Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection

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Abstract

Background. Capillary deposition of complement split product C4d has been suggested to be a valuable marker for humoral rejection. In this retrospective study we evaluated the clinical impact of C4d deposition in renal allografts with special emphasis on associations between C4d staining patterns and histological features of acute rejection.

Methods. One hundred and two allograft biopsies obtained from 61 kidney transplants (1–532 days after transplantation; median 14 days) were examined by immunohistochemistry on routine paraffin sections using a novel anti-C4d polyclonal antibody (C4dpAb).

Results. Forty-two of 102 biopsies showed endothelial C4d deposits in peritubular capillaries (PTC). Histopathological analysis revealed a significantly lower frequency of positive C4d staining in biopsies with rather than in those without acute cellular rejection defined by the Banff grading schema \( P < 0.01 \). For clinical evaluation, patients were classified according to C4d staining in allografts (C4dPTC positive in at least one biopsy, \( n = 31 \) vs C4dPTC negative in all biopsies, \( n = 30 \)). C4dPTC positive patients had significantly higher serum creatinine levels than C4d negative patients. Even in the absence of morphological evidence for rejection, differences in serum creatinine levels between C4dPTC positive and negative recipients were significant (6 months: \( 2.01 \pm 0.75 \) vs \( 1.41 \pm 0.27 \) mg/dl; 12 months: \( 1.95 \pm 0.60 \) vs \( 1.36 \pm 0.34 \) mg/dl; 18 months: \( 1.98 \pm 0.50 \) vs \( 1.47 \pm 0.31 \) mg/dl; \( P < 0.05 \)). All patients with rejection resistant to conventional therapy (\( n = 4 \)) were in the C4dPTC positive subgroup. All recipients with panel reactive antibodies (PRA) > 50\% (\( n = 8 \)) were C4dPTC positive.

Conclusions. Our data indicate that endothelial C4d deposition is associated with inferior graft outcome. We provide evidence that this immunohistochemical finding and its clinical impact are not associated with morphological signs of cellular rejection.

Keywords: C4d; complement activation; endothelium; humoral rejection; immunohistochemistry; kidney transplantation

Introduction

Complement-binding recipient antibodies to donor alloantigens are considered to be the main cause of hyperacute graft rejection. Owing to pre-transplant crossmatch testing, this prototype of humoral rejection is now rarely observed [1–3]. Nevertheless, data are accumulating that humoral immune mechanisms might contribute to other types of allograft rejection [2,3]. High levels of panel reactive antibodies (PRA) indicating humoral presensitization were found to be associated with inferior kidney graft survival [4]. Furthermore the appearance of alloantibodies during the post-transplant period has been reported to predict poor graft outcome [3,5–8]. In some cases, acute rejection resistant to conventional anti-rejection therapy is associated with the occurrence of cytotoxic anti-donor antibodies. Selective removal of recipient IgG by immunoadsorption reversed some of these rejection episodes, which further underscores the contribution of humoral immune mechanisms to rejection [9–11].

Despite increasing evidence for a pathogenetic role of humoral immunity in kidney allograft rejection, current diagnostic and therapeutic efforts are mainly focused on cellular immune reactions. Histopathological classification of acute allograft rejection is largely based on cellular features of alloresponses.
[12]. Only recently, additional morphological features have been proposed for discrimination of acute allograft rejection associated with post-transplantation cytotoxic anti-HLA class I antibodies [8]. Reliable detection of antibody-mediated graft injury is required to govern the application of specific anti-humoral therapeutic strategies. Involvement of humoral immune mechanisms can be suspected by post-transplant crossmatch testing. Complement activation within the graft might indicate antibody-mediated graft injury as well. The complement cleavage product C4d represents a particularly attractive marker for activation of the antibody-dependent classical pathway. Stable covalent binding of C4d to target structures allows its detection in tissue sections over an extended period of time. Capillary C4d deposits in kidney allograft biopsies were associated with poor graft outcome [13,14] and the appearance of post-transplant anti-donor antibodies [15]. These findings together with our recent observation of successful reversal of humoral rejection associated with C4d deposition by immunoadsorption therapy in a pre-sensitized renal allograft recipient [11] prompted us to study the relevance of endothelial C4d deposits in kidney transplantation. In previous studies, patients selected either for humoral rejection [15] or graft dysfunction within the first month [13,14] were examined. The intention of the present study was to analyse the clinical relevance of C4d deposition in biopsies not selected for a specific type of allograft dysfunction. Generation of a peptide-specific anti-C4d polyclonal antibody suitable for paraffin sections permitted a retrospective analysis of routine biopsies.

