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Detection of the Complement Degradation Product C4d in Renal Allografts: Diagnostic and Therapeutic Implications

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Abstract. The immunohistochemical detection of the complement degradation product C4d, a component of the classical complement pathway, offers a new and currently poorly defined tool in the evaluation of renal allograft biopsies. Our retrospective study aims at determining the diagnostic and clinical significance of C4d accumulation in kidney transplants, employing immunofluorescence microscopy. We analyzed 398 diagnostic allograft biopsies ($n = 265$ patients with 1 to 5 biopsies obtained 7 to 7165 d posttransplantation [tx]) and correlated the detection of C4d with 18 histologic changes, panel-reactive antibody titers, response to treatment, and outcome. One hundred twenty-five native kidney and baseline tx biopsies served as controls. Linear deposition of C4d along peritubular capillaries was only found in a subgroup (30%) of allografts post-tx, mainly during the early time-course (median, 38 d post-tx; range, 7 to 5646 d). There was no significant association with infections. C4d staining could change from negative to positive and *vice versa* within days to weeks. The accumulation of C4d was most tightly linked to a morphologic subtype of rejection, transplant glomerulitis ($P < 0.0001$). In addition, tubular MHC class II expression was correlated with C4d deposition ($P < 0.0001$). Both features are signs of "acute active rejection." In comparison with C4d-negative controls, 43% of C4d-positive patients showed increased (>10%) panel-reactive antibody titers (*versus* 19% in the negative group; $P = 0.001$). C4d positivity was frequently associated with higher serum creatinine levels at time of biopsy (compared with C4d-negative group; $P < 0.01$). More C4d-positive patients were treated with polyclonal antithymocyte globulins (ATG) or monoclonal anti-CD_3 antibodies (OKT3) ($P < 0.0001$). Outcome did not significantly differ between C4d-positive and C4d-negative groups. In conclusion, the detection of C4d identifies a humoral alloresponse in a subgroup of kidney transplants, which is often associated with signs of cellular rejection, *i.e.* tx glomerulitis. Allograft dysfunction in C4d-positive rejection episodes is often more pronounced. We provide first evidence that C4d-positive rejection might benefit from intensive therapy, potentially preventing the previously reported high graft failure rate. In addition, we show that a subgroup of C4d-positive cases may not require any immediate therapeutic intervention. The presence of C4d is clinically relevant and should be reported in the histologic diagnosis.

The criterion for establishing a diagnosis of acute renal allograft rejection is the histologic evaluation of a graft biopsy (1). Acute rejection is morphologically characterized by the presence of mononuclear cells in the interstitial compartment, tubulitis, transplant endarteritis, or glomerulitis. The cellular inflammatory component is the most striking feature; therefore, acute rejection is sometimes also referred to as acute cellular rejection (2). However, a humoral response to various donor antigens is serologically well documented, the clinical significance of which is only incompletely understood (3,4). The lack of detectable immunoglobulins or activated complement factors in renal allograft biopsies during acute cellular rejection episodes might in part be explained by their rapid shedding from endothelial cell surfaces. Consequently, in the histologic evaluation of kidney biopsies, humoral components during acute rejection episodes are most likely underestimated (5,6).

A new perspective opened up when Feucht *et al.* (3,7–10) pioneered work on C4d, a complement split product that can be detected in renal allografts. C4d is a degradation product of the complement factor C4, which is typically activated during the classical complement cascade. The classical pathway of complement activation is characteristically initiated by conformational changes in Ig molecules after binding to specific antigens (11,12). After cleavage of most C4 domains during activation and breakdown, only the alpha 2 domain (representing the split product C4d) remains as the most long-lived portion of the C4 molecule near the site of C4 activation. The alpha 2 domain covalently binds to endothelial surfaces and basement membranes via a proteolytically exposed thioester group, making it a stable molecule (in contrast to other cleaved domains) (3). The alpha 2 domain can easily be detected by immunohistochemistry (7–9). Thus, the accumulation of C4d can be regarded as a footprint of a humoral response (3,6,13). Its detection offers

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a new and challenging tool in the evaluation of transplant biopsies.

To date, only few studies have investigated the deposition of C4d in kidney grafts (9,10,14–16). Feucht *et al.* (9) reported that the accumulation of C4d was associated with cellular rejection, circulating donor alloantibodies, and poor 1-yr graft survival. Collins *et al.* (14) found similar results; however, they could not establish an association between C4d deposition and cellular rejection. The interpretation of these studies is, however, limited by small case numbers or a highly selected patient population. To better understand the significance of C4d accumulation in renal allografts, we report histologic and clinical data from 398 graft biopsies not selected for a specific type of allograft dysfunction. Results were compared with 125 controls. Emphasis was placed on histology as well as outcome postbiopsy.

Materials and Methods

Patients and Biopsies

Our retrospective analysis included 265 kidney allograft recipients (157 men, 108 women) who were transplanted and clinically managed at the University Hospital in Basel between 1988 and 1996. One hundred eighty allografts were of cadaveric origin and 85 from living donors (235 first, 25 second, and 5 third transplants). Renal allograft biopsies were performed due to unexplained deterioration of graft function, with a median of 133 d posttransplantation (range, 7 to 7165 d). A total of 398 graft biopsies (232 first, 103 second, 44 third, 16 fourth, and 3 fifth biopsies) were examined.

