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Clinical relevance of antibodies to HLA antigens undetectable by the standard complement-dependent cytotoxicity test

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Abstract Recent literary data suggest that antibodies to HLA antigens undetectable by the standard complement-dependent cytotoxicity test may cause not only chronic, but also acute immunological complications after kidney transplantation. The aim of this study was to investigate the significance of non-cytotoxic antibodies to HLA antigens for the development of immunological complications and a worse graft prognosis after first kidney transplantation. Sera before and early after transplantation from 120 first kidney recipients were analyzed by flow cytometry (FCXM), ELISA and the standard complement-dependent cytotoxicity (CDC) test. Pre-transplant FCXM negativity was related to a lower incidence of rejection episodes in the first post-transplant year ($P < 0.01$). A significant association between acute rejection and the presence of antibodies to HLA class II antigens before and after transplantation was also found ($P < 0.05$). Our study supports the findings of other centers of the detrimental role to the kidney graft played by anti-HLA antibodies undetectable by the classical CDC test.

Keywords Antibodies · HLA · Crossmatch · Flow cytometry · ELISA · Kidney transplantation · C4d

Abbreviations *ACR* Acute cellular rejection · *AHR* Acute humoral rejection · *CDC* Complement dependent cytotoxicity · *ELISA* Enzyme linked immunosorbent assay · *FCXM* Flow cytometry crossmatch · *HLA* Human leukocyte antigens · *LAT* Lambda antigen tray · *PTC* Peritubular capillary

Introduction

Despite major advances in the field of immunosuppressive therapy, transplant rejection remains a significant problem after kidney transplantation [1]. Various details concerning the role of non-cytotoxic antibodies to HLA antigens for the post-transplant incidence of immunological complications have been recently intensively studied [2]. An increasing number of reports suggests that anti-HLA antibodies after transplantation, as detected by flow cytometry (FCXM) and ELISA, are associated with acute or chronic rejection and a generally worse graft prognosis [3, 4, 5]. Recently, C4d positive deposits in peritubular capillaries (PTC) were recognized as a useful in-situ marker of humoral rejection that correlates strongly with the presence of circulating donor-specific antibodies [6, 7]. However, literary data of the predictive value of various techniques relating to the incidence of

acute humoral rejection are still often contradictory, most likely because of variations in the methods of antibody detection and immunosuppressive protocols. The aim of this study was to investigate the relative significance of the FCXM and ELISA tests for antibody detection and analysis with respect to the development of immunological complications and graft prognosis after transplantation. Serum samples from 120 patients before and shortly after first cadaveric kidney transplantation were analyzed against their respective kidney donor lymphocytes by two-color flow cytometry (FCXM), ELISA and the standard complement dependent cytotoxicity (CDC) test. Our study suggests that the application of sensitive methods (flow cytometry, ELISA) for antibody detection might be important in predicting post-transplant immunological complications. This analysis might therefore be also helpful in improving immunosuppressive therapy monitoring and in establishing the prognosis after organ transplantation.

Materials and methods

Patients and immunosuppressive protocols

Patients who had received first cadaveric kidney grafts between May and November 2001 at our center were enrolled in the study. Kidney recipient blood for serum analysis was collected after obtaining patients' informed consent before transplantation, 1 and 2 weeks after transplantation. Fifty-one recipients underwent renal biopsy for deterioration of graft function (increasing /or stagnation of serum creatinine levels, and decreasing of filtration rate) within 4 weeks post-transplantation. Lesions in allograft biopsies were classified according to the Banff classification scale [8]. All biopsies were retrospectively examined immunohistochemically on routine paraffin sections using anti-C4d polyclonal antibody (C4dpAb).

Immunosuppression after transplantation

Eighty-four patients received triple immunosuppression comprising cyclosporine A (CsA), mycophenolate mofetil (MMF), and methylprednisone (MP). Thirteen patients were treated with tacrolimus (FK506), MMF and MP. Eight patients received FK506 /or CsA and azathioprine (Aza), combined with/or without MP. Eleven patients received a combination of Rapamycine (sirolimus), CsA and MP, two patients received CsA /or FK506 with MP, and one patient was treated with FK506 only.