Subjects and methods

Study population

Sixty-one out of 156 consecutive kidney allograft recipients transplanted at the University Hospital of Vienna, Austria, between January 1998 and December 1998 were included. Inclusion criteria were at least one diagnostic allograft biopsy during follow-up. Patients were excluded if adequate renal tissue was not available (no biopsy performed, n = 70; biopsy without cortical tissue, n = 2) or clinical information was incomplete (recipients not followed-up at our institution, n = 23). Fifty-five of the 61 included recipients received a cadaver, four a living-related and two a living-unrelated donor kidney graft. Forty patients underwent first, 14 second, six third and one fifth kidney transplantation.

Immunosuppression

Thirty-two patients received initial triple drug therapy with cyclosporin A (CSA), seven with tacrolimus, together with mycophenolate mofetil (MMF) or azathioprine, and steroids. Nine patients received initial triple drug therapy with rapamycin (initial dose 2 mg/day), CSA and steroids. According to a local protocol, 13 recipients at increased immunological risk (eight of these patients had PRA reactivity >50%, five recipients with PRA reactivity <50% had received a retransplant) received prophylactic therapy with rabbit ATG (Thymoglobuline; Pasteur Merieux S.V., France). Six of these patients additionally received immunoadsorption therapy using the Citem 10 Immunoadsorption System (Excorim, Lened, Sweden; three plasma volumes processed per session, 2–3 sessions per week; duration of therapy according to renal function). Acute rejection episodes were initially treated with steroid bolus therapy (dexamethasone at 100 mg/day for 3 days). In the case of resistance to high dose steroids, therapy with monoclonal (OKT3, Jansen, Raritan, NJ, USA) or polyclonal anti-lymphocyte antibody (ATG) was administered.

Immunological techniques

Cytotoxic crossmatch testing was performed according to the protocol of the Eurotransplant Organization using the standard microcytotoxicity technique described by Terasaki and McClelland [16]. All recipients were transplanted in the presence of a negative pre-transplant cytotoxic crossmatch. Pre-transplant cytotoxic PRA were assessed using a panel of cells from 50 phenotyped donors.

Generation of polyclonal anti-C4d antibody (C4dpAb)

For generation of a polyclonal peptide-specific anti-C4d antibody (C4dpAb), a 15mer peptide (SPTPAPRNP-SDMPQ) corresponding to amino acids 1242–1256 of C4 was synthesized. C-terminally, a cystein was introduced and the peptide was coupled to keyhole limpet haemocyanin. A rabbit was immunized with 100 µg of the peptide dissolved in complete Freund’s adjuvant. The rabbit was boosted three times in 3-weekly intervals with 75 µg of the peptide in incomplete Freund’s adjuvant. IgG fractions were prepared by affinity chromatography on a Protein A column (Pharmacia, Uppsala, Sweden).

