Capillary Deposition of Complement Split Product C4d in Renal Allografts is Associated with Basement Membrane Injury in Peritubular and Glomerular Capillaries: A Contribution of Humoral Immunity to Chronic Allograft Rejection

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Abstract. Endothelial deposition of the complement split product C4d is an established marker of antibody-mediated acute renal allograft rejection. A contribution of alloantibody-dependent immune reactions to chronic rejection is under discussion. In this study, the association of immunohistochemically detected endothelial C4d deposition in peritubular capillaries (PTC) with morphologic features of chronic renal allograft injury was investigated in a large study cohort. C4d deposits in PTC were detected in 73 (34%) of 213 late allograft biopsies performed in 213 patients more than 12 mo after transplantation (median, 4.9 yr) because of chronic allograft dysfunction. Endothelial C4d deposition was found to be associated with chronic transplant glomerulopathy (CG) (P < 0.0001), with basement membrane multilayering in PTC (P = 0.01), and with an accumulation of mononuclear inflammatory cells in PTC (P < 0.001). Furthermore, C4d deposits in PTC (in biopsies with normal glomerular morphology) were associated with development of CG in follow-up biopsies. Other morphologic features of chronic allograft nephropathy (with exception of tubular atrophy) were not associated with C4d deposits in PTC. Analyses of previous and follow-up biopsies revealed that C4d deposits may occur de novo and may also disappear at any time after transplantation. In conclusion, the data suggest that complement activation in renal microvasculature, indicating humoral alloreactivity, contributes to chronic rejection characterized by chronic transplant glomerulopathy and basement membrane multilayering in PTC.

Acute rejection is the most important threat to transplanted kidneys in the early phase after transplantation. Advances in understanding immunologic mechanisms underlying acute allograft rejection enabled the development of efficient diagnostic tools and therapeutic strategies against early immune-mediated graft loss. This led to an increase of 1-yr graft survival rates to more than 90%. Long-term graft survival did not, however, improve to a similar degree (1). A steady decline of renal function over years is still the rule in the majority of cases. Chronic allograft nephropathy (CAN) is a common morphologic finding in late allograft dysfunction. The term CAN (as defined by the Banff classification [2]) does not, however, describe a specific pathologic process; rather, it comprises various types of chronic injuries that may result from a variety of underlying pathogenic mechanisms. Both alloantigen-dependent and alloantigen-independent (e.g., drug toxicity, hypertension, recurrence of disease) mechanisms may be involved (1,3,4). Use of the term CAN reflects the limited ability to discriminate alloantigen-dependent damage from other causes of tissue injury (3). This uncertainty about pathogenic mechanisms of chronic allograft failure is a major obstacle to the development of specific therapeutic interventions. Chronic transplant glomerulopathy (CG) and distinct morphologic changes in arteries and capillaries have been reported as signs of chronic immunologic damage, i.e., true chronic rejection (2,5). The precise nature of the underlying immunologic mechanisms is, however, still unclear (6,7).

Capillary deposition of C4d, reflecting complement activation via the classical pathway (8), is an established marker of antibody-mediated allograft rejection early after transplantation (9–16). In acute rejection, this complement split-product has been shown to be closely associated with the presence of alloantibodies (9–16). C4d was also identified in renal allo-
graft biopsies with morphologic signs of chronic rejection (5,17). Previous studies on C4d in chronically dysfunctioning allografts have suggested a pathogenic role of humoral immunity in a subset of patients (5,18). This finding has been challenged by others (19). Controversial results might at least partially be due to differences in the design of the cited studies, which were performed in highly selected patient groups (5), included small study populations (5,17), did not analyze allograft morphology (20–22), or made no clear distinction between early and late allograft biopsies (19).

In this study, a large series of unselected late renal allograft biopsies was examined immunohistochemically for endothelial deposition of C4d as surrogate marker antibody-mediated alloreactivity in renal allografts. The aim of the study was to systematically analyze the incidence of C4d deposits in late allograft biopsies and to determine the pattern of histomorphologic changes in chronic humoral allograft injury.

Materials and Methods

Study Population

From 1992 to 1998, a total of 1139 individuals were seen with a functioning graft in the outpatient department of our transplant unit. According to local practice, biopsies were not performed on a prospective protocol basis but were based on the clinical course of the patients (e.g., deterioration of kidney function, proteinuria, etc.). Of 1139 recipients, 213 were enrolled in this retrospective cohort study on the basis of the following inclusion criteria: (1) availability of at least one allograft biopsy (index biopsy) performed more than 12 mo after transplantation and during the observation period between July 1992 and September 1998; (2) availability of adequate tissue for histologic diagnosis and immunohistochemistry; and (3) patient follow-up (including all previous and subsequent allograft biopsies) at our institution. The study population consisted of patients transplanted between 1977 and 1997. In this period, 2339 patients were transplanted at our institution (University of Vienna, Austria). For patients with two or more biopsies performed more than 12 mo after transplantation, the first of late biopsies was enrolled as “index biopsy”. Patients were not selected for indication of biopsy, clinical history, or specific diagnoses. Indications for biopsy were a significant increase of serum creatinine levels and/or occurrence of proteinuria (>1.5 g/24 h). In 96 patients, at least one biopsy preceding the index biopsy was available (“previous biopsy”). In 58 patients, “follow-up” biopsies were studied. In cases with more than one “previous” or “follow-up” biopsy, the first biopsy preceding or following the index biopsy was evaluated. In 43 cases, renal cortical tissue samples were available for morphologic analysis of peritubular capillaries (PTC) on electron microscopy (EM). Patient demographics and basic clinical data are listed in Table 1.