In general, two needle biopsy cores were obtained for morphologic work-up. Cores were divided under a dissecting microscope into two parts: one for formalin fixation, and the second one for quick-freezing in optimal cutting temperature (OCT) embedding medium (Miles Laboratories, Elkhart, IN). Formalin-fixed tissue sections were routinely stained with hematoxylin and eosin, periodic acid Schiff, trichrome, elastic van Gieson, and methenamine silver. Fresh frozen tissue was analyzed by immunofluorescence microscopy (IF) using a conventional panel of antibodies directed against IgG, IgM, IgA, C3c, C4c, C1q, fibrinogen (all rabbit anti-human FITC-conjugated polyclonal antibodies; DAKO A/S, Glostrup, Denmark), and a mouse anti-human monoclonal antibody against C5b-9 (Quidel, San Diego, CA). MHC class II expression of tubular epithelial cells was evaluated by direct IF using an FITC-conjugated mouse anti-human monoclonal antibody. The antibody reacts with the β-chain of the gene subregions, DP, DQ, and DR (DAKO A/S; dilution, 1:4 in PBS; 30-min incubation at room temperature). The deposition of C4d was detected by employing the indirect immunofluorescence technique with a primary affinity-purified monoclonal antibody (mouse anti-human; dilution, 1:50; 30-min incubation at room temperature; Quidel San Diego, CA) and an FITC-labeled affinity-purified secondary goat anti-mouse IgG antibody (dilution, 1:40; 30-min incubation at room temperature; Jackson ImmunoResearch Laboratories, West Grove, PA). The C4d antibody binds to an antigen that is expressed in the alpha 2 domain of native C4 and C4d (information provided by the manufacturer). Staining was performed according to standard procedures. Biopsies were reviewed by 2 renal pathologists (VN, MJM), and the findings were scored (Table 1). All biopsies met the Banff criteria for adequacy (17), with 16 glomeruli (median value) available for light microscopy evaluation.

Scoring of Histologic Changes

Eighteen histologic features were analyzed and scored as either present or absent (Table 1). Lesions were defined according to previous reports (18–21). Interstitial cellular rejection (ICR) was classified according to the criteria set by the Cooperative Clinical Trials in Transplantation (CCTT) protocol (19).

Scoring of C4d

C4d was typically detected along peritubular capillaries with a strong linear staining pattern. It was scored as negative, focally positive ($\leq$50% cortical involvement), or diffusely positive ($>$50% cortical involvement). C4d could also be seen with varying degrees of staining intensity in the mesangial areas of most glomeruli. Glomerular staining served as internal quality control; it was not used for scoring.

Control Tissue

One hundred thirteen diagnostic native kidney biopsies and 12 renal graft biopsies taken at the time of transplantation (0-h baseline biopsies) were used as control tissue samples. The 0-h biopsies were obtained after the vascular anastomoses had been completed (postreperfusion).

Clinical Data

The following clinical data were analyzed: (*1*) antirejection therapy subsequent to a biopsy (pulse steroids, antilymphocytic therapy: monoclonal anti-CD_3 antibodies [OKT3], polyclonal antithymocyte

Table 1. Analyzed histologic features (scored as either present or absent)

Vascular features
transplant endarteritis
fibrinoid vascular necrosis
sclerosing transplant vasculopathy (intimal fibrosis with myofibroblastic hyperplasia)
toxic arteriolopathy due to cyclosporin A/tacrolimus
thrombotic microangiopathy
arteriolosclerosis
Glomerular features
transplant glomerulitis
transplant glomerulopathy
glomerulonephritis (membranous, IgA, focal segmental glomerulosclerosis, others)
Tubular features
interstitial cellular rejection and tubulitis
tubular expression of MHC class II (evaluated by IF [22])
toxic tubular injury due to cyclosporin A/tacrolimus (epithelial vacuolization)
ischemic tubular injury (acute tubular injury)
Others
striped interstitial fibrosis
diffuse interstitial fibrosis
pyelonephritis
cytomegalovirus renal graft infection (with viral inclusion bodies)
BK virus nephropathy

globulins [ATG]); (*2*) renal function based on serum creatinine levels at time of biopsy and 1, 3, 6, and 12 mo postbiopsy; (*3*) graft failure defined as return to hemodialysis or transplant nephrectomy (up to 3 yr postbiopsy); (*4*) clinical evidence of infections within 2 wk before biopsy (cytomegalovirus, varicella-zoster virus, sepsis, bacterial infections); and (*5*) induction therapy with antilymphocytic preparations immediately after transplantation.

Detection of Panel-Reactive Circulating Antibodies (PRA)

The presence of cytotoxic antibodies in allograft recipients was determined by cytotoxic crossmatch testing according to the rules of Eurotransplant by using a phenotyped panel of 50 different leukocyte samples. Recipients were tested before transplantation and on a regular bimonthly basis up to 12 wk posttransplantation. PRA tests were later limited to individual cases. Patients revealing cell lysis in $\geq$10% of the leukocyte test samples (*i.e.*, panel reactivity of $\geq$10%) were defined as antibody-positive, and those with panel reactivity of $<$10% as antibody-negative. Only PRA tests performed within 2 wk before a corresponding biopsy were considered for further analysis.

