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Impact of humoral alloreactivity early after transplantation on the long-term survival of renal allografts

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Impact of humoral alloreactivity early after transplantation on the long-term survival of renal allografts.

Background. The contribution of humoral alloreactivity to the rejection of renal allografts is not well defined because humoral antigraft reactions are not easily detectable in transplant biopsies, and serial measurements of circulating alloantibodies in the post-transplantation period are not routinely performed. We have developed diagnostic techniques that improve the assessment of humoral alloreactivity in vivo and in vitro.

Methods. Humoral alloreactivity in transplant biopsies derived from 218 single kidney grafts was detected by assessing the deposition of complement fragment C4d in interstitial capillaries. Circulating alloantibodies were determined in corresponding serum samples by flow cytometry using lymphoblastoid cell lines of donor DR-type as target cells and by a conventional microcytotoxicity test. The impact of capillary C4d and other selected variables on renal graft survival was calculated by univariate and multivariate analysis.

Results. Capillary C4d, present in 46% of biopsies from first grafts and 72% of regrafts, is related to circulating alloantibodies. Grafts with capillary C4d have a markedly shorter survival than grafts without capillary C4d (50% graft survival, 4 vs. 8 years, $P = 0.0001$). Among several risk factors, capillary C4d is the strongest predictor of subsequent graft loss in a multivariate analysis (relative risk, 2.1, 95% CI, 1.4 to 3.1). Humoral alloreactivity detectable within six months after transplantation has a much stronger impact on graft survival than alloreactivity detected beyond this period.

Conclusions. Humoral alloreactivity, manifested by the capillary deposition of complement C4d in about 50% of biopsied renal grafts, exerts a strong impact on graft survival when it operates within six months after transplantation.

Clinical and experimental observations suggest that acute transplant rejections, depending on their severity and type, have a strong impact on the long-term survival of renal allografts because they may be related to the development of chronic rejection [1–3]. Rejections without residual impairment of transplant function are innocuous, whereas rejections followed by organ dysfunction convey an unfavorable long-term prognosis [4, 5]. Likewise, acute vascular, in contrast to interstitial, rejections predicted both early and late graft loss [6]. Since anti-T-cell–directed immunosuppressive therapy in recent years has failed to improve the cumulative long-term survival of renal allografts, not only T-cell–mediated reactions, but other, less well-defined pathogenetic mechanisms may be operative in severe transplant rejections.

Although the association of antidonor antibody and subsequent graft loss has been described for decades [7], the precise role of humoral alloreactivity has remained elusive because humoral immune reactants are usually not detectable in graft biopsies, and serial measurements of circulating alloantibodies in the post-transplantation period are not routinely performed. We have developed diagnostic techniques that improve the detection of humoral antigraft reactions in vivo and in vitro.

Our analysis takes into account the following: (*1*) Endothelial cells within organ grafts form the primary targets for immunologic attacks, but will very rapidly remove deposited antibodies and most complement components from their surface. Conventional immunohistochemistry fails to detect transiently bound humoral immune reactants in graft capillaries. Transient deposition of antibodies can be visualized, however, by the assessment of complement fragment C4d, a stable remainder of classic complement activation within capillaries in vivo [8].

(*2*) Human capillary endothelial cells, in contrast to rodents, express both human lymphocyte antigen (HLA) class I and class II molecules with high density even under normal physiological conditions [8, 9]. Therefore, the analysis of circulating alloantibodies in the clinical situation has to include class II-reactive antibodies. Conventional microcytotoxicity assays have low sensitivity

Key words: graft rejection, complement C4d, antibody, end-stage renal disease, chronic rejection, organ dysfunction.