Histopathology

A total of 367 kidney allograft biopsies were evaluated by two independent observers. Pathohistologic evaluation was performed on formalin-fixed paraffin sections stained with hematoxylin and eosin, periodic acid-Schiff, Methenamin-Silver, and with a trichrome stain (acidic-Fuchsine-orange-G). Histologic lesions were classified and scored according to the Banff 97 classification (2) without previous disclosure of C4d staining results. Additional morphologic changes potentially related to acute humoral rejection (granulocytes or monocytar inflammatory cells in PTC, thrombotic microangiopathy) were also scored semiquantitatively. The diagnosis of chronic cyclosporine toxicity was made only in cases with de novo arteriopathy in absence of severe hypertension or diabetes mellitus.

Immunohistochemistry and Immunofluorescence

Generation, characterization, and staining properties of the monoclonal anti-C4d antibody, C4dpAb (Biomedica, Vienna, Austria), which is suitable for immunohistochemistry on paraffin sections, have been reported previously (9). On frozen sections, staining patterns obtained with C4dpAb are identical to those obtained with a commercially available monoclonal antibody (9). For immunohistochemical detection of C4d, 2-μm sections were deparaffinized and endogenous peroxidase activity was blocked with hydrogen peroxide/methanol. Antigen retrieval was carried out by pressure-cooking for 10 min at 1 bar in citrate-buffer (pH 6.0) as described previously (9). Endogenous biotin was blocked using a Biotin blocking kit (Vector Laboratories, Burlingame, CA). After 30-min incubation with C4dpAb (1:50), bound IgG was visualized using the Supersensitive Kit (BioGenex, San Ramon, CA) according to the manufacturer’s protocol.

PTC staining was categorized semiquantitatively according to the extent and intensity of staining. Scoring was performed without knowledge of histologic diagnoses or clinical history of recipients. Biopsies with circumferential linear endothelial C4d staining in at least 25% of PTC were designated as C4dpAb-positive, all other biopsies as C4dpAb-negative. This definition and the relevance of a cut-off point of 25% for positive staining were validated in a previous study by flow-cytometric detection of circulating antibodies (12).

Table 1. Patient demographics and clinical parameters

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>All Patients (n = 213)</th>
<th>C4d in PTC (Index Biopsy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n = 73)</td>
<td>No (n = 140)</td>
</tr>
<tr>
<td>Male patients, n (%)</td>
<td>123/213 (58)</td>
<td>43/73 (59)</td>
</tr>
<tr>
<td>Recipient age (yr), median (IQR)</td>
<td>45 (34 to 61)</td>
<td>44 (32 to 54)</td>
</tr>
<tr>
<td>Biopsy interval after TXb (yr), median (IQR)</td>
<td>4.9 (2.4 to 7.5)</td>
<td>4.8 (2.4 to 7.0)</td>
</tr>
<tr>
<td>First transplant, n (%)</td>
<td>171/213 (80)</td>
<td>54/73 (74)</td>
</tr>
<tr>
<td>Previous episodes of acute rejection, n (%)</td>
<td>50/213 (23)</td>
<td>13/73 (18)</td>
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</tbody>
</table>

* IQR, interquartile range; PTC, peritubular capillaries; TX, transplantation.
* The first biopsy taken later than 1 yr was included into the study.
* Biopsy-proven episodes of acute rejection.
Immunofluorescent double staining was carried out on paraffin sections of ten C4dPTC-positive and five C4dPTC-negative cases. Sections were pretreated as described above and were incubated with C4dAb (1:10) together with an anti-collagen type IV (1:50) (DAKO, Glostrup, Denmark) or an anti-CD31 (1:20; DAKO) monoclonal antibody for 30' at room temperature. Bound primary antibody was detected with an Alexa-486–conjugated goat anti-rabbit IgG antibody (1:1000; Molecular Probes, Eugene, OR) for C4dAb or an Alexa-594-conjugated goat anti-mouse IgG antibody (1:1000; Molecular Probes) for anti-collagen IV and anti-CD31.

