

Capillary C4d Deposition in Kidney Allografts: A Specific Marker of Alloantibody-Dependent Graft Injury

GEORG A. BÖHMIG,* MARKUS EXNER,[†] ANTJE HABICHT,*
MARTIN SCHILLINGER,[‡] URSULA LANG,[§] JOSEF KLETZMAYR,*
MARCUS D. SÄEMANN,^{||} WALTER H. HÖRL,* BRUNO WATSCHINGER,* and
HEINZ REGELE[¶]

Departments of *Internal Medicine III, [†]Laboratory Medicine, and [‡]Internal Medicine II and Institutes of ^{||}Immunology and [¶]Clinical Pathology, University of Vienna, Vienna, Austria, and [§]Department of Nephrology and Dialysis, Wilhelminen Hospital, Vienna, Austria.

Abstract. Capillary deposition of the complement split product C4d has been discussed as a marker for antibody-mediated kidney allograft rejection. The relationship between C4d staining and posttransplant alloantibody detection remains to be thoroughly investigated, however. In this study, C4d staining in peritubular capillaries (PTC) and the incidence of alloantibody formation, as detected with sensitive techniques, were evaluated among a cohort of transplant recipients who had undergone biopsies and had not been selected for a specific histologic diagnosis. One hundred thirteen biopsies, obtained from 58 cadaveric kidney transplant recipients, were tested. Serum samples obtained at the time of biopsy were evaluated by flow cytometric crossmatch (FCXM) testing and FlowPRA (One Lambda, Inc., Canoga Park, CA) analysis of anti-HLA panel reactivity. Most biopsies with C4d deposits in PTC (C4d_{PTC}⁺, *n* = 21 of 24) were associated with positive post-transplant FCXM results (T and/or B cell FCXM) and/or ≥5% FlowPRA (anti-HLA class I and/or II) reactivity. Approximately 50% of the C4d_{PTC}⁻ biopsies were observed to be associated with donor-specific alloantibodies. Accordingly, high specificity (93%) but low sensitivity (31%) were calcu-

lated for capillary C4d staining (with FCXM testing as the standard method). For clinical evaluation, three patient groups were defined, *i.e.*, a group of recipients with positive C4d staining in at least one allograft biopsy (C4d_{PTC}⁺, *n* = 16) and two C4d_{PTC}⁻ groups, which were discriminated on the basis of posttransplant FCXM results as C4d_{PTC}⁻/FCXM⁺ (*n* = 22) and C4d_{PTC}⁻/FCXM⁻ (*n* = 20) groups. Univariate analyses revealed significant differences between these groups with respect to serum creatinine levels at 12 mo [median, 2.83 mg/dl (interquartile range, 1.93 to 4.2 mg/dl) *versus* 1.78 mg/dl (1.47 to 2.24 mg/dl) *versus* 1.59 mg/dl (1.2 to 1.71 mg/dl), *P* < 0.001]. Of the five immunologic graft losses, four occurred in the C4d_{PTC}⁺ group and one occurred in the C4d_{PTC}⁻/FCXM⁺ group. In a multivariate analysis, C4d positivity was observed to have an independent predictive value for inferior 12-mo graft function (*P* = 0.02), whereas the observed moderate difference between C4d_{PTC}⁻/FCXM⁺ and C4d_{PTC}⁻/FCXM⁻ recipients did not achieve significance. In conclusion, these data demonstrate that positive C4d staining, which is an independent predictor of kidney graft dysfunction, represents a reliable specific marker for antibody-dependent graft injury.

There is increasing evidence for an important role for humoral immune mechanisms in kidney allograft rejection. Alloantibodies not only may cause hyperacute rejection, the most severe type of humoral graft injury, but also may contribute to other types of rejection, such as acute and chronic rejection (1–4). The diagnosis of humoral graft injury is mainly based on serologic test results (posttransplant crossmatch testing) (1–4). In addition, distinct histopathologic features have been reported for acute antibody-mediated rejection (5–7). Recent data suggest that the complement split product C4d may also

be an attractive marker for acute humoral rejection (8–14). Feucht *et al.* (8,9) were the first to demonstrate the occurrence of capillary C4d staining in kidney allografts and its association with inferior graft outcomes. Those findings were confirmed in subsequent studies (11,13,14). Although positive posttransplant crossmatches have been well established to be associated with inferior graft function and histopathologic features of humoral rejection, associations between capillary C4d deposits and posttransplant serologic findings have not been thoroughly studied in larger patient populations not selected for a specific diagnosis. In a recent report, Collins *et al.* (11) reported distinct C4d positivity only in biopsies selected for the presence of acute humoral rejection, as defined by characteristic histologic features and positive posttransplant crossmatching. The same group demonstrated a strong association between C4d staining and positive posttransplant crossmatch results among patients with biopsy-proven rejection (13). Those studies suggested a high specificity of C4d staining for humoral rejection.

Received October 31, 2001. Accepted December 11, 2001.

Correspondence to Dr. Georg A. Böhmig, Division of Nephrology and Dialysis, Department of Internal Medicine III, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria. Phone: +43-1-40400-4390; Fax: +43-1-40400-4499; E-mail: georg.boehmig@nephro.imed3.akh-wien.ac.at

1046-6673/1304-1091

Journal of the American Society of Nephrology

Copyright © 2002 by the American Society of Nephrology

In this analysis, the association of C4d staining with the presence of posttransplant alloantibodies was investigated by using two sensitive flow cytometric techniques, *i.e.*, flow cytometric crossmatch (FCXM) testing and FlowPRA (One Lambda, Inc., Canoga Park, CA) analysis of anti-HLA antibodies. This study was designed to evaluate both the specificity and sensitivity of capillary C4d staining among a cohort of kidney transplant recipients not selected for a specific histologic diagnosis. Clinical outcomes for these patients were evaluated with respect to immunohistochemical and serologic results.

