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Acute Humoral Rejection in Kidney Transplantation: II. Morphology, Immunopathology, and Pathologic Classification

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Abstract. The incidence of acute humoral rejection (AHR) in renal allograft biopsies has been difficult to determine because widely accepted diagnostic criteria have not been established. C4d deposition in peritubular capillaries (PTC) of renal allografts has been proposed as a useful marker for AHR. This study was designed to test the relative value of C4d staining, histology, and serology in the diagnosis of AHR. Of 232 consecutive kidney transplants performed at a single institution from July 1995 to July 1999, all patients ($n = 67$) who developed acute rejection within the first 3 mo and had a renal biopsy with available frozen tissue at acute rejection onset, as well as posttransplant sera within 30 d of the biopsy, were included in this study. Hematoxylin and eosin and periodic acid-Schiff stained sections were scored for glomerular, vascular, and tubulointerstitial pathology. C4d staining of cryostat sections was done by a sensitive three-layer immunofluorescence method. Donor-specific antibodies (DSA) were detected in posttransplant recipient sera using antihuman-globulin–enhanced T cell and B cell cytotoxicity assays and/or flow cytometry. Widespread C4d staining in PTC was present in 30% (20 of 67) of all acute rejection biopsies. The initial histologic diagnoses of the $C4d^+$ acute rejection cases were as follows: AHR only, 30%; acute cellular rejection (ACR) and AHR, 45%; ACR (CCTT types 1 or 2) alone, 15%; and acute tubular injury (ATI), 10%. The distinguishing morphologic features in $C4d^+$ *versus* $C4d^-$ acute rejection cases included the following: neutrophils in PTC, 65% *versus* 9%; neutrophilic glomerulitis, 55% *versus* 4%; neutrophilic tubulitis, 55% *versus* 9%; severe ATI, 75% *versus* 9%; and fibrinoid necrosis in glomeruli, 20% *versus* 0%, or arteries, 25% *versus* 0%; all $P < 0.01$. Mononuclear cell tubulitis was more common in the $C4d^-$ group (70% *versus* 100%; $P < 0.01$). No significant difference between $C4d^+$ and $C4d^-$ acute rejection was noted for endarteritis, 25% *versus* 32%; interstitial inflammation (mean % cortex), 27.2 ± 27% *versus* 38 ± 21%; interstitial hemorrhage, 25% *versus* 15%; or infarcts, 5% *versus* 2%. DSA were present in 90% (18 of 20) of the $C4d^+$ cases compared with 2% (1 of 47) in the $C4d^-$ acute rejection cases ($P < 0.001$). The pathology of the $C4d^+$ but DSA^- cases was not distinguishable from the $C4d^+$, DSA^+ cases. The $C4d^+$ DSA^- cases may be due to non-HLA antibodies or subthreshold levels of DSA. The sensitivity of C4d staining is 95% in the diagnosis of AHR compared with the donor-specific antibody test (90%). Overall, eight grafts were lost to acute rejection in the first year, of which 75% (6 of 8) had AHR. The 1-yr graft failure rate was 27% (4 of 15) for those AHR cases with only capillary neutrophils *versus* 40% (2 of 5) for those who also had fibrinoid necrosis of arteries. In comparison, the 1-yr graft failure rates were 3% and 7%, respectively, in ACR 1 (Banff/CCTT type 1) and ACR 2 (Banff/CCTT type 2) $C4d^-$ groups. A substantial fraction (30%) of biopsy-confirmed acute rejection episodes have a component of AHR as judged by C4d staining; most (90%), but not all, have detectable DSA. AHR may be overlooked in the presence of ACR or ATI by histology or negative serology, arguing for routine C4d staining of renal allograft biopsies. Because AHR has a distinct therapy and prognosis, we propose that it should be classified separately from ACR, with further sub-classification into AHR 1 (neutrophilic capillary involvement) and AHR 2 (arterial fibrinoid necrosis).

Acute rejection of renal allografts is considered primarily a T cell–mediated process (acute cellular rejection, ACR). The role of humoral mechanisms, although well recognized in the setting of hyperacute rejection, ABO-incompatible transplants, and xenograft rejection, has received less attention in the evaluation of acute allograft rejection. In fact, diagnostic criteria for acute humoral rejection (AHR) are not well established in the current classification systems for renal allograft rejection (2,3).

Nearly 20 yr after the observation by Jeannet *et al.* (4) that posttransplant, *de novo*, donor-specific antibodies are associated with a poor outcome, this area is attracting renewed attention. Circulating cytotoxic antidonor HLA class I antibod-

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ies can be detected in about 23 to 38% of patients with ACR (5,6). These rejection episodes typically have an aggressive clinical course. Feucht *et al.* (7,8) first proposed the use of C4d staining of peritubular capillaries (PTC) in the renal allograft biopsies to identify patients with severe cellular rejection and associated C4d with pretransplant panel reactive alloantibodies. Whether C4d was correlated with donor-specific antibodies or pathology in their studies was not established. C4d is a fragment of complement component C4 released during activation of the classical complement pathway by antigen-antibody complexes (9). Because C4d contains an internal thiolester bond, it binds covalently to tissue elements at the local site of activation and is potentially, therefore, a durable marker of antibody-mediated injury.

We recently reported a series of patients selected for circulating *de novo* donor-specific antibodies and biopsy features regarded as typical of AHR, including peritubular capillary/glomerular neutrophils with or without fibrinoid necrosis (10). In all these cases we demonstrated the conspicuous deposition of C4d in PTC of renal allograft biopsies and proposed that C4d is a specific *in situ* marker of antibody-mediated rejection. In that study, the possibility of AHR in the absence of typical morphologic features or positive HLA serology was raised but was not addressed.

Although morphologic features (6,10,11,12,13) may sometimes distinguish AHR from ACR, we suspected that the diagnosis might be missed if the typical features of AHR are focal or if cellular rejection is also present. We designed the current study to test the relative value of C4d staining (immunofluorescence microscopy) as well as histology and serology (circulating *de novo* donor-specific antibodies) in the diagnosis of AHR in all patients who underwent renal allograft biopsies for suspected acute rejection. The serology and the clinical characteristics of these patients have been reported previously (14). In this report, the morphologic features of $C4d^+$ acute rejection are compared with $C4d^-$ acute rejection, and diagnostic criteria for classification of acute rejection in renal allografts are proposed.