Electron Microscopy

Electron microscopy could be performed in 43 of 213 index biopsies. Biopsies were analyzed for the presence of basement membrane multilayering in PTC (MLPTC). Biopsy specimens were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer with 0.1% glutaraldehyde, post-fixed with 1% OsO4 in 0.1 M sodium cacodylate buffer, and stained with 1% aqueous uranyl acetate. Specimens were embedded in epoxy-resin (Serva electrophoresis, Heidelberg, Germany) (polymerization for 36 h at 60°C). Ultrathin sections were prepared on an Ultracut-E Ultramicrotome (Reichert, Austria). After staining with uranyl acetate in methanol and with lead citrate, sections were examined with a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

On the basis of the examination of at least six PTC per biopsy, we categorized MLPTC as being absent if no or only focal splitting of basement membranes (BM) was present, as low-grade MLPTC if three to four BM layers could be observed (with more severe lesions only focally distributed). High-grade MLPTC was defined by five and more BM layers as predominant lesion.

Immune Electron Microscopy

Biopsy specimens obtained from four patients with C4d deposits detected by conventional immunohistochemistry were fixed in freshly prepared 4% formaldehyde in PBS for 8 h and then embedded in Lowicryl K4M (Chemische Werke LOWI, Waldkraiburg, Germany) (23). Ultrathin sections were blocked with 1% bovine serum albumin in PBS for 1 h followed by incubation with C4dAb (diluted 1:1 in 1% BSA, 0.5% Tween-20, 0.1% Triton-X100 in PBS) for 20 h at 4°C. After washing in PBS, slides were incubated for 1 h at room temperature with 10 nm of gold-labeled goat-anti-rabbit IgG (Amersham Pharmacia Biotech, Little Chalfont, UK). Sections were stained with 2% aqueous uranyl acetate and lead citrate (24) and examined with a JEOL JEM 1010 transmission electron microscope.

Statistical Analyses

Data are presented as the median and interquartile range (range from the 25th to the 75th percentile). Percentages were calculated for dichotomous variables. The χ2 test or, if appropriate, Fisher’s exact test were applied for comparison of proportions. The cumulative proportion of CG-free patients in C4d-positive and C4d-negative subjects during the follow-up time interval was calculated by the Kaplan Meier method and compared by means of the log rank test. A P value < 0.05 was accepted as statistically significant; all P values were two sided. Calculations were performed with SPSS for Windows (Version 10.0; SPSS Inc, Chicago, IL).

Results

Linear C4d Staining in Peritubular Capillaries of Late Allograft Biopsies

Seventy-three of the 213 index biopsies (34%) showed linear C4d deposition along endothelial cells of PTC (Figure 1A). C4dPTC-positive cases did not differ significantly from those without C4d deposits regarding gender, age, date of transplantation, time from transplantation to index-biopsy, number of previous transplants, or incidence of acute rejection within the first year after transplantation (Table 1).

Linear C4d Staining in Glomerular Capillaries is Uncommon

In 13 (6%) of 213 index biopsies, linear C4d deposits were also observed among glomerular endothelial cells. In all cases with glomerular endothelial staining, C4d deposits were also present in PTC. In addition, C4dAb detected glomerular immune deposits in 30 cases of immune complex-mediated glomerulonephritis (GN). Our evaluations were based on C4d staining in PTC (C4dPTC).

C4d Staining in PTC of Allograft Biopsies Reflects Endothelial C4d Deposits

To determine the exact localization of C4d deposits in PTC, immune electron microscopy and immunofluorescent double staining were performed in selected C4dPTC-positive biopsies. By immune electron microscopy, antibody-conjugated gold particles were located at the luminal surface of peritubular capillary endothelial cells, between endothelial cells and the basement membrane of PTC and also within some intracellular vesicles (Figure 2). A perfect co-localization of C4d with the endothelial cell marker CD31, demonstrated by immunofluorescent double staining, confirms that C4d is bound to endothelial cells (Figure 1, C through E), whereas double labeling for C4d and collagen IV (as marker for basement membranes) only reveals focal overlap but no true co-localization of staining signals (Figure 1, F through H).

C4d Staining in PTC is Associated with Chronic Transplant Glomerulopathy (CG)

Next, the 213 index biopsies were analyzed for associations of C4d deposition in PTC with morphologic signs of rejection. C4d staining was neither associated with the diagnosis of acute rejection (as defined by the Banff classification) nor with the presence of CAN (as defined by the Banff classification). The former was a rare finding (7% of index-biopsies), whereas the latter was found in the majority of both C4dPTC-positive (92%) and C4dPTC-negative (87%) biopsies (Tables 2 and 3).

Analysis of individual criteria defining Banff chronic allograft nephropathy revealed a highly significant association of C4dPTC staining with CG (Figure 3A) (53% of C4dPTC-positive versus 14% of C4dPTC-negative biopsies; P < 0.0001). Glomerular endothelial C4d staining, a rare finding in general (6%), was observed in 12% of the biopsies showing CG. Tubular atrophy was present in nearly all C4dPTC-positive biopsies (99%) and was less common in biopsies without C4dPTC deposits (89%; 31). Interstitial fibrosis, intimal fibrosis, and mesangial sclerosis of glomeruli were not linked to the presence or absence of C4dPTC staining (Table 2).

