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Capillary deposition of C4d complement fragment and early renal graft loss

HELMUT E. FEUCHT, HELMUT SCHNEEBERGER, GÜNTHER HILLEBRAND, KLAUS BURKHARDT, MAX WEISS, GERT RIETHMÜLLER, WALTER LAND, and EKKEHARD ALBERT

Department of Internal Medicine, Klinikum Innenstadt; Institute of Immunology; Division of Transplant Surgery, Department of Surgery and Nephrology Division, Department of Internal Medicine I, Klinikum Großhadern; Institute of Pathology and Department of Paediatrics, University of Munich, Munich, Germany

Capillary deposition of C4d complement fragment and early renal graft loss. Clinical outcome of kidney grafts that are affected by the complex syndrome of 'early graft dysfunction' is uncertain and rather unpredictable. In this study, an individual prognosis for dysfunctioning allografts ($N = 93$) is attempted by the immunohistological assessment of vascular classical complement activation in graft biopsies. Thus, capillary deposition of complement fragment C4d was observed in the majority ($N = 51$) of early dysfunctioning grafts. In 43 biopsies, abundant deposition of fragment C4d was present in all capillaries, whereas in eight specimens a segmental distribution of capillary C4d was observed. In 42 grafts with early dysfunction no capillary C4d was detectable. Eighteen subsequent graft losses within one year (16 early losses) were recorded in the subgroup with C4d in all capillaries, and three early losses in the group with segmentally distributed C4d. Only four graft losses (3 early losses) were recorded in the C4d-negative group ($P = 0.0027$; Pearson's chi square test). The resulting one-year graft survival rates (72% for the study group) differed markedly between the subgroups. Grafts with generalized or segmental capillary deposition of C4d had 57% and 63% survival, respectively, contrasted by 90% survival in the C4d-negative group. It is of note, however, that also three of the four grafts that were finally lost within the C4d-negative group, showed distinct capillary deposition of C4d in second biopsies. Vascular deposition of complement fragment C4d therefore represents a clinically relevant factor that contributes to early graft dysfunction. Its assessment is helpful for an individual graft prognosis.

The early post-transplantation period is critical for later clinical outcome of renal grafts in allogeneic recipients [1]. During this period, more than one-third of cadaver allografts are either not functioning at all, or show some other degree of dysfunction. Among the most frequent causes of true graft dysfunction (after exclusion of mechanical factors) are: functional impairment owing to detrimental donor conditions, to impaired organ preservation, to rejection episodes, to drug toxicity or to a combination of them [2]. Postoperative biopsies from dysfunctioning grafts are usually performed within two weeks in order to determine reversible or remediable conditions. However, the interpretation of histopathological findings, in particular the distinction between different pathological entities, appears to be especially difficult at that time [3]. Since reliable clinical and laboratory markers for an individual graft prognosis are also lacking, the reasons for early graft losses often remain obscure.

Recently, prominent deposition of 'early' complement split products C4d and C3d has been observed within renal graft capillaries in a substantial number of acute and chronic rejections [4]. Abundant complement deposition in interstitial capillaries was unique to allografts and could not be detected in autologous normal or glomerulonephritic kidneys. Vascular deposits of C4d and C3d, indicating classical pathway activation, were only rarely accompanied by immunoglobulin deposits and terminal complement components C5-9. Because of its association with preformed antibodies to HLA in recipients, vascular presence of complement fragment C4d has finally been assumed to represent an otherwise undetectable humoral immune reaction against graft endothelial cells.

To prove the clinical relevance of that finding, the question of whether such presumed humoral immune reactions were correlated with individual graft outcome is investigated in this study. Thus, biopsies from grafts with early dysfunction were evaluated for the vascular presence or absence of split product C4d as a single criterion. The resulting subgroups were analyzed with respect to graft survival.