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Table 1. Characteristics of renal transplant recipients, donors, and renal grafts included in this study

Variables	Number of cases[a]
Recipient age *≤45/>45 years*	122/95 (1)
Recipient sex *female/male*	83/135
Preformed panel-reactive antibodies *≤5/>5%*	153/56 (9)
Number of transplantations *1/>1*	151/67
Donor age *≤45/>45 years*	128/79 (11)
Donor sex *female/male*	78/131 (9)
Mismatches HLA-A and -B *0/≥1*	35/177 (6)
Mismatches HLA-DR *0/≥1*	135/77 (6)
Cold ischemia time *≤20/>20 hours*	33/175 (10)
Acute tubular necrosis *no/yes*	79/121 (18)
Acute rejection *no/yes*	81/137
CMV infections *no/yes*	189/25 (4)
Graft losses *no/yes*	89/129

[a] Missing values are in parentheses.

and are likely to miss class II-reactive antibodies when peripheral lymphocytes are used as targets. Sensitive cytofluorimetric methods and target cells that also display class II antigens, such as Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs), can greatly improve the detection of circulating alloantibodies in vitro [10].

Our study reports that humoral antigraft responses were detectable in about half of renal transplant recipients who underwent biopsy. We analyzed the survival of grafts with and without capillary deposition of complement C4d and compared the impact of early and late alloimmune reactions.

METHODS

Recipients of renal grafts

This study includes 218 recipients of cadaveric renal grafts transplanted between 1982 and 1997 at the division of transplant surgery, Klinikum Großhadern, Munich, Germany. A subgroup of patients with early graft dysfunction was included in a previous study [11]. All recipients had a negative cross-match with donor lymphocytes prior to transplantation. Further details concerning donor and recipient risk factors are given in Table 1.

Immunosuppressive therapy

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

Transplant biopsies

A total of 310 biopsies from 218 renal transplants had been collected since 1988. Grafts were biopsied because of functional impairment or development of persistent proteinuria after exclusion of mechanical factors by clinical and radiological diagnostic techniques. Biopsy samples were divided and processed for the preparation of cryostat slides and paraffin-embedded sections in parallel. Cryostat slides and paraffin-embedded sections were evaluated independently in the Institute of Immunology and in the Institute of Pathology, respectively. Diagnostic classification of graft biopsies followed standard histopathological criteria [12], and the Banff working classification was applied after 1994 [13].

Assessment of capillary deposition of complement C4d in graft biopsies

Cryostat sections prepared from postoperative graft biopsies were used in indirect immunoperoxidase staining as described in detail previously [11]. In brief, cryostat sections were fixed in ice-cold acetone and washed with phosphate-buffered saline (PBS; pH 7.4). The sections were incubated with a dilution of the indicated first antibody and, after washing, with the peroxidase-labeled secondary antibody. Finally, specimens were stained with 3-amino-9-ethyl-carbazole (Sigma, Deisenhofen, Germany), dimethylsulfoxide, and H_2O_2 and were counterstained with hemalaun before they were embedded in glycerol gelatin (all from Merck, Darmstadt, Germany).

Detection of circulating alloantibodies in serum samples

Corresponding serum samples at the time of biopsy were collected from 48 graft recipients and were stored at −20°C until further analysis. Circulating alloantibodies were measured by two-color flow cytometry and by a conventional microcytotoxicity panel test.

The two-color flow cytometric analysis was performed with EBV-transformed LCLs (Table 2) homozygous for HLA-DR specificities of donor type [14, 15], as described in detail previously [10].The allele assignment of the former HLA-DR specificities 2, 3, 5, and 6 has been specified according to the recent nomenclature. Therefore, the serum reactivity against all of the LCLs listed in Table 2 carrying these previous equivalents of HLA-DR was tested. In brief, 9×10^4 LCLs were incubated with patient serum and, after washing, with fresh human serum as the complement source. Subsequently, cells were incubated with phycoerythrin (PE)-labeled anti-human IgG, and fluorescein isothiocyanate (FITC)-conjugated anti-human complement fragment C4c. A minimum of 8000 cells per sample were then analyzed in a FACScan (Becton Dickinson, Mountain View, CA, USA) with 488 nm Argon Laser excitation wavelength and three