Characterization of C4dpAb

Complement factor C4 was purified from citrated human plasma by ion exchange chromatography on Fast Flow Q-Sepharose (Sigma Chemical Co., St Louis, MO, USA) according to a modification of a previously described protocol [17]. Purification was completed by hydrophobic interaction chromatography on Phenyl-Sepharose High Performance (Pharmacia). Purified C4 was subjected to trypsin digestion to generate fragments resembling C4d (tryptic C4d) for western blot analysis [18]. Purified native C4 or tryptically digested C4 were separated on 12% polyacrylamide gel under non-reducing conditions and subsequently electrotransferred to nitrocellulose. After blocking with 0.5% BSA, C4dpAb was tested at 5 µg/ml in blocking buffer containing 0.2% BSA. C4dpAb bound to a 200 kDa protein band corresponding to native C4. Tryptic digestion of C4 resulted in a labelled protein band at 30 kDa, which is the size of tryptic C4d fragments reported in the literature [18]. For blocking experiments, C4dpAb (5 µg/ml) was pre-incubated overnight at 4°C with purified C4 (9 µg/ml), or with the peptide used for immunization (9 µg/ml). For negative control, C4dpAb was pre-incubated with two different irrelevant peptides or complement components C3 or C5 (each at 9 µg/ml). As shown in Figure 1, pre-incubation of C4dpAb with the peptide used for immunization or with purified C4 completely abolished reactivity to frozen.
Fig. 1. Analysis of antibody specificity on frozen and paraffin sections of renal allograft biopsies. On frozen tissue, staining pattern of C4dpAb (A, ×300) is identical to that observed for anti-C4d mAb (B, ×300), i.e. endothelial reactivity in PTC and diffuse staining along glomerular basement membranes. Pre-incubation of C4dpAb with the peptide used for immunization (C, ×150) completely abolished specific staining signals. Pre-incubation with an irrelevant peptide (D, ×150) did not significantly alter reactivity of C4dpAb. When paraffin sections of the allograft specimens (A–D) were stained with C4dpAb, clear endothelial staining, similar to that observed in corresponding frozen sections, was found in PTC (E, ×240). Anti-C4d mAb failed to detect C4d deposits in paraffin sections (F, ×240). Endothelial staining in glomeruli and PTC in a paraffin-embedded renal allograft biopsy stained with C4dpAb (G, ×480). Glomeruli and PTC in a pre-transplant biopsy are negative for C4d (H, ×480).
sections of a representative C4d positive kidney allograft. Control peptides (Figure 1), C3 or C5 did not affect staining results.

### Histopathology

During follow-up (until July 2000; mean, 691 ± 120 days; range 529–880 days) at least one post-transplant kidney allograft biopsy was performed in each patient due to persistent oligo- or anuria or acute functional impairment. Post- and pre-renal causes of graft dysfunction and toxic CSA (>400 ng/ml) or FK506 (>20 ng/ml) levels were excluded. One hundred and two biopsies obtained from 1–532 days after transplantation (median, 14 days) were evaluated by two independent observers (H.R. and M.E.). Sixty-eight biopsies were performed during the first 4 weeks, 81 during the first 3 months, and 95 during the first year after transplantation. Pathohistological evaluation was done on formalin-fixed paraffin sections. Lesions in allograft biopsies were classified according to the definitions given by the Banff classification [12]. For immunohistochemical studies, 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 previously described [19]. Endogenous biotin was blocked using a Biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). After 30 min incubation with C4dpAb (5 μg/ml) and anti-C4d monoclonal antibody (mAb; Quidel, Alkmaar, Netherlands, up to 20 μg/ml), bound IgG was visualized using the Supersensitive Kit (BioGenex, San Ramon, CA, USA) according to the manufacturer’s protocol.

On frozen sections, a similar protocol was used. Untreated 4-μm sections were incubated with C4dpAb (3 μg/ml) or anti-C4d mAb (2 μg/ml) for 30 min.

### Statistical analysis

Comparisons between groups were performed using the Pearson’s χ² test and the Mann–Whitney U test. Multiple regression analysis was performed using the General Linear Model. *P* values < 0.05 are reported as statistically significant. A commercially available computer program (SPSS 9.0, SPSS Inc., Chicago, IL, USA) was used for all statistical calculations.

### Results

#### C4d staining in allograft and native kidney biopsies

On frozen allograft kidney biopsy sections, immunostaining with C4dpAb revealed staining patterns identical to those obtained with anti-C4d mAb (Table 1, Figure 1). C4d staining on endothelial cells of peritubular capillaries (PTC) was restricted to allograft biopsies. Neither in normal (pre-transplant, n = 25) nor in diseased native kidneys (n = 20) endothelial C4d deposits were detectable (Table 1). In all frozen sections, however, we found C4d staining in glomeruli. On paraffin-embedded sections, C4dpAb retained reactivity to C4d deposits along endothelial cells of PTC (staining intensity slightly reduced) and glomeruli, but lost its reactivity to mesangium and basement membranes of glomeruli. In contrast to C4dpAb, anti-C4d mAb showed no staining at all, when tested on paraffin sections (Table 1, Figure 1).

