection. Dilated PTC appear to have thickened and duplicated basement membranes (arrow). Magnification, ×300 (PAS stain).

five of whom were switched to mycophenolate ± tacrolimus, had a 1-yr graft survival of 100%, better than either of the CR groups before 1998 ($P < 0.03$); one patient returned to dialysis at 14 mo.

Discussion

One goal of our efforts has been to define the pathogenetic mechanisms in the heterogeneous population of patients who have chronic allograft nephropathy. We chose to analyze cases that have arterial and/or glomerular lesions traditionally regarded as being due to CR, which represented 20% of the biopsies taken for chronic allograft dysfunction. Other specific diseases identified in the biopsies for chronic dysfunction were CsAT (14%), recurrent glomerular disease (16%), and *de novo* glomerular disease (13%). The remainder (38%) had nonspecific tubular atrophy and interstitial fibrosis, and this group, if any, deserves the term *chronic allograft nephropathy* until pathogenetic mechanisms are identified.

This study demonstrated PTC C4d deposition in a major subset (61%) of CR, which was highly associated with circulating antidonor HLA antibodies. The minimal frequency of C4d positivity among all of those with chronic graft dysfunction thus can be estimated to be 12% (20% × 61%). The implication is that C4d provides direct, *in situ* evidence for an active humoral immune reaction, which has the potential to separate a substantial fraction of cases with chronic renal allograft rejection from chronic injury of other causes (*e.g.*, CsAT) and, furthermore, indicate ongoing immunologic activity. We propose that this subset be termed *chronic humoral rejection*, analogous to acute humoral rejection.

One of the first reports that antidonor HLA antibodies might be related to CR was a study by Jeannet *et al.* in 1970 (16), who showed that such antibodies detected by reactivity to donor cells were highly correlated with renal allograft loss and chronic allograft arteriopathy. Subsequent studies in renal and cardiac allograft recipients have also noted a strong correlation between circulating antidonor HLA antibodies and chronic

vascular rejection (17,18). For example, posttransplantation IgG antibody to B cells is almost six times more common in renal allograft recipients with CR (86%) than in those with stable function (15%) (19). The problem with the interpretation of these correlative data is that the antibodies may be an epiphenomenon, *e.g.*, from Th2 activation, or caused by the rejection, rather than causing the rejection. It is widely known, for example, that tissue injury can lead to an immune response, as shown by the autoantibodies that sometimes arise after myocardial infarction (20). More direct evidence of antibody participation in CR comes from allograft elution studies, in which antibodies with donor class I and class II specificities, as well as non-HLA alloreactivity, have been eluted from chronically rejected human kidneys (21–23). One of the limitations of these studies is that they are necessarily limited to the end stage of CR, when secondary pathways may obscure the primary mechanism.

The most conclusive evidence for antibody-mediated CR comes from animal studies. Antidonor class I major histocompatibility complex (MHC) antibodies are able to initiate graft arterial intimal fibrosis without participation of T cells. Immunodeficient *scid* mice given repeated doses of anti-MHC class I alloantibody develop fibrous intimal thickening of coronary arteries in cardiac allografts over 1 to 2 mo (24). T cells are sufficient to initiate vascular lesions in B-cell deficient mice, although the lesions do not progress to fibrosis in the absence of antidonor MHC antibody (25). Fibrous lesions are also markedly reduced in strain combinations that fail to elicit a humoral antibody response. Thus antibody is postulated to be a key component in the progression to the stenotic phase of chronic allograft arteriopathy. The exact mechanism of the antibody action is not known but may involve complement, not only as a lytic but also as an activating agent. Terminal complement C5b-9 proteins can elicit signals for cell proliferation by releasing growth factors from cultured human endothelial cells (26). Antibody may require cellular cytotoxic mechanisms to mediate chronic allograft arteriosclerosis, as shown in the aortic allografts into Fas ligand knockout mice, which are largely spared from intimal fibrosis despite high titers of antidonor antibody (27).