Statistical Analyses

The χ^2 test was used for the comparison of frequencies in 2×2 tables (such as histologic changes), and stepwise multiple regression was used for multivariate analyses. The Mann-Whitney *U* test was used for comparing serum creatinine levels between groups.

The statistical analyses were either biopsy-based or patient-based. To compare patient-based outcome between groups, patients were classified as C4d-positive if at least one biopsy revealed accumulation of C4d. The first C4d-positive biopsy was defined as the index biopsy and was used as a reference point for further statistical analyses. C4d-negative patients per definition did not show C4d deposition in any of their biopsies. In this group, the first available biopsy served as the index biopsy. Graft survival was calculated by using the method of Kaplan-Meier. Differences in graft survival were evaluated by using the Peto and Peto generalized Wilcoxon test.

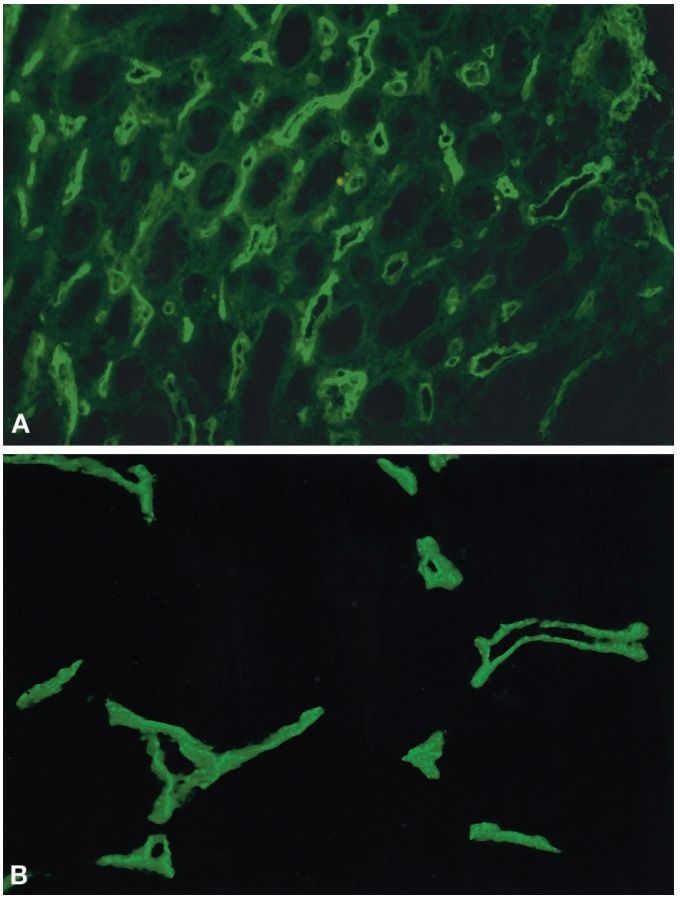

Figure 1. (A) Accumulation of C4d along peritubular capillaries (green, diffuse positivity). Tubules are not staining. (B) Higher magnification illustrating the specific deposition of C4d along capillaries (green). No accumulation is found along tubular basement membranes or in tubular cells. Indirect immunofluorescence microscopy employing a C4d-specific primary antibody. Magnifications: $\times$100 in A; $\times$251 in B.

Results

Detection and Prevalence of C4d in Biopsies/Patients

Characteristic C4d staining was detected in a linear fashion along peritubular capillaries, typically involving an entire capillary circumference (Figure 1). As a rule, many adjacent peritubular capillaries revealed C4d deposits, even in cases of focal staining (always $>$10 capillaries). Staining of only very few scattered capillaries was occasionally seen in areas of fibrosis and considered to be nonspecific. The detection of C4d along peritubular capillaries was not accompanied by any deposition of immunoglobulins or other complement factors. C4d was not found in tubular epithelial cells or normal tubular basement membranes, which only stained for C4d in areas of atrophy and basement membrane thickening. Arteries did not show intimal C4d deposits. Arterioles with hyalinosis revealed C4d accumulation accompanied by IgM and complement factor C3 deposits. Such arteriolar staining profile was considered to be nonspecific.

The overall prevalence of C4d deposition along peritubular capillaries in all renal allograft biopsies was 30% (120 of 398). Twelve percent (48 of 398) of biopsies revealed focal and 18% (72 of 398) diffuse positivity. Further statistical analyses did not reveal differences between focal and diffuse C4d accumulation with regard to associated histologic changes or graft function; therefore, all focal cases were referred to as positive (similar to the approach used by the authors of reference 10). The prevalence of C4d accumulation varied slightly in biopsies from different graft subgroups (cadaveric origin, 29%; living donor origin, 33%; first transplants, 29%; second/third transplants, 38%; differences NS). In general, C4d was detected in biopsies taken during the early time-course posttransplantation; however, it was also seen more than 15 yr after grafting (median, 38 d; range, 7 to 5646 d). The deposition of C4d was a graft-specific event occurring after transplantation, because all control tissue samples from native kidneys and baseline biopsies were C4d-negative. Clinical signs of infections preceding allograft biopsies by 2 wk (n = 119) were not associated with C4d positivity (C4d positivity, 43 (36%) of 119 biopsies; difference to C4d-negative group, NS). Forty of 47 patients had received an induction therapy with antilymphocytic preparations before index biopsy (taken within 20 d after transplantation). C4d positivity was found in 27 of 47 index