Table 2. Allele assignment of the 18 lymphoblastoid cell lines (LCL) used in the test panel

Workshop number	Name	HLA-DR (previous equivalents)
9004	JESTHOM	1
9009	KAS 011	16 (2)
9013	SCHU	15 (2)
9021	RSH	18 (3)
9022	COX	17 (3)
9031	BOLETH	4
9036	SP0010	11 (5)
9038	BM16	12 (5)
9051	PITOUT	7
9060	CB6B	13 (6)
9061	31227ABO	14 (6)
9063	WT47	13 (6)
9068	BM9	8
9070	LUY	8
9075	DKB	9
9078	PMG075	1
9089	BOB	11 (5)
9092	BM92	4

filters for the emitted fluorescence at 530 ± 30 nm, 585 nm ± 42 nm, and >650 nm. Nonreactivity in the two-color cytofluorimetric assay was defined by serum samples taken from normal volunteers. Any patient serum causing an increase in fluorescence intensity of at least 25% was classified as positive.

Panel-reactive antibodies were assessed by the standard microcytotoxicity technique of Terasaki and McClelland [16] using at least 50 typed lymphocyte samples. A panel reactivity of more than 5% was classified as positive.

Antibodies used in flow cytometric analysis and in immunoperoxidase staining

The following mouse monoclonal antibodies were used as primary antibodies: antibody specific for human endothelial cells (Pal-E, IgG2a; Monosan, Uden, Netherlands), anti-human complement fragment C4d (clone M4d2 [17]), and anti-C4d (IgG1; Quidel, San Diego, CA, USA). The following antisera were used as secondary antibodies: peroxidase-conjugated $F(ab')_2$-fragments of rabbit anti-mouse immunoglobulins, $F(ab')_2$-fragments of FITC-conjugated rabbit anti-human complement fragment C4c (all from Dako, Carpinteria, CA, USA), and $F(ab')_2$-fragments of PE-conjugated donkey anti-human IgG (Dianova, Hamburg, Germany).

Statistical analysis

Statistical analysis was performed using the SPSS Processor 7.5 for Windows 95. The death of a graft recipient was documented as graft failure. Graft survival according to dichotomized variables was estimated using the method of Kaplan–Meier and for each set the P value of the log-rank statistic was calculated. A multivariate analysis was performed with variables having a significant impact on graft survival using the Cox-Regression model. For all covariates the proportional hazard was assumed visually. During Cox analysis (forward conditional), the probability for stepwise entry of covariates was $P = 0.05$ and for removal was $P = 0.1$.

RESULTS

Detection of humoral alloreactivity in vivo and in vitro

Figure 1 shows the capillary deposition of complement fragment C4d in graft biopsies. There were three staining patterns: C4d+ indicates staining of all, C4d(+) staining of few, and C4d− absent staining of interstitial capillaries. Because the staining of only few capillaries was a transitional state from C4d+ to C4d− and vice versa, the C4d(+) cases were evaluated as C4d+. If a transplant was repeatedly biopsied and one biopsy (the index biopsy) showed capillary C4d, the transplant was evaluated as C4d+. If all biopsies from a given graft were either positive or negative, the first biopsy was taken as the index biopsy. All data presented here refer to index biopsies. Thus, 46% of first grafts and 72% of regrafts showed capillary C4d.

A comparison with histopathological results showed that capillary C4d occurred predominantly in rejections with vascular involvement (Table 3). Capillary C4d also could be found in other histologic entities such as acute tubular necrosis and different forms of transplant glomerulonephritis.

In 48 recipients, circulating alloantibodies in corresponding serum samples at the time of biopsy were measured using a conventional microcytotoxicity test (panel reactivity) and flow cytometry (LCL reactivity). Table 4 shows that 18 recipients had circulating antibodies reactive against LCL of donor DR type, and 19 recipients showed a panel reactivity of greater than 5%. Capillary C4d in biopsies was significantly associated with LCL reactivity only ($P = 0.008$).