Analyzing individual diagnostic lesions of acute rejection, we found a higher percentage of cases with glomerulitis (20% of C4dPTC-positive versus 9% of C4dPTC-negative
biopsies; \( P = 0.02 \) or (predominantly low-grade) tubulitis (37% of \( C4d_{\text{PTC}} \)-positive versus 24% of \( C4d_{\text{PTC}} \)-negative biopsies; \( P = 0.04 \)) in \( C4d_{\text{PTC}} \)-positive biopsies. Interstitial inflammation and arterial intimal arteritis were not associated with \( C4d \) deposition in PTC. Neither an accumulation of granulocytes in PTC nor thrombotic microangiopathy (both reported as being typical for acute humoral rejection) were found to be more common in biopsies with \( C4d_{\text{PTC}} \) deposits. In our study population, however, these lesions were very rare findings (Table 3).

**C4d Staining in PTC is Associated with Multilayering of Basement Membranes in PTC**

Lamellar splitting and thickening of the BM (Figure 3, C and D) represent the ultrastructural lesions characterizing basement membrane multilayering in PTC (MLPTC). Suitable material
for EM was available for 43 cases of our study population. MLPTC was identified in 28 specimens (Table 4). Twenty-one of 43 biopsies were C4dPTC-positive (Table 4). Analyzing at least six PTC per biopsy, we quantified the lesion on the basis of the number of newly formed lamellae. MLPTC was more common on C4dPTC-positive biopsies (Table 4). In particular, high-grade MLPTC demonstrated a strong association with C4dPTC deposits (15 of 21 in C4dPTC-positive cases versus 3 of 22 in the cases without C4dPTC; \( p = 0.001 \)). MLPTC was also strongly associated with CG (16 of 18 cases with MLPTC also showed CG; \( p = 0.009 \)).

Mononuclear Inflammatory Cells Accumulate in C4d-Positive PTC
A common finding in C4dPTC-positive biopsies was a conspicuous accumulation of mononuclear cells in (frequently dilated) lumina of PTC (Figure 1B) (51% of C4dPTC-positive versus 25% of C4dPTC-negative biopsies; \( p < 0.001 \)). Forty-three percent (31 of 72) of biopsies with accumulation of mononuclear cells in PTC also showed tubulitis. Acute interstitial rejection (Banff I) was, however, diagnosed in only four cases because tubulitis and interstitial inflammation were mild in most biopsies (t1 and i1 according to Banff). In biopsies without increased numbers of inflammatory cells in PTC, tubulitis occurred in only 22% (31 of 141; \( p = 0.02 \)) of cases.

Chronic cyclosporine toxicity showed no association with C4d deposits in PTC (Table 2). Glomerulonephritis (membranous GN, \( n = 16 \); IgA nephritis, \( n = 11 \); mesangial proliferative GN, \( n = 5 \); membranoproliferative GN, \( n = 1 \); crescentic GN, \( n = 1 \)) was more commonly observed in C4dPTC-positive biopsies (25% of C4dPTC-positive versus 11% of C4dPTC-negative biopsies; \( p = 0.02 \)) (Table 3).

C4d Staining is Variable in Sequential Biopsies
To evaluate the dynamics of endothelial C4d deposition over time, we examined allograft biopsies preceding index biopsies (96 cases) and first follow-up biopsies after the index biopsy (58 cases) for the presence of C4dPTC deposits. In 31 cases, all three biopsy types (previous, index, and follow-up) were available. Analysis of all 367 biopsies revealed a time interval from transplantation to the first biopsy with C4dPTC deposits ranging from 1 wk to 17.4 yr. We found C4dPTC to be highly variable over time. In many cases C4dPTC deposits developed de novo several years after transplantation. In some cases, disappearance of C4dPTC deposits was observed in follow-up biopsies (Table 5). Thirty-four of 96 index biopsies for which previous biopsies were available, were C4dPTC-positive. In only eight (24%) of these cases, C4dPTC deposits were present already in a previous biopsy (Table 5). On the other hand, in 5 of 13 patients who had C4dPTC detectable in their previous biopsy (performed within the first year after transplantation), no more C4dPTC was present in the subsequent index biopsy (Table 5). A similar variability of C4dPTC staining was observed between index biopsy and follow-up biopsies (Table 5). Eight (38%) out of 21 patients with a C4dPTC-positive index biopsy were C4dPTC-negative in follow-up biopsies. After index biopsy, immunosuppressive therapy was modified in only one of the eight recipients (conversion to MMF). Furthermore, dosage of immunosuppressive drugs was not increased after index biopsy. The median time interval between C4dPTC-positive and subsequent C4dPTC-negative biopsies was 16 mo (range, 6 to 61). Twelve (48%) out of 25 patients with a C4dPTC-positive “follow-up” biopsy were C4dPTC-negative in their corresponding index biopsy (Table 5). In only three patients (out of a group of 31 with 3 sequential biopsies) C4dPTC was constantly present from the first (previous) to the last (follow-up) biopsy.