biopsies and was not linked to the administration of antilymphocytic preparations (C4d-negative, 16 of 40; C4d-positive, 24 of 40; difference, NS). The accumulation of C4d followed a dynamic pattern, judged by the evaluation of consecutive biopsies from the same patient. C4d staining results could change from negative to positive and *vice versa* within days. A change from negative to positive was observed in 16 consecutive biopsy sets within 64 d (median value; range, 4 to 536 d; lower quartile, 12 d; three shortest intervals, 4, 5, and 8 d). A change from positive to negative staining was observed in 24 consecutive biopsy sets within 76 d (median value; range, 8 to 833 d; lower quartile, 50 d; three shortest intervals, 8, 13, and 18 d).

From a patient-based approach, 35% of all patients (94 of 265) were C4d-positive. The prevalence of C4d positivity was similar in patient subgroups: 34% (81 of 235) in patients with first allografts, 43% (13 of 30) in those with second or third grafts, 35% (63 of 180) in patients with grafts of cadaveric origin, and 36% (31 of 85) in those with grafts of living donor origin (differences, NS). Sixty-five percent of patients (171 of 265) were C4d-negative.

Correlation of C4d with Histologic Changes

The accumulation of C4d was associated with 4 of 18 scored morphologic alterations, all indicative of "active" rejection (Table 2): (*1*) transplant glomerulitis ($P < 0.0001$); (*2*) tubular MHC class II expression ($P < 0.0001$); (*3*) transplant endarteritis ($P = 0.004$); (*4*) interstitial cellular rejection ($P < 0.0001$). The frequency of C4d positivity in these 4 morphologic subgroups ranged from 41% to 57%. Further statistical analysis by stepwise multiple regression with C4d as a dependent variable ($r^2 = 0.133$) demonstrated that tubular MHC class II expression was the morphologic feature that ranked highest, establishing it as the leading parameter linked with C4d accumulation, followed by transplant glomerulitis. Stepwise regression did not show an association between C4d deposition and transplant endarteritis or interstitial cellular rejection. Transplant glomerulitis exclusively correlated with C4d accumulation in a subset of biopsies expressing tubular MHC class II ($n = 246$; C4d positivity in 62% of biopsies with transplant glomerulitis [23 of 37]; $P = 0.01$; all other morphologic changes, NS). The accumulation of C4d was not significantly linked to morphologic lesions characterizing inactive chronic rejection, such as sclerosing transplant vasculopathy, glomerulopathy, or cyclosporine/tacrolimus-induced alterations. In our series, C4d-positive biopsies could not be reliably distinguished from C4d-negative ones on the basis of histologic changes such as (micro)thrombi, marked tubular injury, or polymorphonuclear leukocytes in peritubular capillaries.

Histology associated with persistent deposition of C4d only changed slightly over time as judged by the evaluation of

Table 2. Association between the accumulation of C4d and histologic changes in allograft biopsies (total, $n = 398$; C4d-positive, $n = 120$)[a]

Histologic Features	Number of Cases with Feature (total $n = 398$)	C4d Accumulation Present, *n* (%)	χ^2 *P*
Vascular features			
transplant endarteritis	74	33 (45%)	0.004
fibrinoid necrosis	10	5 (50%)	NA
intimal sclerosis	48	16 (33%)	NS
toxic arteriolopathy	145	40 (38%)	NS
thrombotic microangiopathy	30	12 (40%)	NS
arteriolosclerosis	135	41 (30%)	NS
Glomerular features			
transplant glomerulitis	49	28 (57%)	<0.0001
transplant glomerulopathy	17	9 (53%)	NS
glomerulonephritis	54	14 (26%)	NS
Tubulo-interstitial features			
interstitial rejection	131	56 (43%)	<0.0001
MHC-class II expression	246	102 (41%)	<0.0001
toxic tubular injury	73	19 (35%)	NS
ATN	11	3 (27%)	NA
Others			
striped interstitial fibrosis	225	51 (23%)	NS
diffuse interstitial fibrosis	21	3 (14%)	NS
pyelonephritis	4	2 (50%)	NA
CMV infection	3	1	NA
BK-Virus nephropathy	9	0	NA

[a] Most biopsies showed several changes; NA, not applicable due to small case numbers.

consecutive C4d-positive biopsies. In 20 patients, an initial C4d-positive biopsy (taken 21 d posttransplantation, median value; range, 10 to 3303 d) was followed by a second C4d-positive biopsy (median, 42 d posttransplantation; range, 19 to 3889). In these C4d-positive repeat biopsies, the pattern of histologic changes was unaltered (in comparison with the initial biopsies), with the exception of an increased frequency of transplant endarteritis (25% in the initial biopsies, and 50% in repeat biopsies).