Association of capillary C4d in biopsies with other determinants of transplantation

The distribution of recipient and donor determinants among C4d+ and C4d− transplant biopsies is listed in Table 5. Overall, capillary deposition of C4d is significantly associated with graft losses ($P = 0.001$), retransplants ($P = 0.001$), and preformed panel-reactive antibodies (>5%, $P = 0.005$). Substantially fewer patients received antilymphocyte antibody treatment in the C4d− group. Other factors were not significantly associated with capillary complement deposition (Tables 1 and 5).

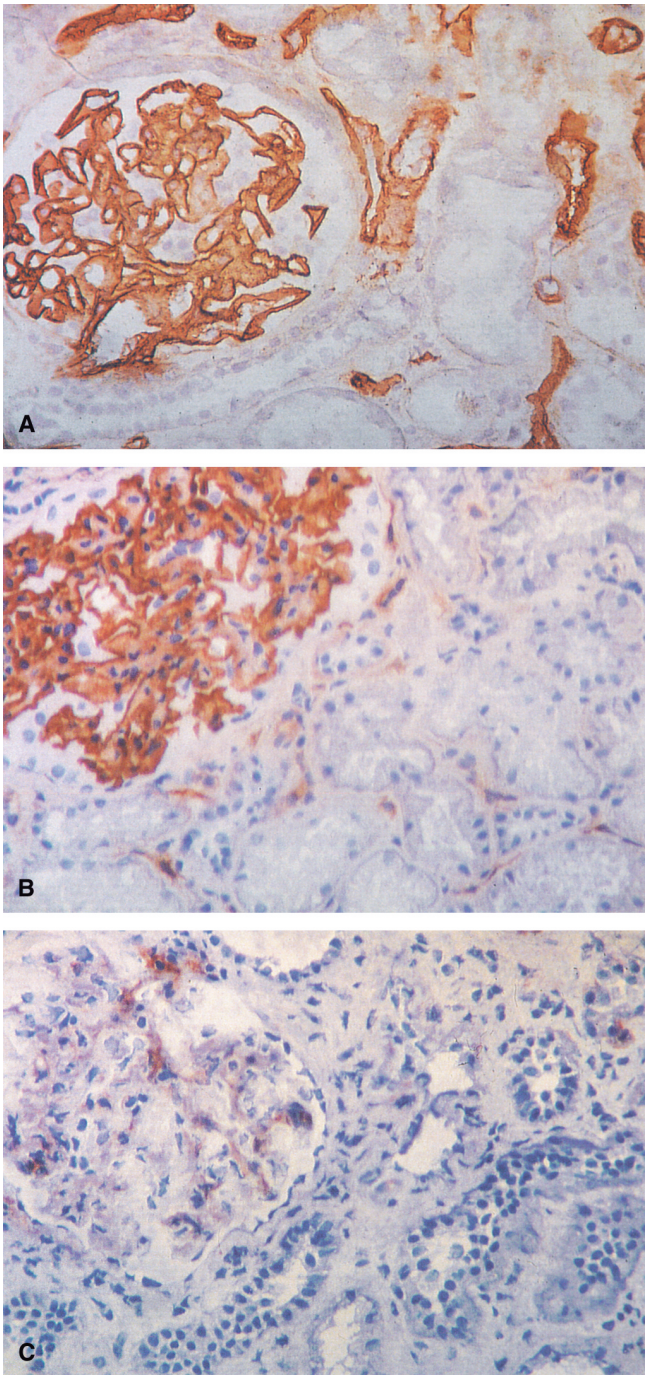

Fig. 1. Staining patterns of complement fragment C4d in renal graft biopsies. (*A*) C4d+: staining of all interstitial capillaries. (*B*) C4d(+): staining of only few capillaries. (*C*) C4d−: absent deposition of capillary C4d (magnification ×120).