In 42/102 (41.2%) of biopsies stained with C4dpAb, C4d was detected in PTC, and 15/42 (35.7%) of these PTC positive specimens (14.7% of all biopsies) had an additional endothelial staining for C4d in glomeruli. No biopsy showed glomerular endothelial staining in absence of C4d deposits in PTC.

#### C4d staining in consecutive allograft biopsies

For 28/61 kidney transplant recipients, two (n = 19) or more (n = 9) allograft biopsies were available. In 15 (initial C4d staining in PTC either positive or negative) or 24 (initial C4d staining along glomerular endothelium either positive or negative) repeatedly biopsied recipients, C4d staining results did not change during follow-up. In some recipients with initially negative C4d staining, one or more subsequent biopsies were classified positive (appearance of peritubular C4d

<table>
<thead>
<tr>
<th>Material</th>
<th>Frozen sections</th>
<th>Paraffin sectionsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C4dpAb</td>
<td>Anti-C4d mAb</td>
</tr>
<tr>
<td>PTC</td>
<td>Glomeruli</td>
<td>PTC</td>
</tr>
<tr>
<td>Native kidneys, normal n = 25</td>
<td>0/25 (0)</td>
<td>25/25 (100)</td>
</tr>
<tr>
<td>Native kidneys, diseasedb n = 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney allografts n = 12</td>
<td>5/12 (41.7)</td>
<td>12/12 (100)</td>
</tr>
</tbody>
</table>

mAb, monoclonal antibody; PTC, peritubular capillaries.

aAnti-C4d mAb is not applicable on paraffin sections.

bNumber of biopsies with positive C4d staining is shown (percentages given in brackets).

cFrozen material was not available. Diagnosed diseases were: immune-complex-mediated glomerulonephritis (n = 8), minimal change nephropathy (n = 4), interstitial nephritis (n = 4) and thrombotic microangiopathy (n = 4).

dOn paraffin sections, positive glomerular staining is confined to glomerular endothelium.
deposits in nine recipients, accompanied by glomerular deposits in three of them). A loss of peritubular C4d staining was observed in four patients. In three of these recipients, first biopsies showed only weak staining in PTC without reactivity to glomeruli. In one patient, complete disappearance of strong C4d reactivity both in PTC and glomeruli was found in a specimen taken 362 days after the first biopsy.

C4d deposition and pathohistological evaluation

In specimens with peritubular C4d deposits, acute rejection (according to Banff criteria) was significantly less common (942, 21.4%) than in C4d negative biopsies (30/60, 50%; P = 0.0035). Correspondingly, the majority of C4d positive biopsies showed no signs of rejection (33/42 vs 30/60 C4d negative biopsies). Analysing each Banff type of rejection individually we found no association of endothelial C4d staining with Banff type I and II. However, all three biopsies graded Banff III showed peritubular C4d deposits. Evaluating C4d deposition along glomerular endothelium we found no significant differences with respect to acute rejection (Table 2).

In biopsies without rejection (n = 63) the histopathologic diagnoses were: Banff Borderline lesion (n = 21), acute tubular damage (n = 17), donor-derived damage (n = 8), CSA toxicity (n = 2), thrombotic microangiopathy (n = 2), chronic allograft nephropathy (n = 2), bacterial infection (n = 1), and minor/non-specific changes (n = 10).

Defining criteria of the Banff scheme [12] were also analysed individually (Table 2). Sixteen of the 102 allograft biopsies showed transplant glomerulitis, 67 interstitial inflammation, 48 tubulitis, and 30 intimal arteritis. Peritubular, but not glomerular C4d staining, was significantly less common in biopsies with tubulitis (P = 0.0064). Thus, only 13/42 (31%) biopsies with positive peritubular C4d staining showed tubulitis as compared with 35/60 (58.3%) biopsies with negative peritubular staining. Intimal arteritis was less common in biopsies with C4d deposits (9/42, 21.4%) than in biopsies without C4d (21.60, 35%). This difference did not reach statistical significance. Remarkably, in biopsies with positive glomerular C4d staining, glomerulitis was observed at significantly higher frequency (P = 0.042).