Materials and Methods

Patients

Between June 1998 and September 1999, 226 cadaveric kidney transplants were performed in our unit. For 124 patients (54.9%), at least one allograft biopsy was performed during the follow-up period (up to July 2001). For a cohort of 58 of the 124 recipients who underwent biopsies, cryopreserved donor splenocytes and serum samples obtained before transplantation and at the time of biopsy were available for retrospective serologic testing. These patients were enrolled in this study. One of the included recipients received a combined kidney/pancreas transplant, and another received a combined kidney/heart transplant. The median patient age in our study cohort (male/female ratio, 32/26) was 51 yr [interquartile range (IQR), 37 to 60 yr]. Twelve recipients had received a retransplant. The median HLA mismatch was 3 (IQR, 2 to 3), and the median cold ischemia time was 13 h (IQR, 9 to 18).

Immunosuppression

Forty of the 58 study patients received initial baseline immunosuppressive therapy consisting of cyclosporin A (CsA) (dose initially adjusted to yield trough levels between 200 and 400 ng/ml), mycophenolate mofetil (2 g/d, administered orally), and steroids. Nine recipients received rapamycin (initial dose, 2 mg/d) together with CsA and steroids. Two retransplanted and sensitized patients [cytotoxic panel-reactive antibody (PRA) reactivity, >10%], one combined kidney/heart transplant recipient, and three recipients of suboptimal grafts (non-heart-beating donor or donor age of >60 yr) received induction therapy with rabbit or horse antilymphocyte serum (ALS). Another three recipients underwent an induction protocol with the anti-interleukin-2 receptor antibody Daclizumab (Hoffman-La Roche, Basel, Switzerland) ($n = 1$) or Basiliximab (Novartis AG, Basel, Switzerland) ($n = 2$). Histologically proven acute rejection episodes and some cases of Banff borderline lesions were initially treated with steroid bolus therapy (dexamethasone administered at 100 mg/d for 3 d). Steroid-resistant graft dysfunction was treated with antilymphocyte polyclonal antibodies (ALS) or, for one patient, antilymphocyte monoclonal antibody (mAb) (OKT3; Jansen, Raritan, NJ). Thirteen patients received tacrolimus rescue therapy. As previously described (15), two patients with characteristic features of humoral rejection (capillary C4d staining, intracapillary granulocytes, and severe graft dysfunction) received immunoadsorption (IA) therapy in addition to ALS (both patients also exhibited features of cellular rejection). Two recipients with clinical and histopathologic diagnoses of hemolytic uremic syndrome (positive C4d staining for one patient) underwent a change to tacrolimus treatment (CsA-induced hemolytic uremic syndrome was suspected) and plasma-exchange therapy.

Renal Allograft Pathologic Analyses

Biopsies were performed because of persistent oliguria or anuria or acute functional impairment, after exclusion of post- and prerenal causes of graft dysfunction and toxic CsA (>400 ng/ml) or FK506 (>20 ng/ml) levels. During the follow-up period (up to July 2001), 113 renal allograft biopsies were performed for the 58 included patients. Thirty-three of these patients underwent more than one biopsy. Biopsies were performed a median of 19 d after transplantation (range, 3 to 681 d). Nineteen biopsies were performed >3 mo after transplantation.

Histopathologic evaluation and C4d staining were performed with formalin-fixed, paraffin-embedded sections. Specimens were stained with hematoxylin and eosin, periodic acid-Schiff stain, methenamine-silver, and the trichrome stain acidic fuchsin-Orange G. Lesions in allograft biopsies were classified according to the definitions provided by the Banff 97 working classification of renal allograft pathologic features (16).

For detection of C4d, we used a novel polyclonal anti-C4d antibody (C4dpAb; Biomedica, Vienna, Austria) generated by immunization of a rabbit with a 15-mer peptide corresponding to amino acids 1242 to 1256 of C4. The generation and characterization of C4dpAb are described elsewhere in detail (14). Specific binding of C4dpAb to purified C4 and tryptic C4d was demonstrated by Western blot analysis. Furthermore, antibody specificity was proven in blocking experiments with tissue sections. Preincubation of C4dpAb with purified C4 or the peptide used for immunization completely abolished reactivity with frozen sections of a representative C4d⁺ kidney allograft. In contrast, irrelevant control peptides or other complement factors (C3 or C5) did not affect the staining results. Identical peritubular staining patterns for C4d with C4dpAb and an anti-C4d mAb (Quidel, Alkmaar, Netherlands) were demonstrated in a series of renal biopsies (12 allografts and 25 normal native kidneys) for which both paraffin-embedded and frozen sections were available. Immunostaining of paraffin-embedded sections with C4dpAb revealed the same peritubular staining pattern as that observed for corresponding frozen sections stained with anti-C4d mAb (14). For immunohistochemical detection of C4d on formalin-fixed, paraffin-embedded sections, we used indirect immunoperoxidase staining. Sections (2 μ m) were deparaffinized, and endogenous peroxidase activity was blocked with hydrogen peroxide/methanol. Antigen retrieval was performed by pressure-cooking (at 1 bar) for 10 min in citrate buffer (pH 6.0). Endogenous biotin was blocked by using a biotin blocking kit (Vector Laboratories, Burlingame, CA). After a 30-min incubation with C4dpAb (5 μ g/ml), bound IgG was observed by using the Supersensitive Kit (BioGenex, San Ramon, CA), according to the protocol provided by the manufacturer. The intensity of endothelial C4d staining was classified as weak, moderate, or strong. Some of the biopsies with low-intensity C4d staining demonstrated an inhomogeneous distribution of C4d [only a few C4d⁺ peritubular capillaries (PTC)]. In biopsies with moderate or strong C4d staining, virtually all capillaries stained positive.