Methods

Patients and graft biopsies

A total of 596 patients in this study received cadaver kidney grafts in the Division of Transplant Surgery between January 1989 and June 1992. In 199 patients postoperative graft biopsies were performed within four weeks because of early graft dysfunction. Early graft dysfunction was defined as either primary non-function (persisting oligo-/anuria) or any other functional impairment within four weeks after transplantation (after mechanical factors have been excluded by clinical and radiological diagnostic techniques). From 100 unselected first biopsies (mean interval after transplantation: 11 days) either snap-frozen tissue specimens ($N = 52$) or cryostat sections ($N = 48$) were transferred to the Institute of Immunology for the immunohistological assessment of complement deposition. From these, 93 specimens could be evaluated. From 16 grafts

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second biopsies were obtained two to four weeks later. Deposition of C4d was assessed in all specimens by indirect immunoperoxidase staining without knowledge of histopathological diagnoses. Diagnostic classification of graft biopsies followed standard histopathological criteria [5, 6].

Immunosuppressive therapy

Immunosuppressive therapy in recipients was started either with a triple drug regimen (cyclosporine, steroids, azathioprine) or, in patients with immunological risk factors (presence of panel reactive antibodies and/or previously rejected grafts), with a quadruple drug regimen (supplemented with polyvalent or monoclonal anti-lymphocyte antibodies). Rejections were treated with high-dose steroids for three days, and if unresponsive, with anti-lymphocyte antibodies (polyvalent or monoclonal preparations).

Crossmatch technique and assessment of panel reactive antibodies

Serological HLA typing has been described in detail elsewhere [7]. All recipient sera produced a negative crossmatch test with donor lymphocytes before transplantation. Crossmatches were performed according to the rules of the Eurotransplant Organization using the standard microcytotoxicity technique of Terasaki and McClelland [8]. The crossmatches were performed with peripheral lymphocytes (mixture of T and B cells) with a prolonged incubation time of two hours with complement. Crossmatches with historic serum samples of the patients were not performed. According to the rules of Eurotransplant the sera of recipients on the waiting list were screened four times a year for the presence of lymphocytotoxic antibodies and positive serum samples were investigated with a total of 150 typed lymphocyte samples in order to determine the antibody specificity. Antibody specificities found in the serum of a prospective recipient led to the exclusion of all kidneys possessing that particular antigen. A panel reactivity of greater than 30% was classified as "immunological risk," and a reactivity greater than 84% as "highly immunized."

Immunoperoxidase staining

Indirect immunoperoxidase staining was performed as described previously [4]. In brief, 4 μm cryostat sections (air-dried, fixed in cold acetone (Merck, Darmstadt, Germany), washed in phosphate buffered saline (PBS) at pH 7.4 were reacted serially with appropriately diluted primary monoclonal antibodies and with peroxidase-conjugated second antibody (rabbit anti-mouse immunoglobulins; Dakopatts, Hamburg, Germany). Sections were finally stained with 3-amino-9-ethylcarbazole (Sigma, München, Germany), DMSO, H_2O_2 (Merck) and counterstained with hemalaun (Merck). For microscopic evaluation, sections were mounted with glycerin-gelatin (Merck). As a control, sections were incubated with supernatants from irrelevant isotype-defined mouse hybridomas or non-immune goat or rabbit Ig.

Monoclonal antibodies

From our own panel of mouse monoclonal antibodies (mAbs) reactive with the C4d (α2) portion of human complement component C4, mAbs M4d2 and M4d3 [9, 10], together with a commercially available mAb against C4d (Byk-Sangtec, München, Germany) were used in parallel in a first series of 30 biopsies. Since identical staining results were obtained, mAb M4d2 was used subsequently throughout the study.

To ascertain the presence of capillaries in a biopsy specimen and to determine their approximate number, one of several commercially available mAbs reactive against vascular endothelial cells were used in parallel sections throughout the study: PAL-E and EN 4 (Sanbio, Uden, The Netherlands); BMA 120 (Behringwerke, Marburg, Germany); anti-human factor-VIII related antigen (Immunotech S.A., Marseille, France).

Data monitoring and statistical analysis

Patient data were monitored with computer assistance using the ENABLE data processing tools (Markt & Technik, Haar, Germany). Graft survival rates were computed by the Cutler-Ederer method [11]. For statistical comparison, Pearson's chi square test, Cochran's test for linear trend and Fisher's exact test were calculated using the BMDP 4F program.