Materials and Methods

Patients

Of 232 consecutive renal transplants performed at the Massachusetts General Hospital over a 4-yr period (July 1995 to July 1999), 81 patients suffered at least one episode of clinical acute rejection in the first 3 mo after transplantation. Clinical acute rejection was suspected in cases with acute allograft dysfunction with normal or subtherapeutic levels of cyclosporine and normal findings by renal ultrasound. Of these, all patients who had undergone a renal biopsy at onset of acute allograft dysfunction with: (*1*) available frozen tissue for immunofluorescence microscopy and (*2*) available posttransplant serum samples within 30 d of the biopsy for donor specific antibody testing were included in this study ($n = 67$). The remaining cases (14 of 81) with clinical acute rejection were excluded because nine patients had not undergone a renal allograft biopsy, four patients lacked frozen tissue for C4d staining, and one patient lacked serum to test for donor specific antibodies.

Clinical data were gathered from our patient and pathology databases and review of medical records. For outcome analysis and clinicopathologic correlation, all patient data up to July 31, 2000, were included. Serum creatinines were observed and compared between the groups at the time of biopsy, at 6 mo, and 1 yr after biopsy. The graft failure rate at 1 yr was calculated. Graft survival was compared between the $C4d^+$ and $C4d^-$ groups.

Histology

Renal allograft biopsies were processed for routine light microscopy. Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained sections of the renal allograft biopsies were examined for (*1*) ACR, using standard diagnostic criteria as outlined by the Cooperative Clinical Trials in Transplantation (CCTT) classification system and incorporated into the revised Banff criteria (2,3) and (*2*) AHR. Morphologic criteria for AHR are not established; therefore, we used provisional criteria of neutrophils in PTC and fibrinoid necrosis, as have been previously observed in AHR (10,11,12). However, we then surveyed a broad range of histologic features that might correlate with independent evidence of AHR (C4d and donor-specific antibodies) in all cases. Coded samples from the initial renal allograft biopsy with available frozen tissue that correlated with acute allograft dysfunction were scored by one of the authors (SM). The criteria used to score the cases included neutrophil counts in PTC per high power field (hpf) in ten ×40 fields (field diameter, 0.55 mm) using an Olympus BX41 microscope (Tokyo, Japan). A case was considered positive for the presence of PTC neutrophils when, on average, ≥2 neutrophils per hpf in PTC were identified in 10 consecutive ×40 hpf. Similarly, a case was considered positive for neutrophilic glomerulitis when, on average, ≥1 neutrophil per glomerulus was identified. The presence of one tubule with intraepithelial neutrophils in 10 high power fields was sufficient for neutrophilic tubulitis. The presence or absence of fibrinoid necrosis and thrombi in glomeruli and arteries, endarteritis, mononuclear cell tubulitis, tubular injury, interstitial infiltrates, interstitial hemorrhage, and cortical infarction were also recorded.

Immunofluorescence

Unfixed frozen sections of the renal allograft biopsies were stained for C4d with a sensitive, three-step immunofluorescence technique developed in our laboratory and described previously (10). Briefly, 4-μm-thick frozen sections were prewashed in phosphate-buffered saline (pH 7.2) and then incubated in 100 mg/ml Avidin D (Vector Labs, Burlingame, CA) to block endogenous biotin. Sections were washed, and excess avidin was bound 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 (1:100) (Vector Labs), and then after washing, with FITC-streptavidin (1:50) (Biomeda, Foster City, CA), each for 30 min. Sections were examined by an Olympus BX60 (Tokyo, Japan) epiillumination fluorescence microscope at ×40 and scored for C4d staining in PTC without knowledge of the clinical or pathologic diagnoses. Staining for C4d was considered positive ($C4d^+$) when the PTC were diffusely (all high-power fields) and brightly stained (excluding areas of necrosis). Focal areas and no staining of PTC for C4d were considered C4d-negative ($C4d^-$). All subsequent biopsies ($n = 11$) on these patients performed up to July 31, 2000, were evaluated for changes in the pattern of C4d staining. Routine immunofluorescence studies were also done by using polyclonal antibodies to IgG, IgM, IgA, C3, fibrin, and albumin by standard methods (15), and staining patterns in PTC were observed; these were negative in PTC of nearly all the acute rejection cases; therefore, they are not further discussed.

Screening for Circulating Donor-Specific Antibodies

Circulating donor-specific antibodies posttransplant were identified by using T and B cell cytotoxicity assays and/or flow cytometry as described previously in detail (14,16). Pretransplant donor-specific antibodies were tested in all patients by antihuman globulin cytotoxicity assay.

Statistical Analyses

Data are expressed as mean ± SD. Statistical significance was assessed by ANOVA for continuous data variables and Fischer's exact test or χ^2 test for nominal data variables. The results were considered significant with $P < 0.05$. Graft survival was estimated by the Kaplan Meier method. All statistical analyses were performed with StatView 4.5 for Windows (Abacus Concepts Inc. Berkley, CA).

Results

By immunofluorescence microscopy, 30% (20 of 67) of all acute rejection renal allograft biopsy samples revealed diffuse and bright C4d deposition in PTC (Figure 1). The initial morphologic diagnoses of the acute rejection cases in the $C4d^+$ and $C4d^-$ groups based on the histology, without knowledge of C4d staining or serology results are shown in Table 1.

The histologic features (Table 2) that distinguished $C4d^+$ acute rejection cases from $C4d^-$ acute rejection included by objective counts neutrophils in PTC and glomerular capillaries, neutrophilic tubulitis, and fibrinoid necrosis of arteries and glomeruli (Figures 1 and 2). The overall mean of the mean number of neutrophils per hpf in PTC was higher in the $C4d^+$ cases compared with the $C4d^-$ cases (4.1 ± 5.2 *versus* 1.0 ± 2.6; $P < 0.001$). More than one neutrophil per peritubular capillary was commonly seen in the $C4d^+$ cases. The overall mean of the mean number of neutrophils per glomerulus was higher in the $C4d^+$ cases compared with the $C4d^-$ cases (1.2 ± 1.0 *versus* 0.2 ± 0.4; $P < 0.001$). Usual mononuclear cell tubulitis was more common in the $C4d^-$ acute rejection cases. No statistically significant difference existed between the $C4d^+$ and $C4d^-$ acute rejection groups for endarteritis, interstitial hemorrhage, cortical infarcts, or the percent cortical involvement by an interstitial mononuclear cell infiltrate (Table 2).