Immunologic Methods

Pretransplant cytotoxic crossmatch testing was performed according to the protocol of the Eurotransplant Organization, using the standard microcytotoxicity technique described by Terasaki and McClelland (17). For inactivation of IgM, sera were pretreated with dithiothreitol. All study patients exhibited negative cytotoxic crossmatch findings before transplantation. Pretransplant cytotoxic PRA reactivity was assessed by using a panel of cells from 50 phenotyped donors.

Fifty-eight pretransplant and 113 posttransplant serum samples obtained at the time of renal biopsy were retrospectively tested for the presence of alloantibodies. For FCXM testing, cryopreserved spleen cells isolated from donor spleens by density gradient centrifugation were used. We used a three-color technique. Donor cells (4×10^5) were incubated with undiluted serum at room temperature for 1 h. The cells were washed and then incubated with a mixture of pretitered, FITC-conjugated, goat anti-human IgG (Accurate Chemical and Scientific Co., Westbury, NY), phycoerythrin-labeled CD3 mAb, and peridin-chlorophyll-A-protein-conjugated CD19 mAb (Becton Dickinson, San Jose, CA) at 4°C for 30 min. Fluorescence intensity was measured by using a FACSCalibur flow cytometer (Becton Dickinson). The lymphocyte population was gated according to forward- and side-scatter characteristics. Recipient reactions against T cells (T cell FCXM) or B cells (B cell FCXM) were analyzed by measuring FITC binding to the cells. According to previous studies (18,19), the FCXM was considered positive when the mean fluorescence intensity was greater than the mean + 2 SD of values for normal control samples (serum samples from four different nonsensitized healthy volunteers). Positivity was graded as FCXM_{high} if mean fluorescence intensity levels were twofold higher than mean control fluorescence intensity levels.

The FlowPRA screening test, a recently established, HLA-specific, flow cytometric technique (20), was performed according to the guidelines provided by the supplier (One Lambda, Inc.). Using fluorescent microbeads coated with purified HLA antigens (either class I or II) from 30 different cell lines, this assay allows measurement of anti-HLA class I and class II PRA reactivity. In brief, 20 μ l of serum were incubated with 5 μ l of FlowPRA class I (FL1-30, lot 6) and 5 μ l of FlowPRA class II (FL2-30, lot 7) beads. After a 30-min incubation period at room temperature, the beads were washed twice and then stained for 30 min with appropriately pretitered, FITC-conjugated, anti-human IgG (Fcy; One Lambda, Inc.). Fluorescence intensity was measured by flow cytometry. The major population of beads was gated in the forward- versus side-scatter dot plot. FlowPRA class I and II beads were discriminated in the FL2 histogram. The marker was set according to the negative control sample, *i.e.*, serum provided by the company (FL-NC; One Lambda, Inc.). The percentage of HLA class I- or class II-coated beads shifted to the right of the cutoff point represents the percentage of FlowPRA class I or class II reactivity. Sera with $\geq 5\%$ FlowPRA class I and/or II reactivity were considered anti-HLA antibody-positive. Anti-HLA class I antibody-positive sera contained 26% (median; IQR, 10 to 83%) FlowPRA class I reactivity, and anti-HLA class II antibody-positive sera contained 37% (median; IQR, 18 to 50%) FlowPRA class II reactivity.

Sera with $\geq 5\%$ FlowPRA reactivity were tested for specific HLA class I (FlowPRA specific HLA class I antibody detection test, FL1SP, lot 006; One Lambda, Inc.) and/or HLA class II (FlowPRA specific HLA class II antibody detection test, FL2SP, lot 004; One Lambda, Inc.) antibodies. The respective bead panels (each composed of four groups containing eight different colored beads with different FL2 fluorescence properties) were composed of 32 microbeads coated with purified HLA (class I or II) antigens. Beads were incubated with serum and stained with FITC-conjugated anti-human IgG as described for the screening test. The major population of beads was gated in the forward- versus side-scatter dot plot. Different beads were discriminated in the FL2 histogram. Markers were set according to the negative control sample (negative control serum, FL-NC; One Lambda, Inc.). Positivity was scored according to the percentage of beads shifted (0 to 20%, negative; 21 to 100%, positive). The specificity of HLA antibodies was calculated from staining patterns, using the software provided by the manufacturer (One Lambda, Inc.).

Statistical Analyses

Continuous data are presented as the median and IQR (range from the 25th to the 75th percentile). Percentages were calculated for dichotomous variables. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated by using standard formulae. Comparisons between groups were performed by using the χ^2 test, the Mann-Whitney *U* test, or the Kruskal-Wallis test, as appropriate. A multivariate logistic regression model was used to assess the independent effects of baseline variables on renal outcomes (serum creatinine levels at 12 mo of >1.755 versus <1.755 mg/dl; patients undergoing dialysis, creatinine levels of >1.755 mg/dl; patient deaths excluded). Variables demonstrating a trend toward a difference in graft function ($P < 0.2$) were entered into the model as possible predictor variables. Results are presented as the odds ratio and 95% confidence interval. The Cox-Snell pseudo- r^2 value was calculated for assessment of the predictive value of the model, and the Hosmer-Lemeshow test was used to assess the model fit. All *P* values are two-sided. *P* values of <0.05 are reported as statistically significant. A commercially available computer program (SPSS for Windows, version 10.0; SPSS Inc., Chicago, IL) was used for all statistical calculations.