The PTC is a target of acute allograft rejection, during which endothelial cells sometimes disappear, leaving only traces of the original basement membrane (28). PTC are also a primary target in xenograft rejection, where Ig and complement are deposited (29). Although cytotoxic T cells likely can mediate endothelial injury in the PTC (30), there is strong evidence to support acute antibody/complement mechanisms as well. The presence of neutrophils in PTC is one of the hallmarks of acute rejection associated with antidonor class I antibodies (4,15). Feucht et al. (5,6) drew attention to C4d in PTC as a marker of severe acute cellular rejection and suggested that it may imply a humoral mechanism. Recently, in a detailed study, we reported that C4d is deposited consistently in PTC in acute humoral rejection but not in acute cellular rejection (4). A strong correlation with circulating antidonor HLA class I or II antibodies taken at the time of the biopsy was identified in the acute humoral rejection cases, confirming a humoral mecha-

Table 3. Correlation of C4d$^+$ chronic rejection with circulating antidonor HLA antibody

	N Tested[a]	Antidonor Antibody		P Value
		Absent	Present	
C4d$^+$	17	2	15 (88%)	0.00004
C4d$^-$	8	8	0	

[a] Peribiopsy sera, taken within 7 wk of tissue sample.

Table 4. Clinical characteristics and follow-up of biopsied patients

Characteristics		Chronic Rejection		Control Allografts	
		C4d$^+$	C4d$^-$	CsAT	TxCIN
N		16	12	21	10
Cadaveric grafts (%)		81	92	57	60
Previous transplants (%)		19	50[a]	5	0
Allograft rejection first 6 mo (%)		63	66	52	30
Time from transplant to biopsy (yr)		7.8 ± 6.4	4.9 ± 3.5	3.7 ± 3.8	5.5 ± 4.9
Age at biopsy (yr)		40 ± 12	42 ± 13	38 ± 13	41 ± 15
Creatinine at biopsy (mg/dl)		4.3 ± 2.4	3.8 ± 1.7	2.9 ± 1.0	2.9 ± 1.1
Proteinuria at biopsy (g/d)		2.0 ± 1.2[b] (10)[d]	2.2 ± 2.0[c] (9)[d]	0.7 ± 1.3 (17)[d]	0.2 ± 0.1 (6)[d]
Mean creatinine of patients with surviving graft at 1 yr after biopsy		3.7 ± 1.2	2.2 ± 1.0	2.2 ± 0.7	1.6 ± 0.5
Graft survival at 1 yr[e] after biopsy (% cases)		62	25[f]	95[g]	80
pre-August 1998 (n = 10)		40[h]			
August 1998 to 1999 (n = 6)		100[i]			

[a] $P = 0.09$ *versus* C4d$^+$ CR; $P < 0.05$ *versus* CsAT or TxCIN.
[b] P = ns *versus* C4d$^-$ CR but <0.05 *versus* CsAT or TxCIN groups.
[c] P = ns *versus* C4d$^+$ CR but <0.05 *versus* CsAT or TxCIN groups.
[d] Numbers in group with proteinuria measured in parentheses.
[e] One-yr follow-up available on all allografts biopsied.
[f] $P < 0.05$ *versus* C4d$^+$ CR.
[g] $P < 0.01$ *versus* C4d$^+$ CR or C4d$^-$ CR.
[h] P = ns *versus* C4d$^-$ CR.
[i] $P = 0.004$ *versus* C4d$^-$ CR; $P = 0.03$ versus pre-August 1998 C4d$^+$ CR cases.

nism. The reason for the susceptibility of the PTC to antibody-mediated damage is unknown, although we speculated that decreased expression of the complement inhibitory proteins might be relevant (4). Whether the C4d-positive CR seen in the present study has any relation to acute humoral rejection remains to be determined. It is notable that one case (1 of 16) had a previous biopsy with acute humoral rejection. None of the C4d-positive CR cases demonstrated the histologic features of acute humoral rejection, *e.g.*, neutrophil accumulation, suggesting that the process is muted or involves different secondary mechanisms. The titers of antidonor antibody were similar to those seen in cases of acute humoral rejection.