Results

Distribution of complement fragment C4d in graft capillaries

Assessment of capillary deposition of complement fragment C4d in graft biopsies revealed three staining patterns. Graft biopsies were subdivided into three groups according to deposition of C4d in interstitial capillaries: biopsies were assigned C4d+ when all capillaries showed deposition of C4d; C4d(+) indicates segmental staining of capillaries; C4d− denotes absent staining in capillaries (Fig. 1).

Table 1 denotes the numerical distribution of first biopsies among the C4d+ ($N = 43$), C4d(+) ($N = 8$) and C4d− ($N = 42$) subgroups. Second biopsies were available from 16 grafts, and six specimens were initially classified as C4d+ and remained so in the repeat samples, whereas 5 out of 10 grafts within the C4d− group clearly changed into C4d+ and (+) patterns, respectively (Fig. 2).

The corresponding histological diagnoses in parallel specimens are given in Table 2. Grafts in the C4d+ group showed more combinations of cellular and vascular rejection and other combined pathological findings, whereas interstitial cell-mediated rejections were more frequent in the C4d− group. Grafts with preservation injury were equally distributed among C4d+ and C4d− groups.

Distribution of donor and recipient risk factors

The distribution of several factors with possible impact on ultimate graft outcome is given in Table 3. There were no apparent differences among the subgroups with respect to donor age, the number of donors with hypotensive episodes or to cold ischemia times. The subgroups also had comparable mismatches in three HLA loci (A, B and DR). The C4d+ group contained more recipients with conventional immunological risk factors (> 30% panel reactivity of preformed antibodies to HLA and/or previously rejected transplants) who received a quadruple immunosuppressive induction therapy ($N = 25$ vs. 7 in the C4d− group). More subsequent anti-rejection treatments with mono- or polyclonal anti-lymphocyte antibodies were applied in the C4d− group, therefore the total number of patients receiving one or two courses of antibody therapy was