Results

Association of Capillary C4d Staining with Posttransplant Serologic Results

Twenty-four of the 113 renal allograft biopsies evaluated (21.2%) demonstrated C4d deposits in PTC (C4d_{PTC}⁺). The prevalence of positive FCXM findings among the 113 tested serum samples obtained at the time of biopsy was 60.2% (*i.e.*, 68 FCXM⁺ sera; T cell reactivity only, $n = 8$; B cell reactivity only, $n = 15$; T plus B cell reactivity, $n = 45$). In a comparison of capillary C4d staining with FCXM results, 21 of the 24 C4d_{PTC}⁺ biopsies (87.5%) and 47 of the 89 C4d_{PTC}⁻ biopsies (52.8%) were associated with FCXM positivity. The 113 posttransplant sera were further tested for the presence of anti-HLA alloantibodies by using the FlowPRA screening test. Fifty-five sera (48.7%) demonstrated $\geq 5\%$ FlowPRA reactivity (anti-HLA class I only, $n = 7$; anti-HLA class II only, $n = 19$; anti-HLA class I and II, $n = 29$). Forty-one sera demonstrated both FCXM positivity and $\geq 5\%$ FlowPRA reactivity. Fourteen sera exhibited $\geq 5\%$ FlowPRA reactivity but negative FCXM results, and 27 sera demonstrated FCXM positivity in the absence of detectable anti-HLA antibodies. Twenty-one of the 24 C4d_{PTC}⁺ biopsies (87.5%) were associated with $\geq 5\%$ FlowPRA reactivity (20 of these sera were also FCXM⁺). The specificity, sensitivity, positive predictive value, and negative predictive value of C4d staining and FlowPRA analysis (FCXM testing was defined as the standard method) are presented in Table 1. Using the FlowPRA specific HLA antibody detection test, we were able to identify HLA specificities for one or more mismatched donor HLA class I and/or class II antigens in 19 of the 55 posttransplant sera with $\geq 5\%$ FlowPRA reactivity. Eleven of those sera were associated with C4d positivity.

Effects of Capillary C4d Staining and Posttransplant Serologic Results on Kidney Allograft Outcomes

Thirty-five of the 58 study patients (60.3%) experienced one or more biopsy-proven rejection episodes, according to the

Table 1. Specificity and sensitivity of capillary C4d staining and posttransplant FlowPRA testing^a

Test	Specificity (%) (95% CI)	Sensitivity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
C4d deposits in PTC	93 (86 to 100)	31 (20 to 42)	87 (73 to 100)	47 (33 to 61)
FlowPRA reactivity of $\geq 5\%$	69 (56 to 82)	60 (48 to 72)	74 (63 to 86)	53 (38 to 69)

^a PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; PTC, peritubular capillaries. FlowPRA reactivity was assessed as detailed in Materials and Methods. Posttransplant flow cytometric crossmatch (FCXM) testing was defined as the standard method for the detection of alloantibody-mediated immunity. By definition, the specificity and sensitivity of a positive posttransplant FCXM result were assumed to be 100%.

Banff classification. Thirteen patients demonstrated Banff type I, 18 Banff type II, and four Banff type III rejection (for patients for whom more than one biopsy was obtained, the rejection episode of highest Banff grade is reported). Histologic diagnoses for the remaining 23 patients were borderline lesions ($n = 10$), acute tubular necrosis (ATN) ($n = 7$), chronic allograft nephropathy ($n = 2$), thrombotic microangiopathy ($n = 1$), donor-derived injury ($n = 2$), and minor/nonspecific changes ($n = 1$). For 28 recipients, graft dysfunction (27 cases of classified Banff rejection and one Banff borderline lesion) was resistant to high-dose steroid treatment, and antilymphocyte antibody therapy was administered. Clinical outcomes for patients with steroid-resistant graft dysfunction are presented in Table 2. Five recipients lost their grafts because of refractory rejection. For another two patients, severe C4d_{PTC}⁺ acute allograft rejection was reversible with combined IA and antithymocyte globulin (ATG) therapy (15). Clinical and immunologic data for patients with severe graft dysfunction are summarized in Table 3. During the follow-up period, five deaths occurred (three resulting from severe septicemia, one resulting from cerebral hemorrhage, and one after aortic valve replacement).

Patients were first divided into two groups according to C4d staining results, *i.e.*, patients with positive C4d staining in at least one allograft biopsy (C4d_{PTC}⁺, $n = 16$) and C4d_{PTC}⁻ recipients ($n = 42$). As calculated in univariate analyses, serum creatinine levels at 12 mo were significantly higher for C4d_{PTC}⁺ patients (2.83 mg/dl; IQR, 1.93 to 4.2 mg/dl) than for C4d_{PTC}⁻ patients (1.6 mg/dl; IQR, 1.32 to 2.15 mg/dl; $P = 0.001$); for calculations, a serum creatinine concentration of 5 mg/dl was presumed for patients undergoing dialysis, and patient deaths (*i.e.*, two C4d_{PTC}⁺ and three C4d_{PTC}⁻ recipi-

ents) were excluded from analysis. Furthermore, C4d_{PTC}⁺ patients experienced more episodes of refractory rejection than did C4d_{PTC}⁻ patients (four of 16 *versus* one of 42 patients, $P = 0.02$). The incidences of Banff rejection were similar for the two groups (10 of 16 *versus* 25 of 42 patients). No significant difference was observed between the two groups with respect to the incidence of steroid-resistant graft dysfunction (nine of 16 *versus* 19 of 42 patients). Two C4d_{PTC}⁺ patients exhibited steroid-resistant rejection associated with characteristic histopathologic features of acute humoral rejection (granulocytes in PTC), which was successfully reversed with IA plus ALS therapy. For multivariate logistic regression analysis, serum creatinine levels at 12 mo were taken as the dependent variable (serum creatinine levels of <1.755 *versus* >1.755 mg/dl; patients undergoing dialysis were included in the calculation). After adjustment of the model for baseline variables with P values of <0.2 (Table 4), *i.e.*, donor age, cold ischemia time, and delayed graft function, C4d staining was observed to be independently associated with inferior allograft function ($P = 0.02$) (Table 5).