Table 3. Distribution of histologic diagnoses and capillary deposition of C4d in 185 graft biopsies

	Capillary deposition of C4d	
	+	−
Borderline changes	7	8
Acute rejections		
Banff grade I, IIA	27	37
Banff grade IIB	12	5
Banff grade III	4	0
Chronic allograft nephropathy	14	8
Other allograft nephropathy		
Acute tubular necrosis	11	8
Vascular hyalinosis	1	2
Interstitial neutrophilic nephritis	0	2
Recurrent/de novo glomerulonephritis	6	4
Combined pathology[a]	19	10

Histological diagnoses refer to the predominant lesion in a given biopsy. Signs under capillary deposition of C4d denote: (+) positive staining; (−) absent staining.

[a] In grafts without predominating lesion but showing any combination of rejection with other pathological findings

Table 4. Capillary deposition of C4d in graft biopsies and antibody reactivity in corresponding serum samples from 48 graft recipients

		Capillary deposition of C4d		
		+	−	*P*
Reactivity against LCL of donor DR-type[a]	positive	14	4	
	negative	11	19	0.008
Panel-reactivity (>5%)	positive	12	7	
	negative	13	16	0.3

Capillary deposition of C4d is defined by: (+) positive staining; (−) absent staining. *P* values are by the Fisher's exact test.

[a] Flow cytometry

Impact of capillary deposition of C4d and other variables on graft survival

The cumulative survival of biopsied renal allografts with and without capillary deposition of complement C4d was analyzed using the method of Kaplan–Meier (log-rank statistic, $P = 0.0001$; Fig. 2). The estimated half-life (50% surviving) of grafts with capillary deposition of C4d was only four years, whereas in the C4d negative group, this interval was extended to eight years after transplantation. To analyze the differential effects of capillary deposition of C4d in early (≤6 months post-transplant) versus late (>6 months post-transplant) biopsies, the cumulative survival was estimated separately for both groups. Grafts with capillary C4d in early biopsies had a markedly reduced survival (75% graft survival at one month and 50% survival at 16 months after transplantation) as compared with grafts without capillary C4d (75% survival at 38 months, 50% graft survival not reached during the observation period; Fig. 3A). Grafts with late biopsies had a comparable survival rate within four years post-transplantation irrespective of the presence or absence of capillary C4d. There was a tendency toward a superior survival of grafts without capillary C4d in the late post-transplantation period (Fig. 3B).

Other well-known variables that could have an impact on graft survival are listed in Table 5. For each factor, the cumulative survival was analyzed separately using the method of Kaplan–Meier, and the *P* value of the log-rank statistic was calculated (Table 6).

Table 5. Capillary deposition of C4d in renal transplant biopsies and distribution of associated transplant determinants in all cases ($N = 218$)

	Capillary deposition of C4d		
	+	−	*P*
Graft losses			
yes	84	45	
no	33	56	0.001
Number of transplantations			
1	69	82	
>1	48 (41%)	19 (18.8%)	0.001
Preformed panel-reactive antibodies (mean ± SD)	16.27% (±28.36)	8.13% (±20.81)	0.005[a]
Anti-lymphocyte antibody treatment			
yes	50	19	
no	67	82	0.0002
Recipient age (mean ± SD)	40.8 years. (±11.1)	44.3 years. (±13.0)	NS
Gender			
male	69	66	
female	50	33	NS
Mismatches (mean ± SD)			
HLA-A	0.97 (±0.72)	0.88 (±0.69)	NS
HLA-B	0.87 (±0.71)	0.93 (±0.74)	NS
HLA-DR	1.35 (±0.71)	0.61 (±0.64)	NS

Capillary deposition of Cd4 is defined as: (+) positive staining; (−) absent staining. Pearson's chi square test was used to calculate the *P* values.
[a] PRA ≤5% vs. >5%