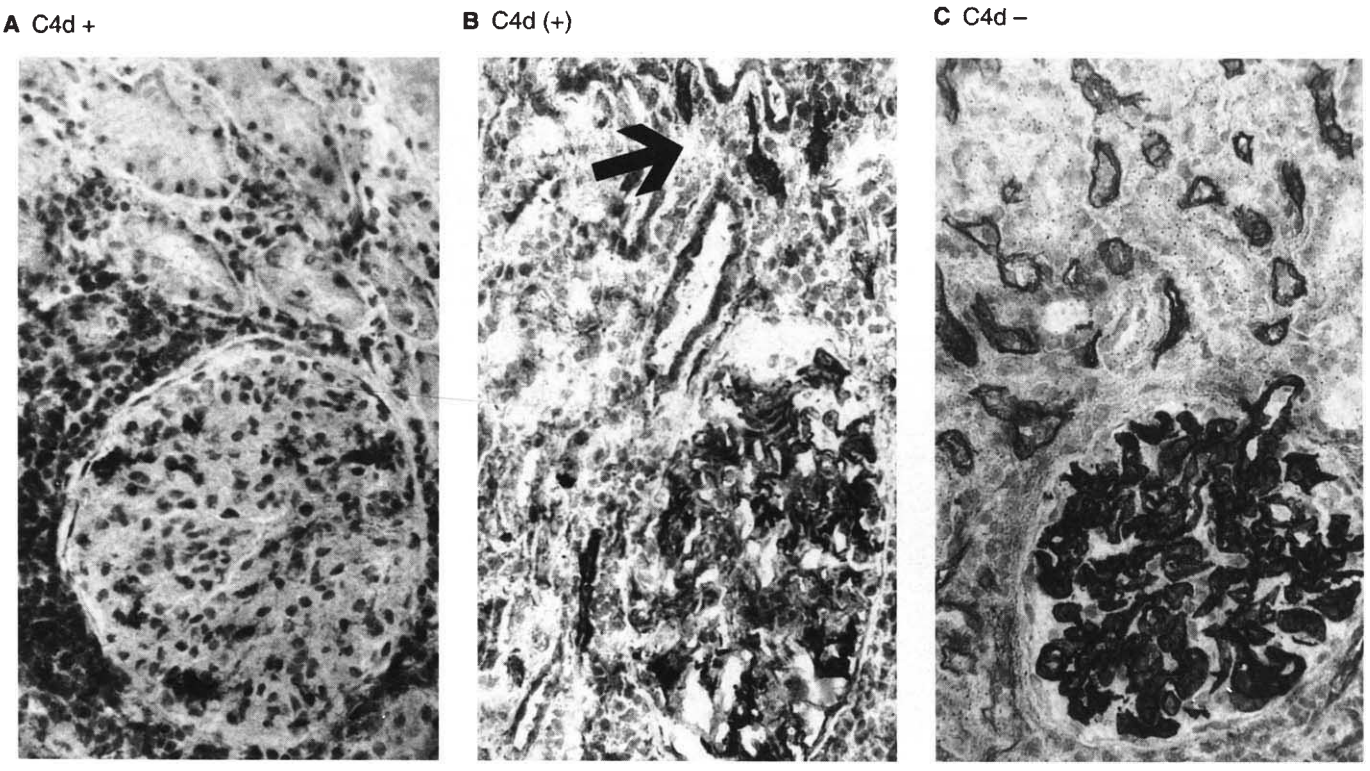

Fig. 1. *Patterns of capillary deposition of C4d in renal transplants with early dysfunction.* (**A**) C4d+ pattern: staining of all interstitial and glomerular capillaries; (**B**) C4d(+): segmental staining of interstitial (arrow) and glomerular capillaries; (**C**) C4d−: interstitial capillaries are negative, glomerular deposits of C4d represent staining of normal glomeruli; (immunoperoxidase staining; magnification × 120).

Table 1. Deposition of complement C4d in 109 biopsies from 93 early dysfunctioning grafts

	Staining pattern of capillary complement deposition		
	C4d+	C4d(+)	C4d−
First biopsies (*N*) mean 11 days after transplant	43	8	42
Second biopsies (2 to 4 weeks later)			
from C4d+ grafts (*N* = 6)	6	0	0
from C4d− grafts (*N* = 10)	2	3	5

comparable within the subgroups. It is interesting to note that seven recipients in the C4d− group were biopsied after quadruple induction therapy and five patients in this group (without quadruple induction) were treated with anti-lymphocyte globulins but remained C4d negative in subsequent biopsies. In the C4d+ group, 10 patients (without quadruple induction) were positive before they received anti-lymphocyte preparations. Neither was capillary C4d associated with the prophylactic application of anti-cytomegalovirus immunoglobulins in 8 recipients.

Mean serum creatinine concentrations at the time of first biopsy did not differ markedly between the groups, indicating comparable degrees of functional impairment. However, as many as 18 graft losses within one year were recorded in the C4d+ subgroup, compared with only four graft losses in the C4d− group. In the small C4d(+) group three grafts were lost. The association of capillary deposition of C4d with subsequent graft loss was found to be statistically significant by calculating both Pearson's chi square test ($P = 0.0027$) and Cochran's test for linear trend ($P = 0.0008$).

Characterization of graft losses

In Table 4, the distribution of immunological risk factors and histological diagnoses among the 25 graft losses is denoted. In the C4d+ group, 13 grafts with primary non-function were recorded, whereas four grafts in the C4d− group initially showed impaired function. From a total of 32 recipients with immunological risk factors, 25 were contained within the C4d+ group, 11 of whom lost their graft (11 re-transplants with 8 recipients having panel reactive antibodies). It should be noted that 14 recipients without any pre-existing risk factors lost their graft, including four patients from the C4d− group, and that all grafts survived in seven recipients receiving quadruple drug therapy in this group. Thus, using Fisher's exact test, there was no significant correlation between the presence of immunological risk factors and graft loss in this study.