In a subsequent analysis, patients were subdivided according to both C4d staining and FCXM results. We defined three patient subsets, *i.e.*, the group of 16 recipients with (presumably alloantibody-mediated) capillary C4d staining (C4d_{PTC}⁺; 13 patients also exhibited FCXM positivity) and two C4d_{PTC}⁻ patient groups, which were discriminated on the basis of posttransplant FCXM results, *i.e.*, C4d_{PTC}⁻/FCXM⁺ recipients ($n = 22$) and C4d_{PTC}⁻/FCXM⁻ recipients ($n = 20$). Immunologic results obtained for these patient groups are presented in Table 6. The proportion of patients classified as FCXM_{high} (definition given in Materials and Methods) was higher for the C4d_{PTC}⁺ group than for the C4d_{PTC}⁻/FCXM⁺ group (12 of 16

Table 2. Clinical outcomes and capillary C4d staining for recipients with steroid-resistant graft dysfunction^a

Patients with Steroid-Resistant Graft Dysfunction	All ($n = 28$ of 58)	C4d _{PTC} ⁺ ($n = 9$ of 16)	C4d _{PTC} ⁻ ($n = 19$ of 42)
Graft loss ^b	5	4	1
Reversible by ALS	21	3	18
Reversible by IA plus ALS	2	2	

^a ALS, antilymphocyte serum; IA, immunoadsorption. Twenty-seven of the patients experienced biopsy-proven rejection, according to the Banff classification. One C4d_{PTC}⁺ recipient exhibited a Banff borderline lesion in a renal allograft biopsy.

^b One recipient with immunologic graft loss received antilymphocyte antibody therapy with OKT3. The other four patients underwent ALS therapy.

Table 3. Patients with severe graft dysfunction^a

Patient No. ^b	Pre-RTX CDC-PRA (%)	≥5% Pre-RTX FlowPRA	Pre-RTX FCXM	Banff Grade	C4d (PTC)	Post-RTX FCXM	Anti-Donor HLA Ab Identified after RTX ^c	Graft Loss
1 ^d	58	Yes	T + B	III	Pos	T + B	Yes (DR52)	Yes
2	8	Yes	T + B	III	Pos	T + B	No	Yes
3	0	No	Neg	II	Pos	Neg	No	Yes
4	8	No	Neg	I	Pos	T	No	Yes
5	0	No	Neg	II	Neg	T	No	Yes
6	0	Yes	T	II	Pos	T + B	Yes (A3, Bw6)	No ^e
7	0	Yes	Neg	III	Pos	B	Yes (DR1, DR10)	No ^e

^a RTX, renal transplantation; CDC-PRA, lymphocytotoxic panel-reactive antibody reactivity; T, positive T cell FCXM; Ab, antibodies; B, positive B cell FCXM; Neg, negative; Pos, positive.

^b Six of seven patients were female (previous pregnancies for three recipients).

^c Antibodies against mismatched donor HLA antigens were assessed by using the FlowPRA specific HLA class I or II antibody test, as detailed in Materials and Methods.

^d Patient 1 had received a third allograft and the other six recipients a first kidney allograft.

^e For patients 6 and 7, severe graft dysfunction (dialysis dependence at the time of rejection) was successfully reversed with IA and antithymocyte globulin therapy.

Table 4. Univariate analyses of variables and their prediction of graft function at 12 mo^a

Parameter	Serum Creatinine Level at 12 mo		P Value
	<1.755 mg/dl (n = 27)	>1.755 mg/dl (n = 26)	
No. with C4d deposits in PTC (%)	2 (7)	12 (46)	0.001
No. female (%)	12 (44)	13 (50)	0.7
No. with previous transplants (%)	7 (26)	5 (19)	0.6
Median no. of HLA mismatches (IQR)	3 (2 to 4)	3 (2 to 3)	0.3
Median CIT (h) (IQR)	13 (8 to 18)	13 (10 to 18)	0.8
Median donor age (yr) (IQR)	46 (38 to 55)	59 (44 to 63)	0.002
No. with DGF (%)	3 (11)	11 (42)	0.01
No. with Banff rejection (I, II, or III) (%)	18 (67)	17 (65)	0.9

^a IQR, interquartile range; CIT, cold ischemia time; DGF, delayed graft function. The five patient deaths were excluded from analysis.

versus 10 of 22 patients). Univariate analyses revealed significant differences among the three patient groups with respect to serum creatinine levels at 12 mo (Figure 1). In addition, the single C4d_{PTC}⁻ case of refractory allograft rejection was classified as C4d_{PTC}⁻/FCXM⁺. In the multivariate logistic regression analysis (confounding factors were donor age, cold ischemia time, and delayed graft function), significantly higher serum creatinine levels at 12 mo were observed for C4d_{PTC}⁺ patients, compared with C4d_{PTC}⁻/FCXM⁻ patients (Table 7). Differences in allograft function between C4d_{PTC}⁻/FCXM⁺ and C4d_{PTC}⁻/FCXM⁻ patients, however, did not achieve statistical significance.

Association of Pretransplant Serologic Findings with Capillary C4d Staining

Eleven of the 16 C4d_{PTC}⁺ recipients (68.8%) exhibited positive pretransplant FCXM findings (T and/or B cell FCXM). Positive pretransplant FCXM findings were signifi-

cantly less common among C4d_{PTC}⁻ patients (7 of 42 patients, 16.7%; $P < 0.0001$). Furthermore, recipient presensitization detected by PRA testing was more common among C4d_{PTC}⁺ patients than among C4d_{PTC}⁻ patients (≥5% FlowPRA reactivity, 10 of 16 versus 15 of 42 patients, $P = 0.08$; ≥5% cytotoxic PRA reactivity, six of 16 versus five of 42 patients, $P = 0.026$).

Discussion

The results of our study demonstrate a strong association between capillary C4d staining in renal allograft biopsy specimens and the presence of alloantibodies detected by sensitive flow cytometric techniques. Our data, which were obtained for kidney transplant recipients not selected for a specific histologic diagnosis, indicate a high specificity but a low sensitivity of capillary C4d deposition as a marker of humoral alloresponses.