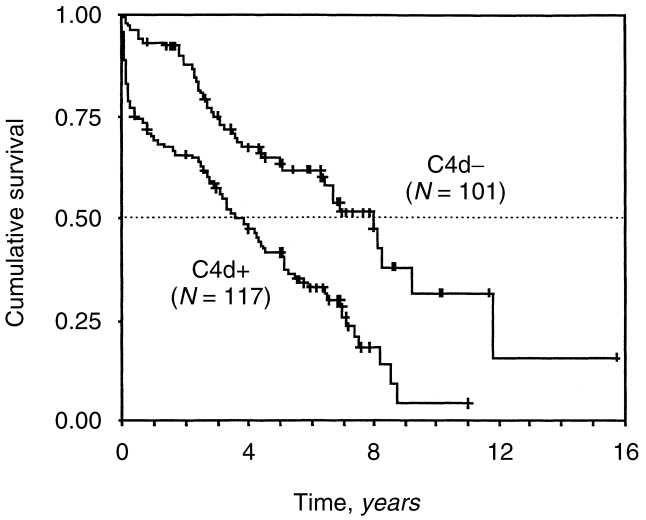

Fig. 2. Capillary C4d in biopsies and cumulative survival of renal grafts (+ censored cases; $P < 0.0001$).

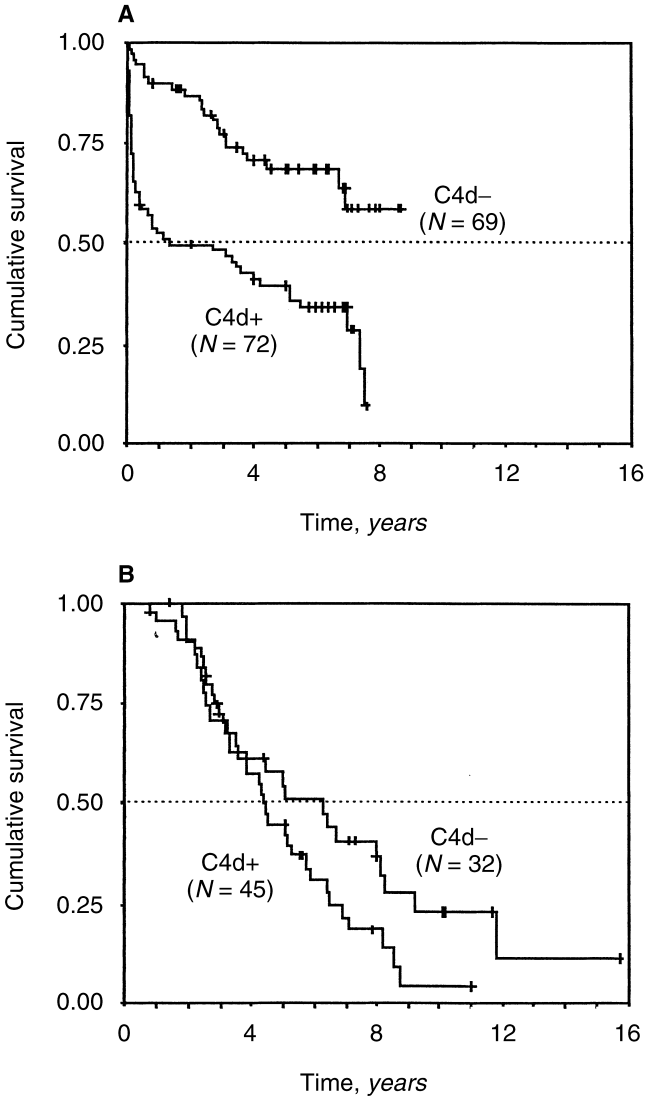

Fig. 3. Survival of renal grafts with index biopsy performed within six months after transplantation (*A*; $P < 0.0001$) and beyond six months after transplantation (*B;* + censored cases).