As was noted in previous reports of acute humoral rejection (4,5), this study also confirms the absence of Ig or C3 staining in PTC in cases of C4d-positive chronic humoral rejection. It has been postulated that humoral rejection probably results from direct antibody-mediated attack against endothelial cells. In experimental models, endothelial cells shed surface immunoglobulins very effectively within 3 to 4 d after a humoral attack (31). Thus, immunoglobulins may not be detectable in PTC at biopsy because of modulation from the surface of

activated endothelium or loss of the target cell itself. Complement split products (C4d) resist modulation by endothelial cells as a result of their covalent binding to tissue structures. Staining for C4d therefore demonstrates *in situ* humoral immune reactions that are not easily detectable otherwise.

The present data identify that the PTC endothelium is also a target of CR, as have some previous studies. The renal microvascular endothelium mostly remains of donor origin, expressing donor antigen even after 25 yr, and thus can always be a potential target of an alloresponse (32). The loss of UEA staining along the PTC in this series of CR cases supports the notion that capillary endothelial injury occurs in CR. A series of papers by Monga and colleagues demonstrated lamination of the PTC basement membrane in CR (33–35). Basement membrane lamination in other sites is regarded as a marker of injury of the overlying cell that synthesizes basement membrane as part of the recovery process (36). The severity of the lesions was associated with the presence of chronic allograft glomerulopathy (37), although there remains debate about the significance and specificity of the lamination. We recently reported that the presence of marked lamination of the PTC by electron

microscopy correlates with the presence of C4d in the PTC (38).

The specificity of the antidonor antibody was demonstrated in this study to be largely anti-HLA. However, 12% of the C4d-positive CR cases had no detectable antidonor lymphocyte antibody. It is possible that such patients may have titers of antidonor HLA antibodies that are too low to measure, as a result of absorption in the graft, or that the specificities were to non-HLA antigens, such as those expressed on the endothelium. It is believed that non-HLA antibodies can mediate acute rejection (39–41), and the same alloreactivity may be relevant to CR. In our experience, approximately 10% of acute humoral rejection cases, as judged by C4d deposition and histology, have no detectable antidonor HLA antibodies (42). Conversely, in the present study, all of the patients with detectable circulating antidonor lymphocyte antibodies did have C4d deposition, which argues that antidonor HLA antibodies, when present, are relevant and usually pathogenetic, at least at levels measurable by the antibody tests performed here. One study detected circulating antidonor antibody in 45% of patients with normal graft function, but no biopsies were performed to determine whether subclinical injury was occurring (17). Circulating antidonor HLA antibodies can precede the clinical onset of CR (17), and the possibility of subclinical episodes of humoral rejection should be considered. In protocol biopsies of allografts with stable function at 2 yr after transplantation, 50% had histologic evidence of CR (43).

In addition to its potential pathogenic significance, C4d in PTC may help distinguish CR from other nonimmune chronic injury. Our data provide argument that isolated interstitial fibrosis without glomerular or arterial lesions is rarely, if ever, due to chronic humoral rejection. In contrast, most of the cases with characteristic glomerular or arterial lesions do have evidence of antibody-mediated injury as judged by C4d deposition. The presence of C4d probably also indicates ongoing immunologic activity of the process. Perhaps this is a reason for their somewhat better prognosis. We do not know how long the C4d persists in the tissue, although in one case of acute humoral rejection, the staining intensity clearly diminished in the biopsy taken 3 wk after therapy.

Treatment and prevention of chronic renal allograft rejection still remains a challenge. Differentiation of chronic immunologic rejection from other causes of chronic allograft dysfunction is especially important on biopsies, because therapy is completely different. In chronic humoral rejection, more potent immunosuppression directed at the humoral limb of the immune system is desirable. Our preliminary results in a small group of patients with C4d-positive CR who were switched to tacrolimus and mycophenolate is encouraging and suggest that this drug combination effectively suppresses antidonor antibody production (Theruvath et al., unpublished data), as previously reported for patients with acute humoral rejection (13). Randomized controlled trials are needed to determine whether the subset of C4d-positive CR is responsive to therapy and which therapy is optimal.

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