In addition, five morphological parameters potentially related to humoral rejection [8] (acute tubular damage, n = 46; thrombotic microangiopathy, n = 4; interstitial bleeding, n = 10; granulocytes in PTC, n = 2; granulocytes in glomeruli, n = 7) were evaluated. With the exception of a weak association of thrombotic microangiopathy with C4d deposition in glomeruli (P = 0.042), we found no statistically significant association of C4d staining with these parameters. However, for some of these criteria, such as accumulation of granulocytes in PTC, only few biopsies were found to be positive, which might explain our failure to detect statistical significances (Table 2).

Immunological risk factors and C4d deposition in renal allograft biopsy

For clinical evaluation, patient subgroups based on differential C4d staining patterns were defined, i.e. recipients with one or more post-transplant biopsies showing C4d deposits in PTC (C4dPTC positive, n = 31) and patients without such deposits in PTC in any of their biopsies (C4dPTC negative, n = 30). As shown in Table 3, age, observation time, cold ischaemia time, gender, and incidence of prior pregnancies were not significantly different between C4d positive and C4d negative subgroups. However, statistically significant

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**Table 2. C4d staining patterns and histopathology**

<table>
<thead>
<tr>
<th>Pathohistology</th>
<th>All biopsies (n = 102)</th>
<th>C4d in PTC</th>
<th>C4d in glomeruli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 102)</td>
<td>Yes (n = 42)</td>
<td>No (n = 60)</td>
</tr>
<tr>
<td>Banff I, II or III, n (%)</td>
<td>39/102 (38.2)</td>
<td>9/42 (21.4)</td>
<td>30/60 (50)</td>
</tr>
<tr>
<td>No rejection or BL lesion, n (%)</td>
<td>63/102 (61.8)</td>
<td>33/42 (78.6)</td>
<td>30/60 (50)</td>
</tr>
<tr>
<td>Banff I, n (%)</td>
<td>11/102 (10.8)</td>
<td>2/42 (4.8)</td>
<td>9/60 (15)</td>
</tr>
<tr>
<td>Banff II, n (%)</td>
<td>25/102 (24.5)</td>
<td>4/42 (9.5)</td>
<td>21/60 (35)</td>
</tr>
<tr>
<td>Banff III, n (%)</td>
<td>3/102 (2.9)</td>
<td>3/42 (7.1)</td>
<td>0/60 (0)</td>
</tr>
<tr>
<td>Glomerulitis, n (%)</td>
<td>16/102 (15.7)</td>
<td>8/42 (19)</td>
<td>8/60 (13.3)</td>
</tr>
<tr>
<td>Interstitial inflammation, n (%)</td>
<td>67/102 (65.7)</td>
<td>25/42 (59.5)</td>
<td>42/60 (70)</td>
</tr>
<tr>
<td>Tubulitis, n (%)</td>
<td>48/102 (47.1)</td>
<td>13/42 (31)</td>
<td>35/60 (58.3)</td>
</tr>
<tr>
<td>Intimal arteritis (v1, v2, v3), n (%)</td>
<td>30/102 (29.4)</td>
<td>9/42 (21.4)</td>
<td>21/60 (35)</td>
</tr>
<tr>
<td>Acute tubular damage, n (%)</td>
<td>46/102 (45.1)</td>
<td>20/42 (47.6)</td>
<td>26/60 (43.3)</td>
</tr>
<tr>
<td>Thrombotic microangiopathy, n (%)</td>
<td>4/102 (3.9)</td>
<td>3/42 (7.1)</td>
<td>1/60 (1.7)</td>
</tr>
<tr>
<td>Intimal bleeding, n (%)</td>
<td>10/102 (9.8)</td>
<td>5/42 (11.9)</td>
<td>5/60 (8.3)</td>
</tr>
<tr>
<td>Granulocytes in PTC, n (%)</td>
<td>2/102 (2)</td>
<td>1/42 (2.4)</td>
<td>1/60 (1.7)</td>
</tr>
<tr>
<td>Granulocytes in glomeruli, n (%)</td>
<td>7/102 (6.9)</td>
<td>4/42 (9.5)</td>
<td>3/60 (5)</td>
</tr>
</tbody>
</table>

BL, Borderline; PTC, peritubular capillaries.