C4d staining in PTC strongly correlated with the presence of posttransplant donor-specific antibodies: 90% (18 of 20) of the $C4d^+$ acute rejection cases had circulating donor-specific antibodies compared with 2% in $C4d^-$ acute rejection cases ($P < 0.001$) (Table 3). In 10% of the $C4d^+$ acute rejection cases, donor-specific antibodies were not detected, but the pathology was similar to other $C4d^+$, donor-specific antibody–positive, acute rejection cases, *e.g.*, abundant neutrophils in PTC and glomeruli. In the $C4d^-$ acute rejection group, 46 of 47 cases were negative for donor-specific antibodies; the remaining patient from this group had weak IgM donor-specific antibodies with focal, weak C4d staining of PTC. All patients had been tested pretransplantation for donor specific antibodies and were negative. Pretransplantation crossmatches were repeated retrospectively in the $C4d^+$ acute rejection cases; only two patients were shown to have weakly positive pretransplantation crossmatches, but all other patients remained negative as discussed in part I (14).

Sensitivity and specificity were calculated with either donor-specific antibodies or C4d as the criterion in diagnosing AHR. When serum donor-specific antibodies were used to define the diagnosis of AHR in these patients with acute rejection, C4d deposition in PTC by immunofluorescence microscopy achieved a sensitivity and specificity of 95% and 96%, respectively (Table 4). In comparison, the presence of neutrophils in PTC or glomeruli was less sensitive and specific for diagnosing AHR. Although the presence of arterial fibrinoid necrosis showed high sensitivity (100%) for diagnosing AHR, its absence did not exclude AHR (specificity, 75%). When C4d was used as the criterion for diagnosis of AHR, the sensitivity of donor-specific antibodies was 90% and the specificity was 98%.

Certain clinical features distinguished the $C4d^+$ and $C4d^-$ groups. The mean serum creatinines at biopsy were higher in the $C4d^+$ *versus* $C4d^-$ acute rejection cases ($P = 0.003$; Table 5). The 1-yr graft loss was 30% (6 of 20) in the $C4d^+$ acute rejection group, compared with 4% (2 of 45) in the $C4d^-$ acute rejection group ($P = 0.007$). The 1-yr graft failure rate (Table 5) was 40% (2 of 5) in those AHR cases that had arterial fibrinoid necrosis in the initial biopsy, compared with 27% (4 of 15) in the remaining AHR cases without arterial fibrinoid necrosis. In contrast, the 1-yr graft failure rate was 7% (1 of 15) and 3% (1 of 30) in ACR cases with and without endarteritis, respectively. The one patient who lost the graft in the ACR group without endarteritis had developed thrombotic microangiopathy secondary to calcineurin inhibitor toxicity subsequent to the rejection episode. In this series, no patient lost a graft due to rejection after the initial biopsy showed only ACR without endarteritis or a humoral component.

The outcome and pathology suggest classifying AHR into two categories (Table 6): those with involvement of PTC and glomerular capillaries by neutrophils (AHR type 1) and those with additional arterial fibrinoid necrosis (AHR type 2). $C4d^+$ cases with either combined AHR and ACR morphology or ACR-only morphology are best grouped with the AHR cases because their outcome correlates with the presence of C4d rather than an additional component of ACR.

The cumulative renal allograft survival was worse in the $C4d^+$ acute rejection group compared with the $C4d^-$ acute rejection group, with a trend for the worse prognosis in those $C4d^+$ AHR cases that had arterial fibrinoid necrosis (AHR 2) (Figure 3). However, the majority of the grafts in all the acute rejection groups that survived the first year after transplantation were stable and functioning at the end of a mean follow-up of 36.1 ± 15.3 mo after transplantation.

Follow-up biopsies in AHR cases ($n = 11$) showed that C4d presence and intensity correlated with persistence of circulating donor-specific antibodies. The time of disappearance of C4d staining after treatment was not systematically determined, because rebiopsies were done for graft dysfunction. Biopsies within 30 d of the initial $C4d^+$ biopsy showed persistent C4d deposition in PTC in 9 of 10 cases; one case was $C4d^-$ 17 d after the initial $C4d^+$ biopsy. Biopsies taken after