Our finding of a high specificity of C4d staining is in line

Table 5. Association of capillary C4d staining and confounding variables with 12-mo allograft function^a

Parameter	OR ^b	95% CI	P Value
C4d deposits in PTC	12.6	1.4 to 110.8	0.02
CIT (per 1-h increment)	1	0.9 to 1.1	0.2
Donor age (per 1-yr increment)	1.1	1.0 to 1.2	0.004
DGF	3.1	0.5 to 18.2	0.2

^a OR, odds ratio; CI, confidence interval; CIT, cold ischemia time; DGF, delayed graft function.

^b Serum creatinine level of >1.755 mg/dl versus serum creatinine level of <1.755 mg/dl, by logistic regression analysis. Cox-Snell pseudo- $r^2 = 0.37$; Hosmer-Lemeshow test model fit; $C = 11.4$, $df = 8$, $P = 0.2$.

with the results of two recently published studies investigating more selected cohorts of kidney transplant recipients (11,13). The finding of three C4d_{PTC}⁺ patients with negative posttransplant FCXM results, however, indicates that capillary C4d staining may not always be associated with the presence or detection of alloantibodies against HLA antigens. C4d deposition among antibody-negative recipients can be speculated to result from the binding of alloantibodies to polymorphic non-HLA antigens that are undetectable by FCXM or FlowPRA testing. A potential pathogenetic role of alloantibodies directed toward non-HLA antigens in kidney transplantation has been demonstrated in numerous studies (21–24). Alternatively, C4d staining could have been produced by very low titers of anti-HLA antibodies escaping detection with flow cytometric techniques. Finally, C4d deposition could have resulted from antibody-independent complement activation. An association of C4d deposition with ischemic injury, in the setting of cardiac allotransplantation, was recently reported (25). In a recently published study, however, we were unable to detect an association between C4d staining and cold ischemia time or the histologic finding of acute tubular damage in kidney transplants (14).

Notably, most FCXM⁺ sera were observed to be both T and B cell FCXM⁺, with a minority containing T or B cell reactivity only. Therefore, the presence of panel reactivity against both HLA class I and II antigens was a frequent finding. Our

results support a pathogenetic role for donor-specific alloantibodies against both HLA classes. Some previous studies focused on a pathogenetic role for anti-HLA class I alloantibodies in acute humoral rejection (5–7); however, other authors also noted a contribution of anti-HLA class II donor-specific antibodies (13,26). Using FCXM testing together with FlowPRA analysis of anti-HLA class I and II antibodies, Piazza *et al.* (26) noted the presence of anti-HLA class II donor-specific antibodies, either alone or together with anti-HLA class I donor-specific antibodies, for as many as 13 of 20 FCXM⁺ kidney transplant recipients.

We noted a substantial proportion of patients who demonstrated no capillary C4d deposits in kidney allograft biopsies but exhibited positive posttransplant FCXM findings and/or significant anti-HLA panel reactivity. These data suggest a low sensitivity of capillary C4d staining. In contrast, in a recent investigation of kidney transplant recipients with steroid-resistant rejection, all C4d_{PTC}⁻ cases were observed to be also antibody-negative (13). There are several possible explanations for this discrepancy, one being differences in patient selection criteria. On the basis of our recent finding that C4d staining may affect graft function independently of histologic features of rejection (14), patients in our study were included irrespective of their histologic diagnoses or clinical courses. This approach led to the inclusion of 23 patients without Banff rejection but also without the typical histologic signs of humoral rejection. Six of those patients exhibited C4d_{PTC} positivity. FCXM positivity was observed for 10 of those patients. Furthermore, in the study reported by Crespo *et al.* (13), some patients were tested only with the less-sensitive standard lymphocytotoxic crossmatch test. In our analysis, all sera were evaluated with sensitive FCXM testing, which allows detection of both complement-fixing and non-complement-binding alloantibodies. Different results regarding the sensitivity of C4d staining may also be attributable to institutional differences in FCXM sensitivity (27). To test this possibility, we used a higher cutoff point between positive and negative reactions (FCXM_{high}). With this cutoff point, we still observed a considerable, although decreased, number of FCXM⁺ cases in the C4d_{PTC}⁻ group.

In our analysis, FCXM testing with donor lymphocytes was used as the standard method for calculating the specificity and sensitivity of C4d staining. The particularly high sensitivity of

Table 6. Serologic findings obtained for three patient groups defined according to C4d staining and FCXM results

	C4d _{PTC} ⁺ (n = 16)	C4d _{PTC} ⁻ /FCXM ⁺ (n = 22)	C4d _{PTC} ⁻ /FCXM ⁻ (n = 20)
No. FCXM ⁺	13	22	
No. FCXM _{high} ^a	12	10	
No. with FlowPRA reactivity of ≥5%	12	9	1
No. with donor-specific anti-HLA antibodies ^b	7	3	1

^a FCXM results graded as FCXM_{high} were defined in Materials and Methods.

^b Antibodies against mismatched donor HLA antigens were assessed by using the FlowPRA specific HLA class I or II antibody test, as detailed in Materials and Methods.

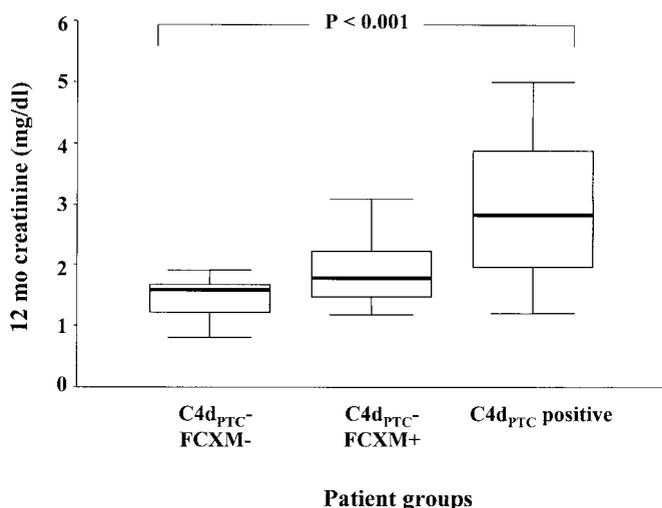


Figure 1. Box plot indicating the median serum creatinine levels at 12 mo (interquartile range and range). For patients undergoing dialysis, a serum creatinine level of 5 mg/dl was presumed. For univariate analysis, the Kruskal-Wallis test was used. The five patient deaths [peritubular capillary C4d deposit-positive (C4d_{PTC}⁺), *n* = 2; C4d_{PTC}⁻/flow cytometric crossmatch-positive (FCXM⁺), *n* = 2; C4d_{PTC}⁻/FCXM⁻, *n* = 1] were excluded from analysis.