C4d and Renal Function/Graft Survival

Biopsy-Based Approach. To analyze the impact of C4d accumulation on graft function, we evaluated s-creatinine levels and graft survival up to 12 mo after biopsy (Table 3; Figures 2 and 3). Although certain C4d-positive cases were associated at time of biopsy with significantly higher s-creatinine levels ($P < 0.01$; Figure 2), this difference was no longer found after 1 mo. In general, the detection of C4d did not indicate pronounced functional deterioration during follow-up, even in biopsies with transplant endarteritis, glomerulitis, or interstitial rejection, *i.e.*, histologic changes associated with the deposition of C4d (Figure 3). One year postbiopsy s-creatinine levels tended to be slightly higher in most of the analyzed C4d-positive groups, although the differences to the corresponding C4d-negative controls were never significant. In certain subgroups (such as biopsies from second or third transplants or biopsies with transplant glomerulitis), this trend was even reversed with more pronounced dysfunction in the C4d-negative patients.

Patient-Based Approach. One year after the index biopsy, neither s-creatinine levels nor graft survival differed significantly between C4d-positive and C4d-negative patients (Table 4 and Figures 4 and 5; C4d-positive patients: s-creatinine, 159 μmol/L [median]; graft loss, 14%; C4d-negative

Table 3. Renal function postbiopsy—C4d-positive *versus* C4d-negative biopsies[a]

Biopsy Groups	Number of Biopsies	S-Creatinine μmol/L Mean ± SD (Median) 1 yr Postbiopsy
All biopsies		
C4d-positive	114	197 ± 106 (169)
C4d-negative	266	198 ± 105 (162)
Biopsies within 38 d posttransplantation		
C4d-positive	55	162 ± 56 (153)
C4d-negative	49	177 ± 81 (149)
Initial biopsies		
C4d-positive	65	173 ± 78 (158)
C4d-negative	158	182 ± 85 (153)
Biopsies from living donors		
C4d-positive	42	181 ± 75 (167)
C4d-negative	86	191 ± 96 (160)
Biopsies from cadaveric donors		
C4d-positive	72	205 ± 120 (172)
C4d-negative	180	201 ± 109 (163)
Biopsies from first transplants		
C4d-positive	98	196 ± 109 (169)
C4d-negative	239	190 ± 97 (159)
Biopsies from 2nd and 3rd transplants		
C4d-positive	16	199 ± 92 (169)
C4d-negative	27	253 ± 140 (224)
Transplant endarteritis		
C4d-positive	32	217 ± 131 (184)
C4d-negative	36	194 ± 111 (163)
Transplant glomerulitis		
C4d-positive	28	231 ± 149 (165)
C4d-negative	20	215 ± 105 (192)
Interstitial rejection		
C4d-positive	55	174 ± 58 (162)
C4d-negative	73	218 ± 134 (173)

[a] The number of biopsies and s-creatinine levels refer to functioning grafts only. Differences between s-creatinine levels C4d positive *versus* negative: not significant.

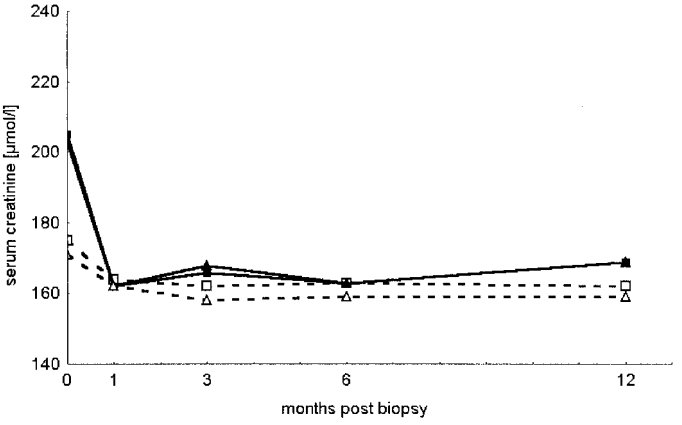

Figure 2. Renal function postbiopsy—C4d-positive *versus* C4d-negative. S-creatinine levels from functioning grafts are listed as median values. After biopsy, renal function did not differ significantly between C4d-positive and C4d-negative groups. Only at time of biopsy, the detection of C4d was associated with higher s-creatinine levels ($P < 0.01$). All biopsies: ■, C4d-positive ($n = 114$); □, C4d-negative ($n = 266$). Biopsies from first transplants only: ▲, C4d-positive ($n = 98$); △, C4d-negative ($n = 239$).