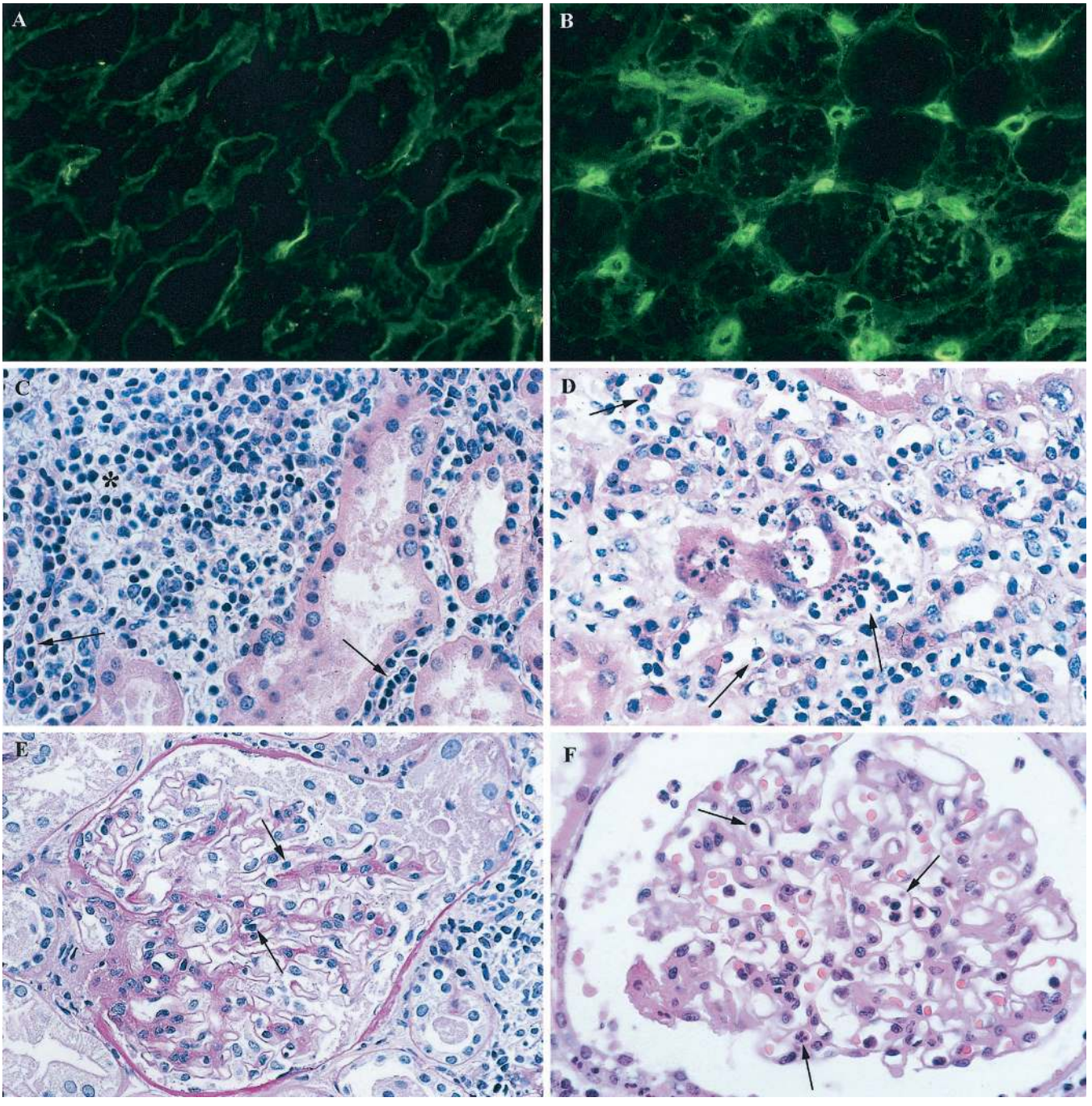

Figure 1. (A) Acute cellular rejection (ACR): no staining for C4d is seen in peritubular capillaries. (B) Acute humoral rejection (AHR): widespread and bright staining for C4d is present in the peritubular capillaries that are interspersed in between the silhouettes of tubules. (C) ACR: mononuclear cells are present in the interstitium (*) and in peritubular capillaries (arrows). (D) AHR: abundant neutrophils are present in dilated peritubular capillaries (arrows). (E) ACR: scattered mononuclear cells are present in glomerular capillaries (arrows). (F) AHR: neutrophils are present in glomerular capillaries (arrows). Staining: C4d-FITC in A and B; Hematoxylin and eosin (H&E) in C, D, and F; and periodic acid-Schiff (PAS) in E. Magnifications: ×400 in A through D; ×450 in E and F.

30 d were negative in 3 of 4 cases (at days 50, 80, 390); one biopsy at 180 d was $C4d^+$ and had chronic rejection. We conclude that C4d is not permanent and disappears within 2 to 3 wk of the loss of donor-specific antibodies. Persistence (or recurrence) of C4d staining in PTC is associated with active, chronic rejection.

Discussion

In this study, we demonstrate that a substantial fraction (30%) of all biopsy-confirmed acute renal allograft rejection episodes have a component of AHR as judged by C4d deposition in PTC. Circulating donor-specific antibodies and characteristic histopathologic morphology are also usually present

Table 1. Histologic diagnoses of $C4d^+$ and $C4d^-$ acute rejection[a]

	$C4d^+$ ($n = 20$)	$C4d^-$ ($n = 47$)
AHR	30%	0
AHR and ACR	45%	9%[b]
ACR 1	10%[c]	68%[d]
ACR 2	5%	23%
ATI	10%	0

[a] AHR, acute humoral rejection; ACR, acute cellular rejection; ACR 1, tubulointerstitial, type 1; ACR 2, with endarteritis; ATI, acute tubular injury.
[b] Patients had ACR 2 and abundant neutrophils in peritubular capillaries (≥2 neutrophils per hpf).
[c] 50% (1 of 2) were "suspicious for ACR" by Banff criteria.
[d] 34% (11 of 32) were "suspicious for ACR" by Banff criteria.

Table 2. Morphology: $C4d^+$ vs $C4d^-$ acute rejection[a]

	$C4d^+$ ($n = 20$)	$C4d^-$ ($n = 47$)	*P*
Neutrophils in			
PTC[b]	65	9	<0.0001
glomeruli[c]	55	4	<0.001
tubules[d]	55	9	0.0001
Fibrinoid necrosis			
arteries[d]	25	0[f]	0.001
glomeruli[d]	20	0	<0.006
Fibrin thrombi			
arteries[d]	0	0	
glomeruli[d]	20	0	<0.006
Endarteritis[d]	25	32	0.7 (ns)
MNC tubulitis[d]	70	100	<0.001
Acute tubular injury[d]	75	9	<0.0001
focal necrotic tubules[d]	40	2	0.0002
Interstitial inflammation[e]	27.2 ± 27	38 ± 21	0.09 (ns)
hemorrhage[d]	25	15	0.4 (ns)
Cortical infarction[d]	5	2	0.5 (ns)

[a] PTC, peritubular capillaries; MNC, mononuclear cells.
[b] Percent (%) cases with an average of ≥2 neutrophils per high-power field in peritubular capillaries in ten ×40 (field diameter, 0.55 mm) fields.
[c] Percent (%) cases with an average of ≥1 neutrophil per glomerulus.
[d] Percent (%) cases.
[e] Mean percent cortex involved.
[f] Fibrinoid necrosis in an arteriole only was seen in one case.

in these patients. However, we also demonstrated that 25% of the AHR cases would not have been recognized without the C4d stain; 15% showed only ACR morphology and 10% acute tubular injury. C4d staining of renal allografts is therefore valuable in the recognition of AHR, especially when features of ACR or only acute tubular injury are present.

The histologic features associated with C4d deposition in PTC in our cases are similar to those reported in patients with acute rejection and circulating class I cytotoxic anti-donor HLA antibodies (11). The differences include more frequent PTC neutrophils in our series (65% *versus* 46%) and fewer cases of cortical infarction and fibrin thrombi than were described in the report by Trpkov *et al* (11). Although a similar number of humoral rejection cases have arterial fibrinoid necrosis in the two series (25% and 24%), more frequent endarteritis was described in their class I antibody–negative acute rejection cases (75%) than in our series of $C4d^-$ acute rejection cases (32%). The increased frequency of AHR in our series (30% *versus* 20%) is due in part to our inclusion of cases with donor-specific antibodies reactive to class II antigens as well as the donor-specific antibody–negative cases.