*P* < 0.01, C4d positive vs C4d negative subgroup. *P* < 0.05, C4d positive vs C4d negative subgroup.
differences were found for recipient pre-sensitization (Table 3). All eight patients with PRA levels >50% belonged to the C4dPTC positive subgroup. Six of these highly sensitized recipients belonged to the group of retransplanted patients (n = 21). Retransplanted recipients were more commonly C4dPTC positive (16/31, 51.6%) than negative (5/30, 16.7%; P < 0.01).

C4d deposition and clinical outcome

Next, we examined the association of C4d deposition with serum creatinine levels in patients with functioning kidney grafts. Serum creatinine levels in patient subgroups are listed in Table 4. Mean serum creatinine was significantly higher in C4dPTC positive than in C4dPTC negative recipients. Even in transplant recipients without histological evidence for acute cellular rejection, serum creatinine levels differed significantly. In patients with morphological evidence for rejection, differences in serum creatinine levels did not achieve statistical significance (Table 4). It has to be mentioned that three C4d positive recipients who lost their graft due to antibody-resistant rejection could not be included into calculation of creatinine levels. Two of the patients had rejection Banff III, one had rejection Banff II at the time of graft loss. Regarding glomerular endothelial staining there were no statistically significant differences in creatinine levels between C4d positive and C4d negative patients (data not shown).

In our series, PRA reactivity >50% was not associated with significantly higher serum creatinine levels. However, in two of three immunological graft losses pre-transplant PRA reactivity was >50%. In retransplanted patients, we found slightly higher serum creatinine levels at 12 (P = 0.048) and 18 months (P = 0.046). Furthermore, all patients with immunological graft loss belonged to the retransplanted patient subgroup. Multiple regression analysis was used to assess the relative importance of positive C4d staining and of retransplantation as predictive variables for serum creatinine levels at 18 months post-transplantation. We found peritubular C4d staining, but not retransplantation, having an independent influence on serum creatinine levels at 18 months post-transplantation (intercorrelation between C4d staining and retransplantation corrected; positive peritubular C4d staining: \( P = 0.015, \eta^2 = 0.114 \); retransplantation: \( P = 0.117, \eta^2 = 0.049 \)).

As shown in Table 5, patient subgroups (C4d positive vs C4d negative) did not differ with respect to incidence of delayed graft function, time of first biopsy and serum creatinine or incidence of dialysis at time of biopsy. The four patients with graft loss during follow-up (in one patient due to surgical complication, in three recipients due to therapy-resistant rejection) were in the C4dPTC positive subgroup. Death occurred in five patients, in one case due to cerebral haemorrhage, in four cases due to septicemia. A causal relationship between the presence of C4d and patient death cannot be assumed.

C4d and antibody-resistant rejection

Forty-nine patients received anti-rejection therapy (also administered to some patients with Banff Borderline lesions). These were similarly distributed between C4d positive (23/31, 74.2%) and C4d negative subgroups (26/30, 86.7%; difference not significant, data not shown). All four recipients with antibody-resistant rejection (two classified type III, one type II and one borderline) belonged to the C4dPTC positive subgroup (\( P = 0.042 \)). Two of these patients also showed endothelial C4d deposits in glomeruli. In one case, acute antibody-resistant humoral rejection classified borderline according to the Banff schema was successfully treated with immunoabsorption therapy [11].

Discussion

The present study provides evidence that deposition of C4d in kidney allograft biopsies is associated with an inferior post-transplant clinical course. Endothelial C4d deposition was found to occur independently of classical morphological signs of acute cellular rejection, indicating that antibody-mediated graft injury can take place in absence of cellular rejection.
Furthermore, we found a clear association between retransplantation, a major risk factor for recipient pre-sensitization, and peritubular capillary C4d staining. In addition, we observed a significantly higher incidence of humoral pre-sensitization, reflected by high PRA reactivity, in C4d positive patients. These results support the role of C4d as a marker of humoral allorecognition and are in line with previous reports [13,14].

A prerequisite for our retrospective analysis was the generation of an anti-C4d polyclonal antibody, which is unique in its suitability for C4d staining in paraffin-embedded tissue. This antibody permitted simultaneous analysis of C4d staining patterns and pathohistological examination on the same tissue block. Endothelial staining in glomeruli and PTC was virtually identical in frozen and paraffin sections. The only difference between frozen and paraffin sections was observed in glomerular mesangial staining which might be explained by a reduced overall sensitivity of the antibody or possibly by a preferential alteration of non-endothelial C4d deposits as a consequence of formalin fixation and/or paraffin-embedding.