C4d Staining Is Associated with Progression to CG in Subsequent Biopsies
C4dPTC deposition in biopsies with unaffected glomeruli showed a significant association with the development of CG in follow-up biopsies (Figure 4). In nine (82%) of eleven allografts (with follow-up biopsy available) showing C4dPTC deposits but no detectable CG in the index biopsy, progression to CG could be observed in the follow-up biopsy. Of 33 C4dPTC-negative cases (in index biopsy), only 9 (27%) developed CG in a follow-up biopsy (log rank test, \( p = 0.04 \)) (Table 6). Similarly, 6 (46%) of 13 patients with C4dPTC-positive previous biopsies (performed during first year after transplantation) developed CG in a subsequent C4dPTC-negative index biopsy. In only 5 (6%) of 82 C4dPTC-negative previous biopsies, CG was detectable in the following (still C4dPTC-negative) index biopsy (log rank test, \( p < 0.0001 \)).

Discussion
Chronic allograft dysfunction represents a major clinical problem after transplantation. It is widely accepted that pathogenic mechanisms underlying CAN are heterogeneous in nature (1–4). Both alloantigen-dependent (true chronic rejection) and alloantigen-independent mechanisms may result in very similar patterns of injury. The term chronic allograft nephropathy (CAN) has been coined for this common but nonspecific morphologic appearance of chronically dysfunctioning allo-
Table 2. C4d<sub>PTC</sub> staining and morphologic signs of chronic allograft injury<sup>a</sup>

<table>
<thead>
<tr>
<th>Pathohistology</th>
<th>Index Biopsies (n = 213)</th>
<th>C4d in PTC (Index Biopsy)</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes (n = 73)</td>
<td>No (n = 140)</td>
</tr>
<tr>
<td>Chronic allograft nephropathy, n (%)</td>
<td>189/213 (87)</td>
<td>67/73 (92)</td>
<td>122/140 (87)</td>
</tr>
<tr>
<td>Chronic cyclosporine toxicity, n (%)</td>
<td>26/213 (12)</td>
<td>8/73 (11)</td>
<td>18/140 (13)</td>
</tr>
<tr>
<td>Chronic glomerulopathy, n (%)</td>
<td>58/213 (27)</td>
<td>39/73 (53)</td>
<td>19/140 (14)</td>
</tr>
<tr>
<td>Interstitial fibrosis, n (%)</td>
<td>207/213 (97)</td>
<td>73/73 (100)</td>
<td>134/140 (96)</td>
</tr>
<tr>
<td>Tubular atrophy, n (%)</td>
<td>197/213 (93)</td>
<td>72/73 (99)</td>
<td>125/140 (89)</td>
</tr>
<tr>
<td>Intimal fibrosis, n (%)</td>
<td>169/213 (79)</td>
<td>56/73 (77)</td>
<td>113/140 (81)</td>
</tr>
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<td>Mesangial sclerosis, n (%)</td>
<td>101/213 (48)</td>
<td>36/73 (49)</td>
<td>65/140 (46)</td>
</tr>
<tr>
<td>Arteriolar hyalinosis, n (%)</td>
<td>139/213 (65)</td>
<td>42/73 (56)</td>
<td>97/140 (69)</td>
</tr>
</tbody>
</table>

<sup>a</sup> PTC, peritubular capillaries.
<sup>b</sup> χ<sup>2</sup> test, C4d<sub>PTC</sub>-positive versus C4d<sub>PTC</sub>-negative subgroup.
<sup>c</sup> Lesions are defined and scored according to the Banff 97 classification.