In our patients, when the antigen could be identified, the alloantibodies reacted with donor HLA class I in 60%, HLA class II in 30%, and combined HLA class I and II in 10%, as previously reported (14). This is consistent with published reports indicating that circulating antibodies in AHR are most commonly to donor HLA class I antigens (5) but that some have only reactivity against HLA class II antigens (17,18). Two $C4d^+$ AHR cases (10%) in our series did not have detectable donor-specific antibodies to lymphocytes; however, their biopsies were pathologically similar to the donor-specific antibody positive, $C4d^+$, AHR cases with abundant neutrophils in PTC and glomeruli. Non-HLA antibodies, antiendothelial antibodies that have been described for example (19), might explain the $C4d^+$, donor-specific antibody–negative cases. No statistically significant correlation between any specific pathologic feature (*e.g.*, fibrinoid necrosis) and HLA reactivity of the donor-specific antibodies was detected; however, such a possibility cannot be excluded. C4d deposition in PTC of renal allograft biopsies may, therefore, have diagnostic value in the absence of demonstrable donor-specific antibodies.

In our series, two patients with widespread C4d staining of PTC showed predominantly acute tubular injury on initial biopsy. Both had circulating donor-specific antibodies. Later biopsies performed in one of these cases showed typical morphologic features of AHR with abundant neutrophils in PTC and glomeruli as well as fibrinoid necrosis. In prospective studies, we have found no (10) or focal (2) staining for C4 d in PTC in 12 cases of acute tubular necrosis (0 of 12; Mauiyyedi S, *et al.*, unpublished data); delayed graft function was present in 58% of these cases. Thus, our data differ substantially from that of Feucht *et al.* (20), who found C4d deposition in PTC of 60% of their ATN cases and 60% of recurrent glomerulonephritis. Their series also had a higher frequency of $C4d^+$ acute rejection. We believe that the differences are due in part to the inclusion of cases with "focal" C4d staining of PTC in their "positive for humoral rejection" group. Our criteria for "positive for C4d" interpretation requires widespread and bright staining of PTC, which avoids this potential pitfall. Other technical factors in the performance of the test may also be contributory (such as fixation of tissue, variable intensity of staining in immunohistochemistry, and antibody titer).

The present data permit a better definition of AHR as a pathologic entity that can be incorporated into the diagnostic

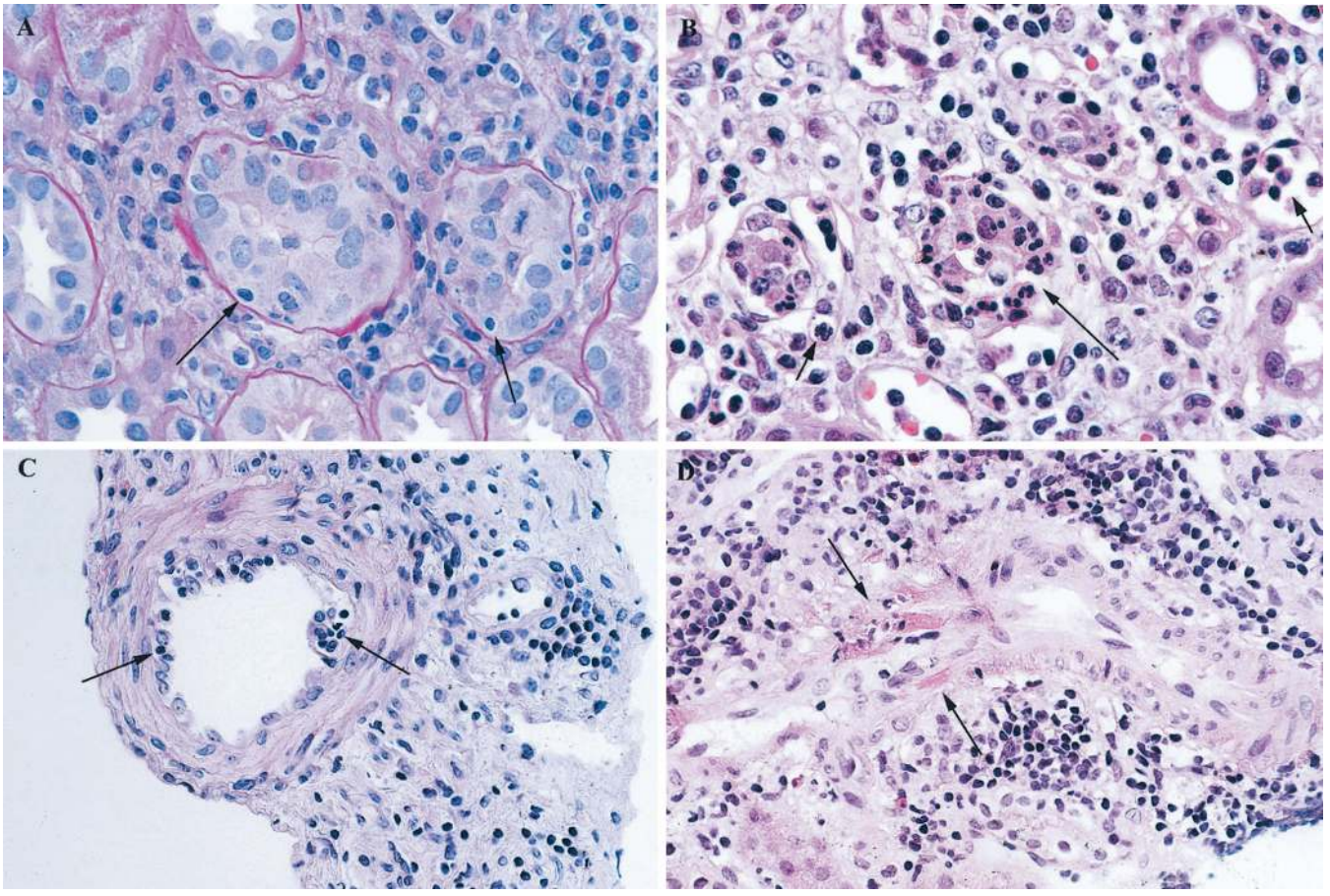

Figure 2. (A) ACR: mononuclear cell tubulitis with intraepithelial lymphocytes in tubules (arrows). (B) AHR: neutrophilic tubulitis with neutrophils invading tubular epithelium (long arrow) and neutrophils in peritubular capillaries (short arrows). (C) ACR: endarteritis with mononuclear cells underneath the endothelium of an artery (arrows). (D) AHR, fibrinoid necrosis of arterial wall (arrows). Staining: PAS in A; H&E in B through D. Magnifications: ×450 in A and B; ×400 in C and D.

Table 3. C4d deposition correlates with donor-specific antibodies in acute rejection

Acute Rejection	*n*	Donor-Specific Antibodies
$C4d^+$	20	18 (90%)
$C4d^-$	47	1 (2%)[a,b]

[a] Weak IgM anti-donor antibodies.
[b] $P < 0.0001$.