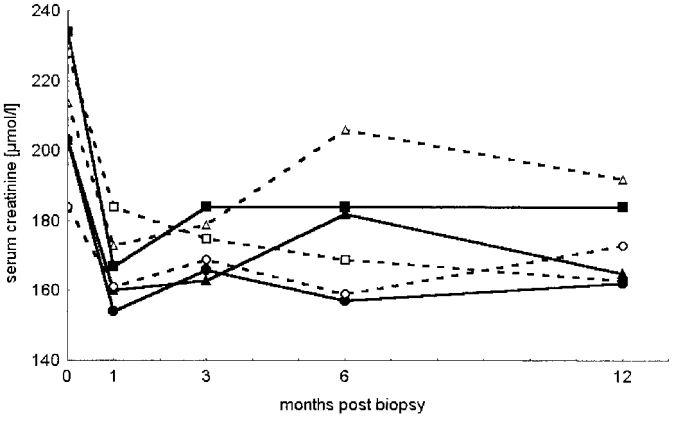

Figure 3. Renal function postbiopsy in different histological subgroups—C4d-positive *versus* C4d-negative. S-creatinine levels from functioning grafts are listed as median values. Differences between C4d-postive and C4d-negative groups, NS. Biopsies with transplant endarteritis: ■, C4d-positive ($n = 32$); □, C4d-negative ($n = 36$). Biopsies with transplant glomerulitis: ▲, C4d-positive ($n = 28$); △, C4d-negative ($n = 20$). Biopsies with interstitial cellular rejection: ●, C4d-positive ($n = 55$); ○, C4d-negative ($n = 73$).

patients: s-creatinine, 153 µmol/L [median]; graft loss, 10%). Most C4d-positive patients showed a trend to less favorable graft function and outcome; however, the differences were never significant. Even persistent C4d deposition observed in initial and repeat biopsies in 20 patients was not associated with poor outcome. Renal function (s-creatinine, 199 µmol/L [median]) and the graft failure rate (4 of 20; 20%) 1 yr after the index biopsy did not differ significantly from a control group of 48 patients with 2 subsequent C4d-negative biopsies (s-creatinine, 183 µmol/L [median]; graft failure rate, 8 of 41 [20%]).

Correlation of C4d with Circulating Panel-Reactive Circulating Antibodies (PRA)

Biopsy-Based Approach. The detection of C4d was associated with elevated titers of PRA. A total of 188 sets (antibody titer test performed within 2 wk before corresponding biopsy) were evaluated. Forty-three percent of C4d-positive biopsies (35 of 82) were accompanied by elevated antibody titers exceeding 10% panel-reactivity, in contrast with only 19% (20 of 106) in the C4d-negative group (χ^2, $P = 0.0012$). An evaluation of certain subgroups (first biopsies, biopsies taken within 38 d posttransplantation, biopsies from 1. transplants) revealed similar frequencies (data not shown).

Patient-Based Approach. Forty patients were PRA-positive at time of index biopsy (1-yr graft survival, 77%), and 91 patients were PRA-negative (1-yr graft survival, 85%; difference, NS). The C4d status did not have any effect on outcome: (*1*) PRA-positive patients: C4d positivity, $n = 28$; 1-yr graft survival, 79%; C4d negativity, $n = 12$; 1-yr graft survival, 75%; difference, NS; (*2*) PRA-negative patients: C4d positivity, $n = 39$; 1-yr graft survival, 85%; C4d negativity, $n = 52$; 1-yr graft survival, 85%; difference, NS.

Treatment

Although, the prevalence of transplant endarteritis in index biopsies was similar in C4d-positive and C4d-negative patients (Table 5; C4d-positive, 20 [21%] of 94; C4d-negative, 23 [13%] of 171; difference, NS), C4d positivity more frequently resulted in antilymphocytic treatment with ATG or OKT3 (C4d-positive, 51 [54%] of 94; C4d-negative, 27 [16%] of 168; $P < 0.0001$). The frequency of antirejection therapy with bolus steroids did not differ (C4d-positive, 26 of 94 [28%]; C4d-negative, 62 of 168 [36%]; difference, NS). More patients in the C4d-negative group remained without antirejection therapy after index biopsy: 79 (47%) of 168 compared with 17 (18%) of 94 in the C4d-positive group ($P < 0.0001$). In general, patients receiving antilymphocytic treatment with ATG or OKT3 presented with higher s-creatinine levels at time of biopsy (>225 µmol/L) compared with the bolus steroid treatment group (<180 µmol/L) and the no-treatment group (<155 µmol/L). C4d-positive patients without antirejection treatment at time of index biopsy ($n = 17$) showed a trend toward impaired graft function during 1-yr follow-up (compared with C4d-negative controls); however, differences never reached statistical significance.

The evaluation of histologic subgroups (Figure 3) with respect to therapy revealed a higher frequency of treatment with antilymphocytic preparations in C4d-positive patients (C4d-positive *versus* C4d-negative: transplant endarteritis, 69% *versus* 64% [NS]; transplant glomerulitis, 61% *versus* 45% [NS]; interstitial cellular rejection, 65% *versus* 45% [$P < 0.04$]).

Discussion

This study evaluates the significance of the detection of C4d in the interpretation of diagnostic renal allograft biopsies. Previous reports suggested the potential pathophysiologic importance of C4d accumulation. The interpretation of those findings was, however, difficult due to a highly selected case

Table 4. Outcome after index biopsy—C4d-positive *versus* C4d-negative patients[a]