In contrast to previous reports [13,15], we studied all patients biopsied during a defined observation period and did not select biopsies based on indication or timing. This led to the inclusion of a considerable number of biopsies showing no morphological signs of rejection. A high proportion of biopsies without signs of cellular rejection showed an endothelial C4d staining pattern. Humoral graft injury without signs of cellular rejection was recently considered to represent pure antibody-mediated rejection [3,6,15]. The observation made by us and by others that antibody-mediated rejection can occur in absence of cellular rejection suggests that morphologic criteria as defined by the Banff classification might not allow identification of antibody-mediated graft injury. The present study revealed no association between signs of cellular rejection (tubulitis and intimal arteritis) and C4d deposits in PTC. A similar observation was earlier reported by others [8] who found a significantly lower frequency of distinct features of cellular rejection, i.e. tubulitis and mild intimal arteritis, in rejecting antibody-positive as compared to antibody-negative rejection.

These results suggest that cell- and antibody-mediated graft injury may occur independently. Our finding that all three biopsies showing Banff v3 lesions (arterial wall necrosis) stained positive for C4d is in line with the assumption that arterial wall necrosis reflects antibody-mediated graft injury [8,12].

Evaluating sequential biopsies, we found in some instances that C4d deposits are not detected in a first biopsy, but occur later during the post-transplant course. This finding might point to a pathogenetic role of humoral immune mechanisms triggered by the allograft. A loss of peritubular C4d staining was
observed only in a few cases. In most patients, however, C4d staining results did not change over time. The exact time course of C4d deposition can only be evaluated in a prospective study testing protocol biopsies. Our study, however, was done on biopsies performed due to impaired renal function. The incidence of C4d deposits in allografts with stable normal function can, therefore, not be deduced from our data. Furthermore, we cannot differentiate whether C4d deposition reflects prior or ongoing humoral rejection.

In our study population, endothelial C4d deposition was associated with inferior clinical outcome. All patients with antibody-resistant rejection or graft failure were in the C4dPTC positive subgroup. Furthermore, in patients with functioning grafts, mean serum creatinine levels were significantly higher in the C4dPTC positive than in the C4dPTC negative subgroup at all tested time points. Importantly, differences in creatinine levels between C4dPTC positive and negative subgroups were significant even when patients with histological evidence for acute cellular rejection had been excluded from calculation. This fact suggests that C4d staining might predict clinical outcome independently of morphological signs of rejection. Our data are in line with numerous reports demonstrating that rejection episodes with post-transplant occurrence of donor-specific antibodies are associated with inferior graft outcome [2,3,6–8]. C4d positive patients with a history of rejection defined by the Banff classification had a somewhat lower serum creatinine than C4d positive patients without rejection. Differences in current creatinine levels achieved statistical significance ($P = 0.038$). The slightly lower serum creatinine values in C4d positive patients with acute cellular rejection might be a result of the administration of conventional anti-rejection therapy. Indeed, it was previously suggested that conventional anti-rejection therapy might target also antibody-mediated immune mechanisms [3,8].

A few recent uncontrolled studies demonstrate reversal of some antibody-mediated graft rejection episodes by immunoadsorption therapy [9,10]. Indeed, in one C4d positive allograft recipient from our study population, humoral rejection (without signs of cellular rejection) was completely reversed by a 3 weeks course of immunoadsorption therapy [11].

More than 50% of biopsies performed during or shortly after treatment with ATG showed no C4d deposition, which argues against direct induction of C4d deposits by anti-lymphocyte antibody as previously concluded from a study evaluating post-transplant biopsies in heart allograft recipients [20]. In fact, a substantial number of patients with C4d positive biopsies under ATG treatment were found to be C4d positive already in biopsies taken before initiation of ATG therapy.

In conclusion, our retrospective analysis indicates that endothelial C4d deposition in PTC is associated with inferior graft outcome. We provide evidence that the appearance of C4d deposition and its clinical impact are not related to morphological signs of cellular rejection. Prospective studies on sequential (protocol) biopsies are required to establish the overall incidence of C4d deposition in kidney allografts and to thoroughly study its kinetics on endothelial cells.

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