To evaluate the most important independent risk factors for the survival of biopsied renal allografts, a multivariate analysis was performed. Variables having a significant impact on graft survival (log rank statistic, $P = 0.01$) were analyzed using the Cox regression model (Table 6). Thus, during Cox regression, including all cases, three independent variables remained. Capillary deposition of C4d in renal allografts is accompanied by a 2.1-fold relative risk of subsequent graft loss, followed by a 1.7-fold relative risk for a donor age above 45 years, and a 1.7-fold relative risk for patients with preformed panel-reactive antibodies (>5%). In the subgroup of biopsies performed within six months after transplantation, only two independent variables remained during Cox regression analysis. In these cases, the deposition of C4d in capillaries is accompanied by a 3.1-fold relative risk of subsequent graft loss, followed by any mismatch at the HLA-DR locus (relative risk, 1.8). When grafts were biopsied later than six months after transplantation, no variable had a significant impact on graft outcome.

DISCUSSION

The present study elucidates the role of the humoral immune system on the survival of renal allografts. In a

Table 6. Impact of selected variables on the survival of biopsied renal allografts

Variable[c]	Univariate analysis P[ad]	Multivariate analysis Cox regression model		
		P[e]	Relative risk	95% CI
All cases ($N = 218$)[b]				
Capillary deposition of C4d	0.0001	0.0002	2.1	1.4–3.1
Preformed panel-reactive antibodies	0.0001	0.007	1.7	1.2–2.5
Donor age	0.001	0.004	1.7	1.1–2.5
First-/retransplants	0.002	—[g]	—	—
Mismatches HLA-DR	0.03			
Cold ischemia time	0.04			
Mismatches HLA-A and -B	0.3			
Acute tubular necrosis	0.6			
Acute rejections	0.8			
Renal allografts with biopsy within 6 months after transplantation ($N = 141$)[h]				
Capillary deposition of C4d	0.0001	0.0001	3.1	1.8–5.3
First-/retransplants	0.0006	—	—	—
Preformed panel-reactive antibodies	0.0007	—	—	—
Mismatches HLA-DR	0.007	0.02	1.8	1.1–2.9
Donor age	0.008	—	—	—
Cold ischemia time	0.03			
Mismatches HLA-A and -B	0.3			
Acute rejections	0.4			
Acute tubular necrosis	0.6			

Statistical analysis: SPSS-Processor 7.5 for Windows95, included in the multivariate analysis were only variables with a significant impact on graft survival ($P \leq 0.01$ during univariate analysis)

[b] Due to missing values 17 cases were excluded

[c] Dichotomized variables, see also Table 1

[d] P value of the log-rank statistic

[e] Significance, Cox regression model

[f] CI confidence interval

[g] Variables excluded during multivariate analysis

[h] Due to missing values 5 cases were excluded

retrospective approach, a subgroup of renal allografts necessitating biopsies was analyzed. Humoral alloreactions operating within the first six months after transplantation apparently have a much stronger effect than those beyond this period. The comprehensive assessment of humoral alloreactivity is accomplished by analyzing the residues of classical complement activation in graft capillaries and by including HLA class II-reactive alloantibodies in the analysis of serum reactivity. Capillary deposition of C4d in graft biopsies represents an immunohistochemical marker of humoral alloreactivity during the course of acute and chronic rejections.

It has been observed previously that rejections in the presence of circulating alloantibodies show a high incidence of severe vascular lesions, whereas severe tubulitis predominates in the absence of antibodies [18]. Notably, in these studies, graft capillaries were devoid of immunoglobulins and complement components by conventional immunofluorescence staining despite the presence of circulating, donor-specific anti-HLA class I antibodies. In the present study, capillary deposition of C4d was detectable in 46% of first grafts and 72% of regrafts that underwent biopsy and was prevalent in rejections showing vascular involvement. As is depicted in Table 3, capillary C4d is represented in various other histologic categories as well. Whether capillary C4d in these cases is predictive of subsequent vascular rejection has to be addressed in a larger study with serial biopsies.