Table 4. Sensitivity and specificity of C4d in PTC, histology, and donor-specific antibodies in the diagnosis of acute humoral rejection[a]

	Sensitivity	Specificity
Serum donor-specific antibodies as criterion for AHR		
C4d in PTC	95%	96%
neutrophils in PTC	76%	86%
neutrophils in glomeruli	47%	91%
arterial fibrinoid necrosis	100%	75%
C4d in PTC as criterion for AHR		
Donor-specific antibodies in serum	90%	98%

[a] PTC, peritubular capillaries.

schema of acute rejection. Three types of acute rejection are currently recognized by the CCTT and the revised Banff classifications, based entirely on histologic criteria: type 1 is characterized by a mononuclear cell interstitial infiltrate and tubulitis, type 2 with endarteritis, and type 3 with fibrinoid arterial necrosis (2,3). Types 1 and 2 are believed to be T cell–mediated on the basis of immunophenotype, experimental studies, and response to anti-T cell agents, *e.g.* OKT3 (21,22,23). Animal studies, including T cell transfer experiments, and the ability of B cell knockout mice to develop typical endarteritis in allografts support this hypothesis (22). In contrast, fibrinoid necrosis of arteries, which is highly associated with circulating anti-donor antibodies in this and previous studies (11), typically does not respond to anti-T cell therapy and has a substantially worse prognosis (21). In the past this was the only form of AHR widely recognized (24). In addition to this category, we now delineate another morphologic type of acute rejection that is humorally mediated and is centered on graft peritubular and glomerular capillaries.

Table 5. Clinical follow-up after biopsy diagnosis of acute rejection[a]

Variable	C4d$^-$		C4d$^+$	
	Without Endarteritis	With Endarteritis	No Arterial Fibrinoid Necrosis	With Arterial Fibrinoid Necrosis
n	32[b]	15	15	5
Post Tx day of bx	14 ± 9.3	15 ± 14.2	12.6 ± 7.4	20.2 ± 16.3
Bx Cr (mg/dl)	3.0 ± 1.0	3.9 ± 3.5	7.0 ± 4.5	5.8 ± 3.3
6m Cr[c] (mg/dl)	1.6 ± 0.6	1.2 ± 0.3	1.8 ± 0.6	1.5 ± 0.3
12m Cr[c] (mg/dl)	1.4 ± 0.4	1.2 ± 0.2	1.5 ± 0.4	1.6 ± 0.4
GF at 1 yr (% cases)	3	7	27	40

[a] C4d$^-$, acute cellular rejection; C4d$^+$, acute humoral rejection; Tx, renal transplant; Bx, at renal allograft biopsy; Cr, mean creatinine; 6m, at 6 months after biopsy; 12m, at 12 months after biopsy; GF, graft failure at 1 year after transplantation.
[b] One patient died with functioning graft, and one patient was lost to follow-up <1 mo after renal transplantation.
[c] Excluding failed grafts.

Because AHR has a distinctive pathogenetic mechanism (antibody/complement), comprises about 30% of the acute rejection biopsies, and has a substantially worse prognosis, we believe that AHR deserves to be classified separately. Our observations suggest that the diagnostic categories can be grouped by pathogenesis and prognosis. The proposed classification (Table 6) retains the morphologic criteria used by the revised Banff/CCTT for ACR types 1 and 2 and moves the former type 3 acute rejection to the more appropriate and specific AHR group. Thus, two forms of AHR can be recognized histologically: type 1 AHR with capillary inflammation, *i.e.*, neutrophils in peritubular and glomerular capillaries, and type 2 AHR with arterial fibrinoid necrosis. The proposed classification adds immunophenotypic criteria to the above, with ACR being C4d$^-$ and AHR being C4d$^+$. One might propose a separate category for C4d$^+$, donor-specific antibody–positive cases with acute tubular necrosis, but in our experience to date, this has been a transient precursor to typical AHR, and we include these in type 1 AHR. The frequency and outcome of the biopsies so classified in this study are given in Table 6. The presence of C4d in PTC clearly alters the prognosis of what may otherwise appear to be ordinary ACR. We believe therefore that the subset of the C4d$^+$ acute rejection biopsies that show morphologic features of both ACR as well as AHR should be classified as AHR because their prognosis resembles "pure" AHR rather than "pure" ACR.

Table 6. Classification, frequency, and outcome of acute renal allograft rejection

Classification	*n*	Frequency[a]	Graft Loss at 1 yr
Acute cellular rejection[b]			
type 1 (tubulointerstitial)	32	48%	3% (1 of 30)[c]
type 2 (endarteritis)	15	22%	7% (1 of 15)
Acute humoral rejection[d]			
type 1 (capillary)[e]	15	22%	27% (4 of 15)
type 2 (arterial fibrinoid necrosis)	5	7%	40% (2 of 5)

[a] Frequency in patients with biopsies of first rejection episode. In the present series, overall 31% (72 of 232) of patients had an episode of acute rejection diagnosed on biopsy.
[b] C4d negative in peritubular capillaries.
[c] One patient died with functioning graft, and one patient was lost to follow up <1 mo after renal transplantation.
[d] Includes patients with combined humoral and cellular rejection.
[e] Includes two patients whose initial biopsy showed acute tubular injury.

The criteria for AHR we proposed previously were acute graft dysfunction, C4d in PTC, and circulating donor-specific antibodies (14). These criteria identified 90% of the AHR cases but failed in 10%, as discussed above. We did not use histologic criteria then because no one feature could be ascribed to AHR consistently. However, for a purely pathologic classification, relevant morphologic features from the renal biopsy are essential even if limited. We now propose that combining histologic and immunopathologic data gives the most sensitive and specific diagnosis of AHR according to the pathologic criteria in Table 7. Because of the confusing data in the literature that C4d deposition (at least focally) can occur in ischemic injury (20), we recommend that, for now at least, the definitive diagnosis of AHR requires the demonstration of circulating donor-specific antibodies, even though this is not as sensitive as C4d in our series. If only two of the three criteria are met, then the diagnosis would be considered "suspicious for AHR." The threshold number of neutrophils needed to diagnose AHR on the basis of morphology has not been analyzed in detail. For the purposes of this article and the diagnostic criteria, we used an average of ≥2 neutrophils per high-power field in PTC in 10 consecutive high-power fields and an average of ≥1 neutrophil per glomerulus to diagnose AHR. It is, however, clear that, even though on average the mean neutrophils per hpf in PTC is increased in AHR, there is some overlap. Fibrinoid necrosis should be in an artery as an indicator of AHR, because arteriolar fibrinoid necrosis can be seen in other processes such as thrombotic microangiopathy and severe hypertension. The histologic criteria of acute in-