Table 3. C4d<sub>PTC</sub> staining and histomorphology<sup>a</sup>

<table>
<thead>
<tr>
<th>Pathohistology</th>
<th>Index Biopsies (n = 213)</th>
<th>C4d in PTC (Index Biopsy)</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes (n = 73)</td>
<td>No (n = 140)</td>
</tr>
<tr>
<td>Banff I, II, or III, n (%)</td>
<td>13/213 (6)</td>
<td>6/73 (8)</td>
<td>7/140 (5)</td>
</tr>
<tr>
<td>Glomerulitis, n (%)</td>
<td>27/213 (13)</td>
<td>15/73 (20)</td>
<td>12/140 (9)</td>
</tr>
<tr>
<td>Interstitial inflammation, n (%)</td>
<td>135/213 (63)</td>
<td>49/73 (67)</td>
<td>86/140 (61)</td>
</tr>
<tr>
<td>Tubulitis, n (%)</td>
<td>62/213 (29)</td>
<td>28/73 (37)</td>
<td>34/140 (24)</td>
</tr>
<tr>
<td>Intimal arteritis, n (%)</td>
<td>5/213 (2)</td>
<td>3/73 (4)</td>
<td>2/140 (1)</td>
</tr>
<tr>
<td>Granulocytes in PTC, n (%)</td>
<td>8/213 (4)</td>
<td>5/73 (7)</td>
<td>3/140 (2)</td>
</tr>
<tr>
<td>Mononuclear cells in PTC, n (%)</td>
<td>72/213 (34)</td>
<td>37/73 (51)</td>
<td>35/140 (25)</td>
</tr>
<tr>
<td>Thrombotic microangiopathy, n (%)</td>
<td>5/213 (2)</td>
<td>3/73 (3)</td>
<td>2/140 (1)</td>
</tr>
<tr>
<td>Glomerulonephritis, n (%)</td>
<td>34/213 (14)</td>
<td>18/73 (25)</td>
<td>16/140 (11)</td>
</tr>
</tbody>
</table>

<sup>a</sup> PTC, peritubular capillaries.
<sup>b</sup> χ<sup>2</sup> test, C4d-positive versus C4d-negative subgroup.
<sup>c</sup> Lesions are defined and scored according to the Banff 97 classification.

grafts (2,3). Reliable identification of the various conditions that lead to morphologic and functional damage is a prerequisite for specific therapeutic interventions. In most patients with chronic allograft dysfunction, however, estimation of the relative contribution of individual damaging processes to a given injury (3) is a very difficult task.

It is generally accepted that alloimmune mechanisms are among the major causes of chronic allograft injury (1,7). The exact nature of chronic allograft injury and its contribution to graft injury are, however, far from being defined, and reliable diagnostic criteria are lacking (2,3). Several studies emphasize an important role of alloantibody-mediated processes in chronic rejection (5,7,17,20–22,25). Some of these studies employed C4d staining as a marker of humoral immunity (5,17). A recent report, however, failed to confirm a link between C4d and chronic allograft injury (19).

A novel anti-C4d antibody (9) suitable for detection of C4d on paraffin sections enabled this retrospective study on archival biopsies. Protocol biopsies of kidneys with stable function are not available at our center; the study was therefore by definition limited to patients with functional impairment of the renal allograft. In this study population, C4d deposits were detected in 34% of late allograft biopsies. Previously, Mauiyeddi et al. (5) reported C4d deposits in 61% of cases with histomorphologic evidence of chronic rejection (i.e., transplant glomerulopathy or arteriopathy), which is quite similar to our observation of 53% C4d positivity in biopsies with transplant glomerulopathy. Only 13% of C4d<sub>PTC</sub>-positive biopsies were observed by same group in a separate analysis of unselected cases of chronic allograft dysfunction (26). The true incidence of C4d deposits cannot be deduced from our data. This issue has to be addressed in a study employing protocol biopsies. However, the results of this study clearly demonstrate a close link between complement activation on capillary endothelial cells of renal allografts and morphologic signs of chronic rejection.

C4d deposits on endothelial cells of renal microvessels can be observed in a fraction of allograft biopsies but not in native kidneys specimens (9,27). Co-localization of C4d with CD31 in immunofluorescent double staining confirms the endothelial
localization of C4d deposits. As shown by immune electron microscopy, C4d deposits are restricted to the surface (i.e., the luminal and also the abluminal cell membrane), and sometimes to intracellular vesicles of endothelial cells (possibly indicating a transcytotic process). This suggests targeted activation of complement rather than passive precipitation of serum components. Several lines of evidence suggest a close relation of immunohistochemically detectable C4d and the presence of donor-specific antibodies; C4d deposits in renal allograft biopsies are associated with pretransplant (9,12,14) and posttransplant (12–14,16) detection of circulating anti-HLA antibodies. In addition, removal of circulating anti-donor antibodies by immunoadsorption leads to restoration of renal function in otherwise therapy-resistant C4d-positive acute rejection (10,11).

Endothelial C4d deposits seem, however, to be unevenly distributed throughout the renal microvasculature. The infrequent detection of C4d deposits on glomerular endothelial cells (even in cases with CG) might be due to biologic mechanisms that counteract the persistence of complement deposits on glomerular endothelial cells, rendering endothelial C4d deposits undetectable by immunohistochemistry. A possible explanation could be an enhanced glomerular endothelial regeneration, resulting in a higher rate of endothelial cell turnover, which leads to a more efficient elimination of complement-loaded endothelial cells in glomeruli than in PTC (28).