FCXM testing may minimize the possibility of negative results attributable to low titers of anti-donor antibodies, which may escape detection with less sensitive crossmatching methods. However, when crossmatch testing with donor lymphocytes is used as the reference method, it must be remembered that, in some instances, this test may produce false-positive results (4). False-positive findings may result from high background levels attributable to nonspecific antibody binding to Fc receptors expressed on donor B cells. Furthermore, in some cases, data may be affected by IgG autoantibody binding to donor lymphocytes.

We also investigated the effects of pretransplant immunologic results on the subsequent occurrence of capillary C4d deposits. We observed an impressive association between flow cytometric detection of pretransplant alloantibodies (a finding that has been well established to be associated with inferior graft outcomes) (18,19,28–31) and posttransplant C4d deposition. Indeed, significant presensitization against donor antigens in the presence of negative standard lymphocytotoxic crossmatch findings was observed for approximately 70% of

C4d_{PTC}⁺ patients. Our data confirm that thorough serologic, histologic, and immunohistochemical evaluation is necessary after transplantation, especially for recipients with detectable presensitization. Timely diagnostic assessments may help identify recipients who may benefit from antihumoral immunosuppressive therapy (15,32).

In line with previous studies (9,11,13,14), we observed an association of C4d positivity with poor kidney allograft outcomes. Four of five immunologic graft losses and two episodes of severe acute rejection with morphologic features of acute humoral rejection, which were successfully reversed with IA in addition to antilymphocyte antibody treatment, were observed in the C4d_{PTC}⁺ patient group. Furthermore, 1-yr serum creatinine levels were substantially higher in C4d_{PTC}⁺ cases, compared with C4d_{PTC}⁻ cases. Combined immunohistochemical and serologic testing allowed the definition of three distinct groups, *i.e.*, C4d_{PTC}⁺, C4d_{PTC}⁻/FCXM⁺, and C4d_{PTC}⁻/FCXM⁻ patients. Univariate analyses revealed significant differences among the three groups with respect to serum creatinine levels at 1 yr. Serum creatinine levels were by far the highest among C4d_{PTC}⁺ patients. Only a slight difference in graft function was observed between C4d_{PTC}⁻/FCXM⁺ and C4d_{PTC}⁻/FCXM⁻ patients. In a multivariate analysis, this modest difference did not achieve statistical significance. For the C4d_{PTC}⁻ case of refractory rejection, a pathogenetic role for alloantibodies can be proposed, because FCXM testing revealed a positive posttransplant crossmatch. Our data suggest particular clinical relevance of posttransplant C4d staining. Detection of complement activation within the graft, as indicated by C4d deposition in PTC, may allow identification of a subgroup of alloantibody-positive patients with poor prognoses. It can be speculated that at least some of these patients might benefit from timely institution of specific antihumoral therapy (15). Our observation that long-term graft outcomes were clearly less affected among FCXM⁺ but C4d_{PTC}⁻ patients emphasizes the particular diagnostic value of C4d staining for humoral graft injury, which may be superior to that of posttransplant FCXM testing alone.

In conclusion, our data confirm the deleterious effects of alloantibodies in kidney transplantation. Capillary C4d staining (a specific marker for the presence of posttransplant alloantibodies) may allow discrimination of harmful humoral immune responses resulting in complement activation within the graft. The clinical data presented in this study demonstrate that positive capillary C4d staining, an independent predictor of

Table 7. Posttransplant immunologic results and 12-mo allograft function^a

Patient Groups	OR ^b	95% Confidence Interval	<i>P</i> Value
C4d _{PTC} ⁻ /FCXM ⁻ (<i>n</i> = 20)	1.0		
C4d _{PTC} ⁻ /FCXM ⁺ (<i>n</i> = 22)	1.7	0.3 to 9.0	0.6
C4d _{PTC} ⁺ (<i>n</i> = 16)	15.4	1.6 to 149.3	0.02

^a OR, odds ratio.

^b Serum creatinine concentrations of >1.755 mg/dl versus serum creatinine concentrations of <1.755 mg/dl, calculated as the dependent variable.

inferior allograft outcomes, represents a reliable specific marker for humoral graft injury.

Acknowledgments

We thank Nicole Huttary for excellent technical assistance and Professor H. Hofmann for valuable scientific support. The study was supported by Grant 1652 from the Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien (to Dr. Böhmig) and by a grant from the Else Kroener-Fresenius Stiftung (to Dr. Regele).