The predominant histological entity in the C4d+ group was a combination of cellular and vascular rejection, that was also highly associated with subsequent graft loss ($P = 0.0001$; Fisher's exact test). Interestingly, four specimens in the C4d+ group were initially classified as preservation injury, but three clearly changed in pathology and developed rejection in later biopsies (2 cellular rejections, 1 combined cellular and vascular rejection). Likewise, two grafts with combined pathological

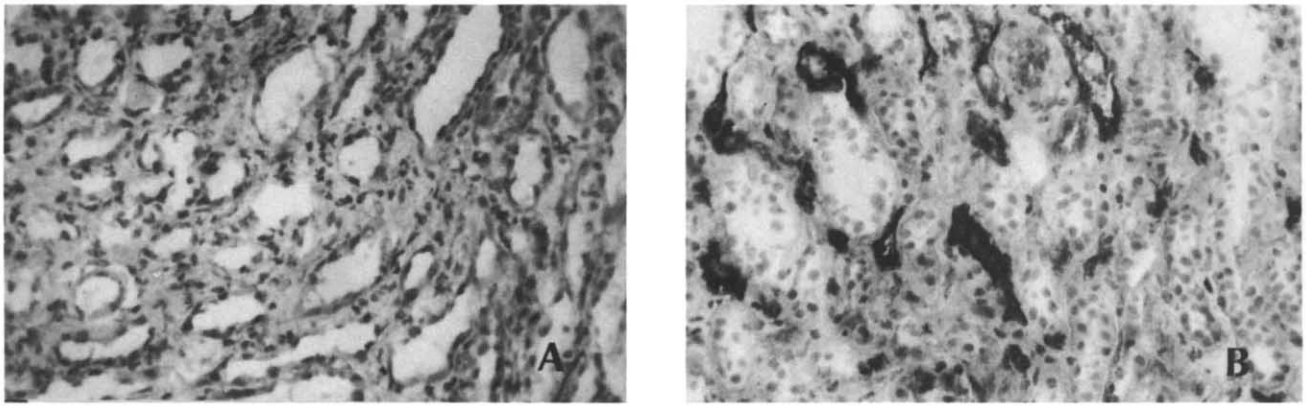

Fig. 2. *Deposition of C4d in consecutive biopsies from one graft with early dysfunction and subsequent loss because of progressive rejection.* **(A)** Absence of C4d 10 days after transplantation; **(B)** prominent deposition of C4d in peritubular capillaries 24 days after transplantation (× 250).

Table 2. Histological diagnoses[a] and C4d staining patterns in 93 graft biopsies

	Capillary complement staining		
	C4d+	C4d(+)	C4d−
First biopsies *N*	43	8	42
Acute rejections			
Cell-mediated interstitial	8	6	25
Vascular	2	0	0
Cellular + vascular rejection (including acute cellular arteritis)	9	0	2
Preservation injury	11	0	8
Combined pathological findings[b]	13	2	7

[a] Histological diagnoses refer to the predominant pathological lesions in a given biopsy

[b] In grafts without predominating lesion but showing any combination of cellular/vascular rejection with preservation injury or cyclosporine toxicity

findings in that group changed into predominant rejection (1 cellular, 1 combined cellular plus vascular rejection). It should finally be noted that from four grafts that were lost in the C4d− group because of rejection, one clearly changed into the C4d+ (Fig. 2) and two into the C4d(+) pattern. Thus, only one graft out of 25 was lost without capillary deposition of complement fragment C4d.

Graft survival rates in relation to capillary deposition of C4d

The one-year graft survival rate of all allogeneic kidney transplantations (N = 596) performed during the study period was 82%, whereas a less favorable one-year survival of 71% was recorded for all grafts that were biopsied within four weeks because of early dysfunction (N = 199). The 93 unselected patients in the complement study showed an almost identical one-year graft survival rate of 72% (Fig. 3).

When these patients were further subdivided according to the distribution of capillary C4d in first biopsies, marked differences appeared with a one-year graft survival rate of only 57% in the C4d+ group (N = 43), 63% in the C4d(+) group (N = 8), in contrast to the C4d− group (N = 42) that reached a one-year graft survival of 90% (Fig. 4).