This study established a close link of endothelial C4d deposition with chronic transplant glomerulopathy (CG) and multilayering of basement membranes in PTC (MLPTC). MLPTC has previously been described in association with antibody-mediated immune mechanisms (18,29). Presumably, the association of C4dPTC with CG was not detected in previous studies either because CG was one of the inclusion criteria (5,17) or late allograft biopsies were analyzed together with early allograft biopsies (with a very low incidence of CG), thus obscuring the association between C4dPTC and CG (19).

CG and MLPTC are commonly regarded as signs of chronic allograft rejection in humans as well as in experimental models (5,29–33). These lesions tend to occur simultaneously (29,30,33,34) and show similar patterns of basement membrane thickening and reduplication, which are believed to result from endothelial injury (29,30,33). Complement activation (reflected by endothelial C4d deposition) is likely to play a major causative role in endothelial injury of allografts. It might thus represent the pathogenic link between CG and MLPTC, thereby explaining the frequently observed simultaneous occurrence of these two morphologic lesions. In view of the close relation of endothelial C4d deposition with serologically detectable alloantibodies (5,10,12–14) it is very likely that endothelial-bound alloantibodies (inducing complement-mediated development of CG and MLPTC) represent an important driving force in chronic rejection.

Other morphologic signs of CAN (with the exception of tubular atrophy being more common in C4dPTC-positive cases) were not associated with C4dPTC. This is also true for arterial intimal fibrosis, a lesion being potentially related to chronic rejection (2,5). Several non-alloantigen-related conditions (e.g., hypertension, donor-derived arteriosclerosis) may, however, also contribute to intimal fibrosis.

There also was no association of C4dPTC staining with acute rejection (defined according to the Banff classification), which is of course rare in late allograft biopsies. Analysis of individual morphologic criteria for acute cellular and/or humoral rejection revealed only tubulitis (mostly as low-grade lesion and therefore not sufficient for diagnosis of acute rejection Banff type I) and glomerulitis to be more common in C4dPTC-positive biopsies.

A remarkable finding was a frequently observed accumulation of mononuclear cells (but not of granulocytes as observed in acute humoral rejection) in PTC of C4dPTC-positive biopsies. A study on ultrastructural alterations of PTC in antibody-mediated rejection (mainly dealing with the acute phase of rejection) reported a similar accumulation of not further specified mononuclear inflammatory cells in PTC in a few biopsies performed 8 mo after transplantation (29). We failed, however, to establish an association between either inflammatory cells or C4dPTC deposition and interstitial rejection. The pathogenic or
diagnostic relevance of inflammatory cells accumulating in PTC is therefore currently not clear.

In acute humoral rejection, endothelial C4d deposition mostly develops within few days or weeks after transplantation (9,12,14,15). There is scarce information, however, about the dynamics of C4d deposits in later phases after transplantation. C4d deposits were found to occur de novo at virtually any time after transplantation. In certain cases, C4d PTC deposition was detectable in sequential biopsies over years. On the other hand, there were a considerable number of C4dPTC-positive cases without C4d PTC depositions in subsequent biopsies. The trigger for the occurrence of alloantibodies late after transplantation is yet unclear; however, the indirect pathway of allorecognition could be one attractive candidate. In this pathway recipient CD4\(^+\) T cells are activated by alloantigen when presented by self antigen-presenting-cells (35). Its relevance in renal transplant recipients with chronic allograft dysfunction has been shown earlier (36), and it was suggested that indirect allorecognition is crucial for effector mechanisms of allograft destruction to occur (i.e., DTH, alloantibodies, cytotoxicity) (37). Recent studies in animal models for cardiac
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Table 4. C4d\(_{PTC}\) staining and peritubular capillary basement membrane lesions in electron microscopy\(^a\)

<table>
<thead>
<tr>
<th>Morphological Lesion</th>
<th>All Biopsies (n = 43)</th>
<th>C4d in PTC</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes (n = 21)</td>
<td>No (n = 22)</td>
</tr>
<tr>
<td>MLPTC (low- and high-grade), n (%)</td>
<td>28/43 (65)</td>
<td>18/21 (86)</td>
<td>10/22 (45)</td>
</tr>
<tr>
<td>MLPTC (only high-grade), n (%)</td>
<td>18/43 (42)</td>
<td>15/21 (71)</td>
<td>3/22 (14)</td>
</tr>
</tbody>
</table>

\(^a\) MLPTC, basement membrane multilayering in PTC; PTC, peritubular capillaries.

\(^b\) \(\chi^2\) test, C4d\(_{PTC}\)-positive versus C4d\(_{PTC}\)-negative subgroup.