Patient Groups	Number of Patients	S-Creatinine (μmol/L) 1 yr after Index Biopsy Mean ± SD (Median)	Graft Failure 1 yr after Index Biopsy % (n^b)
All patients			
C4d-positive	94	172 ± 66 (159)	14% (86)
C4d-negative	171	181 ± 88 (153)	10% (147)
Index biopsies within 38 d posttransplantation			
C4d-positive	51	161 ± 58 (142)	18% (45)
C4d-negative	38	173 ± 84 (137)	27% (30)
Index biopsies within 180 d posttransplantation			
C4d-positive	65	164 ± 53 (153)	15% (58)
C4d-negative	91	184 ± 100 (154)	12% (62)
Index biopsies after 180 d posttransplantation			
C4d-positive	29	191 ± 86 (168)	15% (26)
C4d-negative	80	178 ± 75 (150)	4.1% (73)
Patients with cadaveric transplants			
C4d-positive	63	179 ± 75 (161)	16% (57)
C4d-negative	117	183 ± 91 (153)	13% (104)
Patients with transplants of living donor origin			
C4d-positive	31	158 ± 41 (156)	21% (29)
C4d-negative	54	177 ± 82 (152)	5% (43)

[a] The s-creatinine levels refer to functioning grafts only. %, rate of graft failure. Index biopsy, patient's first available C4d-positive or C4d-negative biopsy. S-creatinine levels and graft failure rates between C4d-positive and C4d-negative patients: differences not significant.
[b] Evaluable number of patients 12 mo after index biopsy.

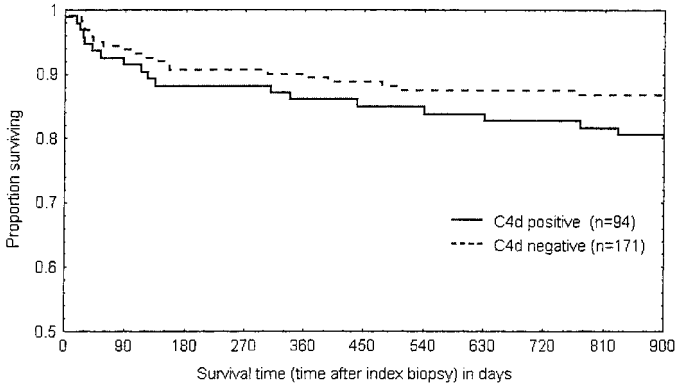

Figure 4. Graft survival of C4d-negative *versus* C4d-positive patients. Difference between C4d-positive and C4d-negative patients, NS.

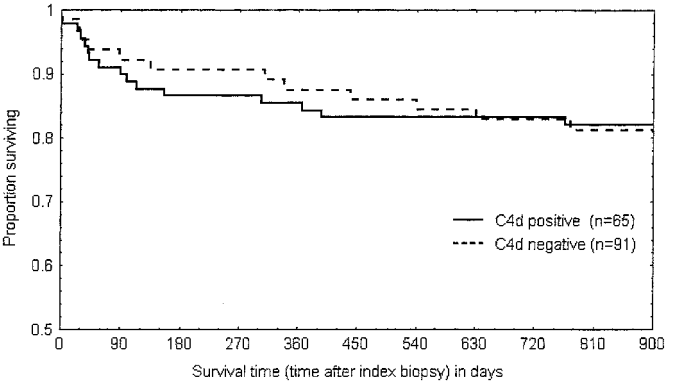

Figure 5. Graft survival of C4d-negative *versus* C4d-positive patients (index biopsy, <180 d post tx). Difference between C4d-positive and C4d- negative patients, NS.

population (9,14,15), small case numbers (14) or unclear case selection (10). In contrast, our observations were made in the largest group of diagnostic kidney transplant biopsies reported to date. Our biopsies were not selected for a specific type of allograft dysfunction. We show that C4d is a useful adjunct marker molecule to diagnose acute rejection episodes with humoral components that likely require intensive antirejection therapy. Our extensive histologic analysis shows a close association between C4d and transplant glomerulitis as well as tubular MHC class II expression. Both these features are signs of acute cellular rejection (2,18,22–25). The correlation between C4d and transplant glomerulitis is unique. For comparison, tubular MHC class II expression is most closely correlated with interstitial cellular rejection and tubulitis (22). Additional, although weaker, links were found between C4d and transplant endarteritis and interstitial cellular rejection. Similar to previous reports (3,10,13), we also found a correlation between C4d and humoral components of acute rejection, *i.e.*, elevated titers of panel-reactive antibodies. This observation confirms the role of C4d as an indirect marker for humoral mediators of rejection. We demonstrate for the first time that the detection of C4d follows a dynamic course with presumed build-up and breakdown within days to a few weeks—similar to the time-course of acute rejection episodes. C4d was only detected in renal allografts posttransplantation and not in native

Table 5. Therapy and outcome after index biopsy—C4d-positive *versus* C4d-negative patients[a]

Patient Groups	Number of Patients	S-Creatinine (μmol/L) 1 yr after Index Biopsy Mean ± SD (Median)	Graft Failure 1 yr after Index Biopsy % (n[b])
Treatment with ATG or OKT3			
C4d-positive	51	167 ± 65 (150)	20% (49)
C4d-negative	27	229 ± 140 (179)	27% (26)
Treatment with bolus steroids only			
C4d-positive	26	169 ± 37 (172)	14% (21)
C4d-negative	62	175 ± 79 (149)	14% (51)
No specific antirejection treatment			
C4d-positive	17	187 ± 95 (167)	13% (16)
C4d-negative	79	170 ± 68 (149)	9% (69)

[a] The s-creatinine levels refer to functioning grafts only. %, rate of graft failure. S-creatinine levels and graft failure rates between C4d-positive and C4d-negative patients: differences not significant.
[b] Evaluable number of patients 12 mo after index biopsy.

kidney or baseline transplant biopsies taken at time of surgery. There was no evidence that C4d was associated with nonspecific types of acute tubular injury, infections (including BK virus nephropathy), or the administration of antilymphocytic preparations before biopsy. No significant correlation was found between C4d accumulation and inactive chronic sclerosing lesions, such as sclerosing transplant vasculopathy or cyclosporine-induced arteriolopathy. All these observations stress the importance of C4d in the setting of acute/active rejection.