References

1. Feucht HE, Opelz G: The humoral immune response towards HLA class II determinants in renal transplantation. *Kidney Int* 50: 1464–1475, 1996
2. Baldwin WM III, Halloran PF: Clinical syndromes associated with antibody in allografts. In: *Kidney Transplant Rejection*, edited by Racusen LC, Solez K, Burdick JF, New York, Marcel Dekker, 1998, pp 127–147
3. Crespo M, Delmonico F, Saidman S, Tolkoff-Rubin N, Williams W, Colvin R, Cosimi AB, Pascual M: Acute humoral rejection in kidney transplantation. *Graft* 3: 12–17, 2000
4. McKenna RM, Takemoto SK, Terasaki PI: Anti-HLA antibodies after solid organ transplantation. *Transplantation* 69: 319–326, 2000
5. Halloran PF, Wadgymar A, Ritchie S, Falk J, Solez K, Srinivasa NS: The significance of the anti-class I 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 response. II. Clinical and pathologic features of renal transplants with anti-class I-like antibody. *Transplantation* 53: 550–555, 1992
7. 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
8. Feucht HE, Felber E, Gokel MJ, Hillebrand G, Nattermann U, Brockmeyer C, Held E, Riethmüller 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
9. Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Riethmüller G, Land W, Albert E: Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int* 43: 1333–1338, 1993
10. Lederer SR, Schneeberger H, Albert E, Johnson JP, Gruber R, Land W, Burkhardt K, Hillebrand G, Feucht HE: Early renal graft dysfunction: The role of preformed antibodies to DR-typed lymphoblastoid cell lines. *Transplantation* 61: 313–319, 1996
11. Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N, Cosimi AB, Colvin RB: Complement activation in acute humoral renal allograft rejection: Diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 10: 2208–2214, 1999
12. 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
13. Crespo M, Pascual M, Tolkoff-Rubin N, Mauyyedi 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
14. Regele H, Exner M, Watschinger B, Wenter C, Wahrman M, Österreicher C, Säemann MD, Mersich N, Hörl WH, Zlabinger GJ, Böhmig GA: Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. *Nephrol Dial Transplant* 16: 2058–2066, 2001
15. Böhmig GA, Regele H, Exner M, Derhartunian V, Kletzmayer J, Säemann MD, Hörl WH, Druml W, Watschinger B: C4d-positive acute humoral renal allograft rejection: Effective treatment by immunoadsorption. *J Am Soc Nephrol* 12: 2482–2489, 2001
16. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Crocker 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
17. Terasaki PI, McClelland JD: Microdroplet assay of human serum cytotoxins. *Nature (Lond)* 204: 998–1000, 1964
18. Talbot D, Cavanagh G, Coates E, Givan AL, Shenton BK, Lennard TWJ, Proud G, Taylor RMR: Improved graft outcome and reduced complications due to flow cytometry crossmatching and DR matching in renal transplantation. *Transplantation* 53: 925–928, 1992
19. Lazda VA: Identification of patients at risk of inferior allograft outcome by a strongly positive B cell flow cytometry crossmatch. *Transplantation* 57: 964–969, 1994
20. Pei R, Wang G, Tarsitani C, Rojo S, Chen T, Takemura S, Liu A, Lee J: Simultaneous HLA class I and class II antibodies screening with flow cytometry. *Hum Immunol* 59: 313–322, 1998
21. Wood NL, Schook LB, Studer EJ, Mohanakumar T: Biochemical characterization of human vascular endothelial cell-monocyte antigens defined by monoclonal antibodies. *Transplantation* 45: 787–792, 1988
22. Yard B, Spruyt-Gerritse M, Claas F, Thorogood J, Bruijn JA, Paape ME, Stein SY, van Es LA, van Bockel JH, Kooymans-Coutinho M, Daha MR, van der Woude FJ: 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
23. Sumitran-Karuppan S, Tyden G, Reinholdt F, Berg U, Moller E: Hyperacute rejections of two consecutive renal allografts and early loss of the third transplant caused by non-HLA antibodies specific for endothelial cells. *Transplant Immunol* 5: 321–327, 1997
24. Perrey C, Brenchley PE, Johnson RW, Martin S: An association between antibodies specific for endothelial cells and renal transplant failure. *Transplant Immunol* 6: 101–106, 1998
25. Baldwin WM III, Samaniego-Picota M, Kasper EK, Clark AM, Czader M, Rohde C, Zachary AA, Sanfilippo F, Hruban RH: Complement deposition in early cardiac transplant biopsies is associated with ischemic injury and subsequent rejection episodes. *Transplantation* 68: 894–900, 1999
26. Piazza A, Poggi E, Borrelli L, Servetti S, Monaco PI, Buonomo O, Valeri M, Torlone N, Adorno D, Casciani CU: Impact of donor-specific antibodies on chronic rejection occurrence and graft loss in renal transplantation: Posttransplant analysis using

- flow cytometric techniques. *Transplantation* 71: 1106–1112, 2001
27. Scornik JC, Bray RA, Pollack MS, Cook DJ, Marrari M, Duquesnoy R, Langley JW: Multicenter evaluation of the flow cytometry T-cell crossmatch. *Transplantation* 63: 1440–1445, 1997
 28. Garovoy MR, Rheinschmidt MA, Bigos M, Perkins H, Colombe B, Feduska N, Salvatierra O: Flow cytometry analysis: A high technology crossmatch technique facilitating transplantation. *Transplant Proc* 15: 1939–1944, 1983
 29. Mahoney RJ, Ault KA, Given SR, Adams RJ, Breggia AC, Paris PA, Palomaki GE, Hitchcox SA, White BW, Himmelfarb J, Leeber DA: The flow cytometric crossmatch and early renal transplant loss. *Transplantation* 49: 527–535, 1990
 30. Kerman RH, Van Buren CT, Lewis RM, DeVera V, Baghdasarian V, Gerolami K, Kahan BD: Improved graft survival for flow cytometry and antihuman globulin crossmatch-negative retransplant recipients. *Transplantation* 49: 52–56, 1990
 31. Ogura K, Terasaki PI, Johnson C, Mendez R, Rosenthal JT, Ettenger R, Martin DC, Dainko E, Cohen L, Mackett T, Berne T, Barba L, Lieberman E: The significance of a positive flow cytometry crossmatch test in primary kidney transplantation. *Transplantation* 56: 294–298, 1993
 32. Böhmig GA, Regele H, Säemann MD, Exner M, Druml W, Kovarik J, Hörl WH, Zlabinger GJ, Watschinger B: Role of humoral immune reactions as target for antirejection therapy in recipients of a spousal-donor kidney graft. *Am J Kidney Dis* 35: 667–673, 2000