Table 5. C4d\(_{PTC}\) staining in previous and follow-up biopsies\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Index Biopsy, n (%)</th>
<th>Time to/from Index Biopsy (mo), median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C4d(_{PTC})-Positive</td>
<td>C4d(_{PTC})-Negative</td>
</tr>
<tr>
<td>Previous biopsy(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4d(_{PTC})-positive, n (%)</td>
<td>13/96 (14)</td>
<td>8/13 (62)</td>
</tr>
<tr>
<td>C4d(_{PTC})-negative, n (%)</td>
<td>83/96 (86)</td>
<td>26/83 (31)</td>
</tr>
<tr>
<td>Follow-up biopsy(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4d(_{PTC})-positive, n (%)</td>
<td>25/58 (43)</td>
<td>13/25 (52)</td>
</tr>
<tr>
<td>C4d(_{PTC})-negative, n (%)</td>
<td>33/58 (57)</td>
<td>8/33 (24)</td>
</tr>
</tbody>
</table>

\(^a\) IQR, interquartile range; PTC, peritubular capillaries.

\(^b\) Latest previous biopsy (taken within the first year) and earliest follow-up biopsy were investigated, patients with previous biopsies available are not necessarily identical with patients with follow-up biopsies.

Figure 4. Kaplan Meier curve displaying the cumulative frequency of continuously chronic transplant glomerulopathy (CG)–free patients after the early biopsy (until the index biopsy) and after the index biopsy (until the late follow-up biopsy) according to the absence or presence of C4d.

Table 6. Chronic transplant glomerulopathy in index biopsies and follow-up biopsies\(^a\)

<table>
<thead>
<tr>
<th>Index Biopsy</th>
<th>n (%)(^a)</th>
<th>Follow-up Biopsy, (b) n (%)</th>
<th>Time from Index Biopsy (mo), median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4d(_{PTC})-pos. CG-pos.</td>
<td>10/21 (48)</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>C4d(_{PTC})-pos. CG-neg.</td>
<td>11/21 (52)</td>
<td>9/11 (82)</td>
<td>2/11 (18)</td>
</tr>
<tr>
<td>C4d(_{PTC})-neg. CG-pos.</td>
<td>4/37 (11)</td>
<td>2/4 (50)</td>
<td>2/4 (50)</td>
</tr>
<tr>
<td>C4d(_{PTC})-neg. CG-neg.</td>
<td>33/37 (89)</td>
<td>9/33 (27)</td>
<td>24/33 (73)</td>
</tr>
</tbody>
</table>

\(^a\) CG, chronic allograft nephropathy; IQR, interquartile range; PTC, peritubular capillaries.

\(^b\) Follow-up biopsies were performed 8, 9, 15, and 45 mo after index biopsy.
allograft vasculopathy elegantly demonstrate that indirect allorecognition and CD4(+) T cells are crucial for the development of transplant arteriosclerosis and that alloantibody production is dependent on CD4(+) T cell help after indirect allorecognition (38–40). Higher frequencies of indirectly activated CD4(+) T cells were reported in patients with chronic allograft nephropathy, whereas significant titers of alloantibodies could only be detected in a minority of patients (41). It is thus tempting to speculate that deposition of C4d in biopsies of patients with chronic allograft rejection may represent a remaining trace of alloantibodies, originally produced via the help of indirectly primed allo-specific CD4(+) T cells.

Given the strong association of C4dPTC and CG, we were particularly interested in the follow-up of cases showing normal glomerular morphology despite the presence of C4dPTC deposits. Interestingly, progression to CG in a subsequent biopsy was significantly more common in C4dPTC-positive than in C4dPTC-negative cases. This further underscores a close association of endothelial complement activation and the development of CG. CG (42) and severe proteinuria (43–45) (being usually associated with CG) are predictors of an unfavorable outcome. Detection of C4d in allograft biopsies could therefore be of diagnostic and therapeutic relevance, because complement activation (presumably reflecting antibody-mediated alloreactivity) precedes the evolution of structural damage in glomeruli and PTC. In recent studies, plasmapheresis together with tacrolimus-mycophenolate or treatment with immunoadsorption were proposed as anti-humoral therapy in acute rejection (46). The feasibility of a therapy for chronic C4d-positive kidney allograft rejection is not yet established. Recently, Theruvath et al. (17) demonstrated that rescue therapy with tacrolimus and mycophenolate mofetil effectively reduces posttransplant DSA levels in chronic humoral rejection. The clinical efficacy of this approach will have to be evaluated in a larger prospective study.

In conclusion, this study reveals a close association of endothelial C4d deposits with CG and MLPTC (both being regarded as signs of chronic allograft rejection) and with an accumulation of mononuclear inflammatory cells in PTC. These findings support the previously suggested contribution of humoral immune mechanisms to chronic rejection. In most cases, C4dPTC deposition in late allograft biopsies does not reflect persistence of early onset humoral injury but rather may occur de novo at any time after transplantation. C4dPTC deposition seems to be a reliable marker of humoral alloreactivity; its detection might therefore be a simple and inexpensive way to identify patients who are likely to benefit from anti-humoral therapy.

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See related editorials, “C4d and the Fate of Organ Allografts” (pp 2417–2419) and “Capillary C4d Deposition as a Marker of Humoral Immunity in Renal Allograft Rejection” (pp 2420–2423).