The detection of C4d was also associated with several unusual features. First, the specificity of C4d for peritubular capillaries was an unexpected finding, particularly because all arteries were spared. Second, approximately 50% of biopsies with histologic signs of acute active rejection and half of those accompanied by elevated panel-reactive antibody titers were C4d-negative. How might these findings be explained? We speculate that the humoral components associated with the deposition of C4d are directed specifically against the endothelium of peritubular capillaries with MHC class I, class II, or other endothelial antigens as potential targets (4,26–28). The pattern of antigen presentation might differ from that expressed on the endothelium of arteries, even those with endothelialis. Thus, on one hand we can have a humoral alloresponse directed against capillary targets (*i.e.*, C4d positivity), and on the other, a cellular alloresponse directed against the endothelium of arteries (*i.e.*, transplant endarteritis) or tubules (*i.e.*, tubulitis). Humoral and cellular alloresponses might affect a transplanted kidney either independently or concurrently. The endothelium is a target for both cellular and humoral responses (4,29); therefore, the significant association between C4d and transplant glomerulitis and transplant endarteritis (especially in repeat biopsies) is not surprising. Due to the fact that PRA cytotoxicity tests only measure complement-dependent cell lysis of leukocytes, only a moderate correlation with capillary-specific C4d deposition can be expected. We are perhaps dealing with 2 or even more independent humoral mechanisms. Future studies will need to specifically examine this aspect.

Collins *et al.* (14) reported the lack of any significant C4d deposition in 14 typical cases of acute cellular rejection. The authors concluded that acute cellular rejection was morphologically distinct from cases showing C4d positivity, for which they coined the term "acute humoral rejection." In subsequent reports from the same group (2,15), it was suggested that C4d was a useful marker to specifically diagnose chronic rejection. These observations differ from our findings as well as those reported by others (6,9,30). It is conceivable that some of the discrepancies are due to the small case numbers studied by Collins *et al.*, which resulted in erroneous data. Here, we clearly show that approximately 40% to 50% of biopsies with histologic signs of acute cellular rejection are C4d-positive. In most of our patients, C4d-positive biopsies could not be reliably distinguished from C4d-negative ones on the basis of standard light microscopy examination. Because the turnover of C4d follows a dynamic course, and because all specific statistical analyses were unrevealing, we do not believe that C4d is a marker of chronic rejection. Instead, C4d marks an active and often transient humoral response, which can be superimposed over various histologic changes, including—occasionally—chronic rejection. Most often, however, C4d deposition is found in association with acute cellular rejection. The following clinical observations support our view. C4d-positive patients generally presented with more pronounced allograft dysfunction, which, in our center, often resulted in intensive antirejection treatment with antilymphocytic preparations. Under this type of acute rejection therapy, renal function improved. The immediate administration of antilymphocytic preparations was in many cases directly influenced by the knowledge about C4d positivity. In Basel, the detection of C4d has been traditionally used as an indirect marker for transplant endarteritis and, thus, more severe acute rejection. This notion was based on the original report by Feucht *et al.* (9), which showed a high prevalence of C4d positivity in vascular rejection (82%) and a high graft failure rate.

For the first time, favorable outcome data are presented in a large cohort of C4d-positive renal allograft recipients. This

good result is most likely due to our aggressive therapeutic approach. Thus, we provide initial evidence that intensive treatment of C4d-positive acute rejection episodes might be beneficial. Anecdotal observations similar to ours have also been made by others (15,31). Future studies, however, have to be designed to define optimal treatment protocols. Currently, we believe that two large groups of C4d-positive patients have to be considered: (*1*) C4d-positive patients presenting with pronounced allograft dysfunction or showing histologic signs of allograft rejection (last but not least, interstitial rejection). These patients seem to benefit from aggressive antirejection therapy; (*2*) a small group of C4d-positive patients who present with only mild allograft dysfunction and no histologic signs of rejection. These patients do not appear to significantly benefit from antirejection therapy. These untreated C4d-positive patients in our series notably did not include cases with histologic features suggestive of an antibody mediated type of rejection, *i.e.*, no prominent polymorphonuclear leukocytes in peritubular capillaries and no fibrin thrombi (1,32).

In conclusion, we show that C4d is a useful immunohistochemical marker molecule in the evaluation of renal allograft biopsies. We propose to avoid the term "acute cellular rejection." It is misleading, not recognizing the significance of humoral alloresponses. Considering our current understanding, we believe that a diagnosis should best be reported on the basis of the histologic changes (*i.e.*, tubulo-interstitial, vascular, glomerular) amended to the presence or absence of C4d.

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