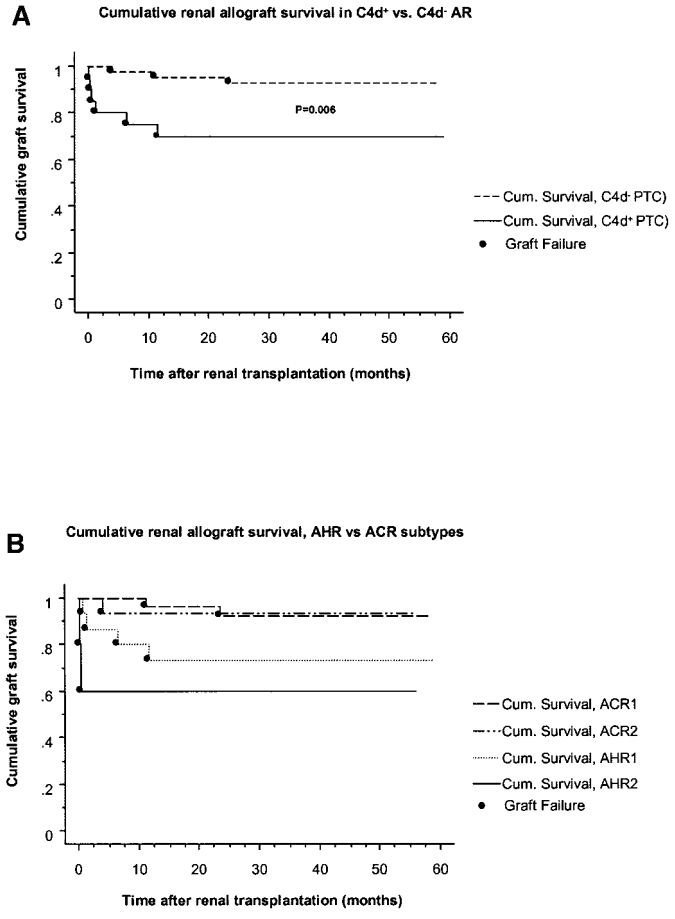

Figure 3. Cumulative renal allograft survival estimated in $C4d^+$ and $C4d^-$ acute rejection groups diagnosed within 3 mo of transplantation (Kaplan Meier method). (A) Worse renal allograft survival in the $C4d^+$ group compared with $C4d^-$ group (●, graft failure at mo after transplantation; $P = 0.006$). (B) Cumulative renal allograft survival of AHR and ACR subtypes reveal a trend for the worse survival in the AHR 2 group (●, graft failure at mo after transplantation; ACR *versus* AHR, $P = 0.006$; ACR 1 *versus* ACR 2, $P = 0.9$; AHR 1 *versus* AHR 2, $P = 0.4$).

flammation or injury separate acute from chronic humoral rejection because the latter lacks these features (25).

Several special caveats of C4d staining should be emphasized. First, only PTC staining should be considered, because C4d in normal kidneys can always be found in glomeruli and often in arterial intima and the tubular basement membranes. In contrast, PTC uniformly lack C4d. Second, infarcted tissue can be negative for C4d despite positive staining for C4d in viable areas. Third, PTC neutrophils or arterial fibrinoid necrosis can rarely occur with absent or only focal staining of PTC for C4d; such cases should probably be classified as suspicious for AHR. For example, weak C4d staining was identified in one of our cases that had PTC neutrophils in the initial biopsy and arterial fibrinoid necrosis in a subsequent biopsy, with weak IgM donor-specific antibodies in serum of undetermined specificity. The nature of such findings remains unknown, but it may represent AHR mediated by antibodies to antigens predominantly expressed in arteries. The presence of PTC neutrophils alone as a diagnostic criteria will lead to overcalling AHR, as seen in 9% of our $C4d^-$ ACR cases.

Table 7. Pathologic criteria for acute humoral rejection[a]

1. C4d deposition in peritubular capillaries[b]
2. At least one of the following:[c]
   a. Neutrophils in peritubular capillaries
   b. Arterial fibrinoid necrosis
   c. Acute tubular injury
3. Circulating donor specific antibodies

[a] Cases that also meet the criteria of type 1 or 2 acute cellular rejection (Banff/CCTT) are considered to have both processes.
[b] Bright and diffusely positive staining for C4d in peritubular capillaries.
[c] Neutrophils in peritubular capillaries: on average ≥2 neutrophils per high power field in peritubular capillaries in 10 consecutive ×40 (field diameter, 0.55 mm) fields; fibrinoid necrosis in an artery: larger than an arteriole; acute tubular injury: loss of brush borders, flattened epithelium, apoptosis.
If only two of the three numbered criteria are present, the term "suspicious for AHR" is recommended (for example, when donor specific antibodies are not tested).

In our series of patients, the overall graft loss at 1 yr is 4% for ACR and 30% for AHR. These data are quite compatible with that of Halloran *et al.* (6), who reported 3.9% graft loss after a rejection episode without anti-class I antibodies and 38.5% after rejection associated with class I antibodies. It is notable that 75% (6 of 8) of the overall 1-yr graft losses in our patients are in the AHR group. Renal allograft function after successful treatment of AHR was similar to that observed in the ACR cases, confirming the observation of Halloran's group that those with rejection associated with anti-class I antibodies who recover have a similar prognosis as those without antibodies (6). The relatively better 1-yr graft survival rate (70%) in our AHR group, compared with others that have reported poor graft survival rates of about 16 to 50% (11,26), may be due in part to our treatment approach with plasmapheresis, tacrolimus, and mycophenolate mofetil (13,14). Chronic rejection is associated with donor-specific antibodies, and we have detected C4d and donor-specific antibodies in patients with morphologic evidence of chronic rejection (25). Whether AHR promotes chronic rejection is unknown. Further studies are warranted to answer this question.

In summary, the present pathologic data support the recognition of a distinct and common type of acute renal allograft rejection mediated by specific anti-donor antibodies that react with graft endothelium leading to the deposition of complement, notably C4d. This form of rejection, termed "acute humoral rejection," has a typical but variable morphology and a distinctly worse prognosis compared with T cell–mediated rejection. Incorporation of AHR as a diagnostic category, including its two variants (capillary and arterial), should be important in clinical management and the analysis of clinical trials.