Table 3. Distribution of donor and recipient risk factors, therapeutic regimens and graft losses among C4d+, C4d(+) and C4d− early dysfunctioning grafts (N = 93)

	Capillary complement staining		
	C4d+	C4d(+)	C4d−
First biopsies *N*	43	8	42
Mean donor age *years*	41 ± 15	42 ± 11	37 ± 13
Donors with hypotensive episodes	16	3	21
Cold ischemia time *hours*	25 ± 7	26 ± 4	25 ± 6
Mismatches in HLA-loci (*mean*)			
A	0.8	0.9	0.8
B	0.9	0.7	0.7
DR	0.4	0.4	0.3
Immunosuppressive therapy			
Induction with quadruple drug[a]	25	0	7
Induction with triple drug	18	8	35
Subsequent anti-rejection treatments	36	8	35
Steroids only	8	2	3
Plus antibodies	28	6	32
Total of patients receiving antibodies	38	6	36
Serum creatinine *mean conc. μmol/liter*			
at time of first biopsy	676 ± 188	636 ± 280	629 ± 246
end of follow-up (functioning grafts)	200 ± 141	143 ± 22	164 ± 83
Graft losses (within one year)	18[b]	3[b]	4[b]

[a] Recipients with immunological risk factors (panel reactive Ab to HLA and/or previously rejected grafts)

[b] P = 0.0027 Pearson's chi square test

Discussion

Abundant deposition of 'classical' complement split products in graft capillaries as shown in this and in an earlier study [4] is a novel finding. It warrants re-evaluation of the role of complement, of a major biological clearance and defence system [12], in transplantation. A favorable graft outcome in the absence of complement deposition, compared with a significantly worse outcome in its presence, indicates that vascular complement

Table 4. Complement staining patterns, immunological risk factors and histological diagnoses in 25 graft losses

	Capillary complement staining		
	C4d+	C4d(+)	C4d−
Graft losses (N = 25)	18	3	4
Early losses (<3 months)	16	3	3
Primary non-function	13	2	0
Later losses (3–12 months)	2	0	1
Pre-existing immunological risk			
Re-transplants	11	0	0
with panel reactivity >30%	6	—	—
with panel reactivity >84%	2	—	—
Histological diagnosis in first biopsy			
Cell-mediated interstitial rejection	2	2	2[a]
Vascular rejection	1	0	0
Cellular + vascular rejection	8	0	1
Preservation injury	4	0	0
subsequent change in pathology	3	—	—
Combined pathology	3	1	1[a]
subsequent change in pathology	2	1	1

[a] Grafts (N = 3) that changed from C4d− into C4d+/(+) staining patterns in later biopsies

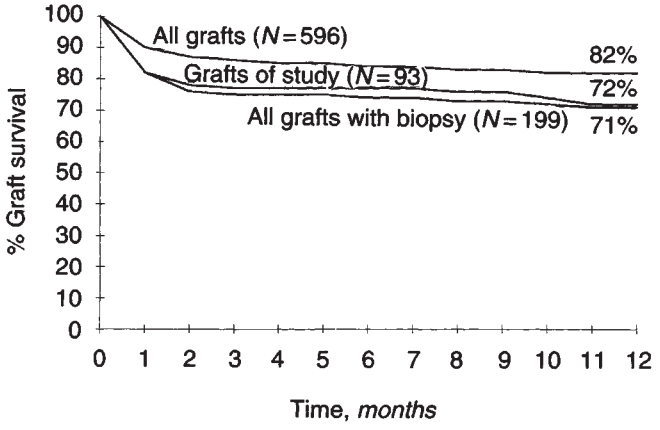

Fig. 3. *Survival rates of all allogeneic cadaver kidney grafts transplanted during study period, of all grafts that were biopsied within four weeks and of transplants included in complement study.*

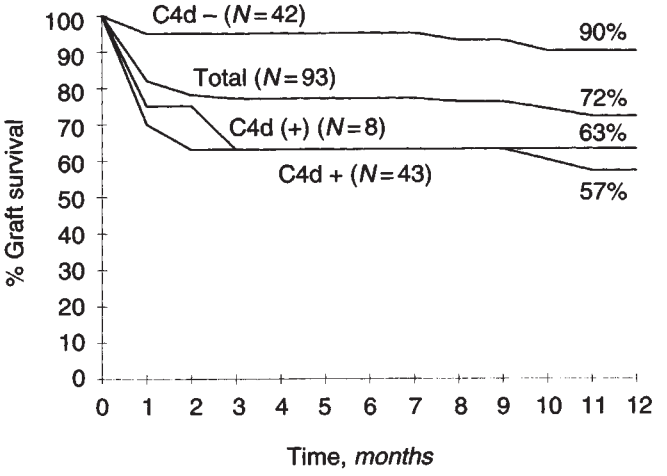

Fig. 4. *Survival rates of early dysfunctioning grafts with complement staining pattern C4d+, C4d(+) and C4d−.*