Altogether, humoral alloreactions have a strong impact on the survival of biopsied renal allografts, resulting in a half-life (50% of grafts surviving) of four years of grafts with capillary deposition of C4d, contrasted by a half-life of eight years of grafts without C4d (Fig. 2). In a multivariate analysis using the Cox regression model, capillary C4d turned out to be the strongest independent predictor of subsequent graft loss, followed by the presence of preformed panel-reactive antibodies (>5%) and a donor age above 45 years. Other well-known determinants of graft survival, such as the occurrence of acute rejections and/or acute tubular necrosis, had no influence in this study with biopsied transplants.

Inspection of the survival graphs (Fig. 2) suggested an influence of humoral immunity during the early post-transplantation period and also during a later phase when chronic rejection usually develops. However, subdivision into early (within 6 months) and late (beyond 6 months) humoral alloreactions revealed striking differences (Fig. 3). Humoral alloreactivity operative early after transplantation had a much stronger impact than alloreactivity during later periods. Detection of capillary deposition of C4d within six months after transplantation was associated with a drastically reduced graft survival. In a multivariate analysis, it was the strongest predictor of subsequent graft loss (relative risk, 3.1), followed by any mismatch at the HLA-DR locus (relative risk, 1.8). Humoral alloreactivity in later periods added minimal risk. It can be deduced that the immunopathogenic mechanisms leading to adverse graft outcomes are confined to the early post-transplantation period. Capillary deposition of C4d heralds early graft loss within the first year but has also an impact beyond this period.

The immediate and the delayed effects of humoral immune reactions soon after transplantation again emphasize the critical importance of the early postoperative period. Other examples of early events with long-term consequences would be the occurrence of delayed graft function [19, 20] or the degree of reperfusion injury [21]. Also, in these instances, the progressive deterioration of organ function follows an early inciting event.

Humoral reactions late after transplantation, although clearly detectable in serum samples and in biopsies, obviously do not significantly reduce graft survival, as the survival rate of C4d+ grafts is only slightly worse than that of C4d− grafts. This finding is surprising and warrants further investigation. We have not yet determined whether early and late humoral attacks differ in strength

or in other immunobiological properties of the immune response. For instance, differential modes of allorecognition, for example, preferential reactivity against donor HLA class I or class II molecules, allorecognition via direct or indirect pathways [22] or development of TH1 versus TH2 responses [23] have to be considered. Indirect recognition can result in the generation of T-cell help (TH1) for cell-mediated cytotoxic reactions, but also in help (TH2) for alloantibody production by B cells [24]. Thus, it has to be taken into account that antibody production might be a marker for a primary T-cell response.

It is also notable that the survival of C4d negative grafts in the late post-transplantation period is considerably worse than that of complement-free grafts in the early period. Thus, grafts that necessitated biopsy late after transplantation seem to be uniformly affected by nonhumoral forces that constrain their survival. These forces may represent alloantigen-independent factors such as arterial hypertension, drug nephrotoxicity, chronic viral infection, hyperlipidemia, or delivery of insufficient nephron mass [25]. There is still the theoretical possibility that C4d negative grafts in the late period nevertheless suffered from an early humoral attack that escaped detection because either biopsies were not performed or cryostat slides for the assessment of capillary deposition C4d were not available.

In conclusion, demonstration of humoral reactivity against renal allografts and the associated reduced survival point to the humoral immune system as a potentially important target for immunosuppressive interventions. Using the techniques described, immunosuppressive therapies in transplant recipients can now be selected on the basis of individual demand, with anti-B-cell–directed immunosuppression being most promising during the early postoperative period. Appropriate therapeutic interventions have been proposed recently [26]. Future efforts should concentrate on the precise characterization of allogeneic endothelial cell antigens, since they represent the primary in vivo targets of humoral attacks.

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