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References

1. Mauiyyedi S, Crespo M, Pascual M, Collins AB, Saidman S, Rubin N, Williams W, Cosimi AB, Schneeberger E, Colvin RB: C4d deposition in peritubular capillaries. The morphology and immunophenotype of acute humoral rejection. *Transplantation* 69: S402, 2000
2. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Yamaguchi Y: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55: 713–723, 1999
3. Colvin RB, Cohen AH, Saiontz C, Bonsib S, Buick M, Burke B, Carter S, Cavallo T, Haas M, Lindblad A, Manivel JC, Nast CC, Salomon D, Weaver C, Weiss M: Evaluation of pathologic criteria for acute renal allograft rejection: reproducibility, sensitivity, and clinical correlation. *J Am Soc Nephrol* 8: 1930–1941, 1997
4. Jeannet M, Pinn VW, Flax MH, Winn HJ, Russell PS: Humoral antibodies in renal allotransplantation in man. *N Engl J Med* 282: 111–117, 1970
5. Halloran PF, Wadgymar A, Ritchie S, Falk J, Solez K, Srinivasa NS: The significance of the anti-class I antibody response. I. Clinical and pathologic features of anti-class I-mediated rejection. *Transplantation* 49: 85–91, 1990
6. Halloran PF, Schlaut J, Solez K, Srinivasa NS: The significance of the anti-class I antibody response. II. Clinical and pathologic features of renal transplants with anti-class I-like antibody. *Transplantation* 53: 550–555, 1992
7. Feucht HE, Felber E, Gokel MJ, Hillebrand G, Nattermann U, Brockmeyer C, Held E, Riethmuller G, Land W, Albert E: Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. *Clin Exp Immunol* 86: 464–470, 1991
8. Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Riethmuller G, Land W, Albert E: Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int* 43: 1333–1338, 1993
9. Chakravarti DN, Campbell RD, Porter RR: The chemical structure of the C4d fragment of the human complement component C4. *Molecular Immunology* 24: 1187–1197, 1987
10. Collins AB, Schneeberger EE, Pascual M, Saidman SL, Williams WW, Tolkoff-Rubin N, Cosimi AB Colvin RB: Deposition of C4d in peritubular capillaries is a marker of acute humoral renal allograft rejection. *J Am Soc Nephrol* 10: 2208–2214, 1999
11. Trpkov K, Campbell P, Pazderka F, Cockfield S, Solez K, Halloran PF: Pathologic features of acute renal allograft rejection associated with donor-specific antibody. Analysis using the Banff grading schema. *Transplantation* 61: 1586–1592, 1996
12. Colvin RB: Renal transplant pathology. In: *Heptinstall's Pathology of the Kidney*, edited by Jennette JC, Olson JL, Schwartz MN, Silva FG, Philadelphia, Lippincott-Raven, 1998, pp 1409–1540
13. Pascual M, Saidman S, Tolkoff-Rubin N, Williams WW, Mauiyyedi S, Duan JM, Farrell ML, Colvin RB, Cosimi AB, Delmonico FL: Plasma exchange and tacrolimus-mycophenolate rescue for acute humoral rejection in kidney transplantation. *Transplantation* 66: 1460–1464, 1998
14. Crespo M, Pascual M, Tolkoff-Rubin N, Mauiyyedi S, Collins AB, Fitzpatrick D, Farrell ML, Williams WW, Delmonico FL, Cosimi AB, Colvin RB, Saidman SL: Acute humoral rejection in renal allograft recipients: I. Incidence, serology and clinical characteristics. *Transplantation* 71: 652–658, 2001
15. Collins AB: Immunofluorescence. In: *Diagnostic Immunopathology*, edited by Colvin RB, Bhan AK, McCluskey RT, New York, Raven Press, 1995, pp 699–710
16. Fuller TC, Phelan D, Gebel HM, Rodey GE: Antigenic specificity of antibody reactive in the antiglobulin-augmented lymphocytotoxicity test. *Transplantation* 34: 24–29, 1982
17. Scornik JC, Bray RA, Pollack MS, Cook DJ, Marrari M, Duquesnoy R, Langley JW; Multicenter evaluation of the flow cytometry T-cell crossmatch: Results from the American Society of Histocompatibility and Immunogenetics-College of American Pathologists proficiency testing program. *Transplantation* 63: 1440–1445, 1997
18. Russ GR, Nicholls C, Sheldon A, Hay A: Positive B cell crossmatch and glomerular rejection in renal transplant recipients. *Transplant Proc* 19:785–788 1987
19. Yard B, Spruyt-Gerritse M, Class F, Thorogood J, Bruijn JA, Paape ME, Stein SY, van Es LA, van Bockel JH, Kooymans-Coutinho M: The clinical significance of allospecific antibodies against endothelial cells detected with an antibody-dependent cellular cytotoxicity assay for vascular rejection and graft loss after renal transplantation. *Transplantation* 55: 1287–1293, 1993
20. Lederer SR, Kluth-Pepper B, Schneeberger H, Albert E, Land W, Feucht HE: Impact of humoral alloreactivity early after transplantation on the long term survival of renal allografts. *Kidney Int* 59: 334–341, 2001
21. Nickeleit V, Vamvakas EC, Pascual M, Poletti BJ, Colvin RB: The prognostic significance of specific arterial lesions in acute renal allograft rejection. *J Am Soc Nephrol* 9: 1301–1308, 1998
22. Russell PS, Chase CM, Colvin RB: Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: Lack of progression to sclerotic lesions in B cell-deficient mice. *Transplant* 64:1531–1536, 1997
23. Schroeder TJ, Weiss MA, Smith RD, Stephens GW, First MR: The efficacy of OKT3 in vascular rejection. *Transplantation* 51: 312–5, 1991
24. Busch GJ, Reynolds ES, Galvanek EG, Braun WE, Dammin GJ: Human renal allografts. The role of vascular injury in early graft failure. *Medicine* 50: 29–83, 1971
25. Mauiyyedi S, Della Pelle P, Saidman S, Collins AB, Pascual M, Tolkoff-Rubin N, Williams WW, AB. Cosimi, Schneeberger EE, Colvin RB: Chronic humoral rejection: Identification of antibody mediated chronic renal allograft rejection by c4d deposits in peritubular capillaries. *J Am Soc Nephrol* 12: 574–582, 2001
26. Lobo PI, Spencer CE, Stevenson WC, Pruett TL: Evidence demonstrating poor kidney graft survival when acute rejections are associated with IgG donor specific lymphocytotoxin. *Transplantation* 59: 357–360, 1995