deposition is representing not merely an epiphenomenon but rather a clinically relevant factor.

Assessment of capillary complement fragment C4d is attained by a simple and rapid immunohistological staining technique. Using one of the various antibodies to vascular endothelial cells in parallel, the percentage of C4d-positive capillaries can be reliably estimated even in very small or ill-preserved biopsy specimens.

Thus, complement was present in the majority of a representative cohort of grafts with early dysfunction, including all but four subsequent graft losses. It can be deduced that immediate humoral anti-graft reactions are quite frequent in this complex clinical setting. That vascular deposition of complement was the result of antibody treatment is highly unlikely, since in 22 recipients there was no association with the therapeutic application of mono- or polyclonal anti-lymphocyte preparations. Instead, association with circulating panel-reactive antibodies [4] and the dynamics of complement deposition point to the presence of preformed anti-endothelial cell antibodies that may cause complement precipitation. It should be noted, however, that complement deposition also occurred in the absence of any measurable anti-HLA activity. The precise antigenic specificity of these antibodies escaping conventional crossmatch and lymphocyte panel testing remains to be determined [13]. Conversely, complement deposition did not occur despite the presence of conventional immunological risk factors. Thus, vascular absence of C4d denotes a subgroup with favorable graft prognosis even within the 'immunological risk' group, known to have generally a poor prognosis [14]. As a consequence, there seems to be a stronger association of graft loss with complement deposition than with other risk factors.

In this context it is also worth noting that even within the original C4d-negative group, three of four grafts showed distinct capillary complement deposition in second biopsies before they were lost. This suggests an ongoing humoral anti-graft reaction following transplantation. (Whether the small group with segmental deposition of complement C4d also belongs in this category is not clear at the moment.)

In this study, only short-term effects with respect to capillary complement deposition are recorded. Longer follow-up periods are required to denote the long-term sequelae in grafts that showed initial complement deposition but survived the first year, and perhaps even more importantly, in grafts that were subject to later humoral reactions.

By conventional HLA typing, there were no significant differences with respect to HLA mismatches between the C4d-positive and the C4d-negative subgroups. However, the HLA class II molecules including HLA-DR, -DP, -DQ, that are important constitutive and inducible antigens on endothelial cells [15], deserve special attention. It will be interesting to determine, for example, whether previously unrecognized mismatches for HLA-DR that can now be revealed by improved DNA typing [16] would account for some of the humoral immune reactions reported herein.

Despite differing numbers of quadruple induction therapies in the groups, there were no apparent differences with respect to the overall frequency and modality of subsequent anti-rejection

treatments. The favorable graft outcome in the complement-negative group permits speculation that split product C4d separates 'pure' cell-mediated rejections responsive to therapy from 'mixed' rejections that may be less responsive. Likewise, grafts that showed initially either preservation injury or combined pathological findings but developed rejection later, could be identified on that basis. Thus, deposition of C4d in capillaries might indeed serve as an early and sensitive marker of impending vascular injury, since the histopathological criteria of vascular rejection [5, 6] usually refer to changes in arterioles or other larger vessels that can be missing in a given specimen.

In conclusion, capillary deposition of complement fragment C4d appears to reveal clinically relevant, but otherwise undetectable humoral anti-graft reactions. Its assessment provides a valuable prognostic marker for individual graft outcome and helps to define patients that may require additional or alternative forms of immunosuppressive therapy.

Acknowledgments

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Reprint requests to Dr. H.E. Feucht, Medizinische Klinik, Klinikum Innenstadt, Universität München, Ziemssenstraße 1, D-8000 München 2, Germany.

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