Analysis of antibodies

1. Flow cytometry crossmatch (FCXM): two-color flow cytometry analysis was performed as described previously [9, 10]. Mononuclear cells were isolated from donor spleen cells by Ficoll gradient centrifugation (Ficoll-Hypaque density 1.077 g/ml) and T lymphocytes were enriched by nylon wool separation. Donor T lymphocytes ($0.5–1.10^6$ lymphocytes per / test serum) were incubated with patient sera /or negative control serum (from one male AB Rh-negative individual) for 30 min at 20°C. Detection of patient IgG antibodies bound to respective donor cells was performed using fluorescein isothiocyanate (FITC)-labeled $F(ab)_2$ goat anti-human IgG antibody (30 min, 20°C) (Jackson ImmunoRes. Lab, West Grove, Pa., USA). T cells were stained by phycoerythrin (PE)-conjugated anti-CD3 monoclonal antibody (Becton Dickinson, San Jose, Calif., USA). Samples were analyzed after cell fixation on a FACScalibur flow cytometer (Becton Dickinson). Data analysis was performed using the CellQuest sofware; FITC (FL1) fluorescence of patient samples was compared with the mean FL1 fluorescence of negative control samples plus 2 standard deviations (cutoff point).
2. ELISA (LAT test): mixed class I&II trays (LATM) and LAT Class I & II (88 Ag and 40 Ag panel) kits for analysis of anti-HLA specificity were purchased from OneLambda (Canoga Park, Calif., USA), and tests were performed according to the manufacturer's instructions. Diluted patient sera (1:2 for LATM tests and 1:5 for LAT1240 and LAT1288 tests) were incubated for 1 h at 20°C in Terasaki microplates pre-coated with various HLA antigens isolated from EBV cell lines. After washing, bound IgG molecules were detected by anti-human IgG antibody conjugated to alkaline phosphatase (45 min 20°C). Optical density (OD) after addition of BCIP substrate was measured on an ELX 800NB reader and results were analyzed using the LAT software. Positivity of ELISA results was defined by the LAT sofware by comparison of the OD measured in sample wells with the OD of negative control wells.
3. Complement-dependent cytotoxicity (CDC) test: the complement-dependent cytotoxicity (NIH) test was performed according to the EFI (European Federation of Immunogenetics) accreditation standards protocol [11, 12].

Immunohistochemistry

Immunohistochemical staining of paraffin embedded tissues was performed using anti C4d polyclonal antibody (Biomedica, Austria). After deparaffinization and rehydratation, slides were treated in a pressure-cooker (10 min, 10 mmol/l citrate buffer, pH 6.0). Endogenous peroxidase was blocked for 30 min in 0.3% H_2O_2 in 70% methanol. Endogenous biotin was blocked using a Biotin blocking system (DakoCytomation, Denmark). Non-specific background staining was blocked by Protein Block (DakoCytomation). After incubation with antibody (diluted to 6.7 mg/l, 30 min), C4d antibodies were detected with a horse-radish peroxidase-labeled biotin-streptavidin system (LSAB Plus Kit, DakoCytomation) according to manufacturer's protocol. Finally, the sections were incubated with 3,3-diaminobenzidine (10 min), counterstained with Harriśs hematoxylin and mounted in Entellan (Merck, Germany).

Statistical analysis

The association between the positivity of the FCXM /or ELISA tests with the incidence of acute rejection episodes was evaluated by the chi square test; $P < 0.05$ was considered significant. With respect to rejection, the test (FCXM /or ELISA) was considered as "true positive" (TP), "false positive" (FP), "true negative" (TN), and "false negative" (FN), as indicated in Table 1.

The sensitivity of the FCXM and ELISA tests for prediction of acute rejection and their specificity as "confirmatory tests" for the absence of acute rejection in the first year after transplantation was calculated using the following formulas:

Table 1 Evaluation of the FCXM and ELISA tests with respect to the incidence of rejection

Acute rejection	Test	
	Positive (+)	Negative (–)
Yes (+)	True positive (TP)	False negative (FN)
No (–)	False positive (FP)	True negative (TN)

$$Sensitivity = \frac{TP}{TP + FN}\%$$

$$Specificity = \frac{TN}{FP + TN}\%$$

The relationship between the positivity in FCXM /or ELISA and median serum creatinine levels up to 1 year after transplantation was estimated using the Mann-Whitney test.

Results

Flow cytometry crossmatch (FCXM), ELISA-LAT tests and relation to acute rejection after transplantation

Serum samples from 120 patients before and shortly (1 and 2 weeks) after first cadaveric kidney transplantation were analyzed. Patients were followed up for the occurrence of rejection episodes, kidney graft function (as expressed by serum creatinine levels) and graft survival up to 1 year post-transplant. During the first month after transplantation, morphological features of allograft cellular rejection (ACR) were detected in 16 of 51 cases (31.3%), grade I in 3/51, grade II in 13/51. In two cases originally diagnosed as ACR, it was not possible to perform C4d staining due to lack of tissue. Biopsies with diffuse linear C4d deposits in PTCs were considered as positive (Fig. 1). A focal C4d positive pattern was evaluated as negative. There was C4d positive staining in 6 biopsies, in 3 of them simultaneously with ACR (21.4%). In 3 other cases there was C4d positivity alone. All C4d positive cases showed neutrofils in PTCs or glomerular capillaries and/or in both of these localities. An excellent correlation between C4d staining and the results of the FCXM test performed after transplantation was found (Table 2). No relationship was observed between the FCXM test performed before transplantation and C4d staining (results not shown).

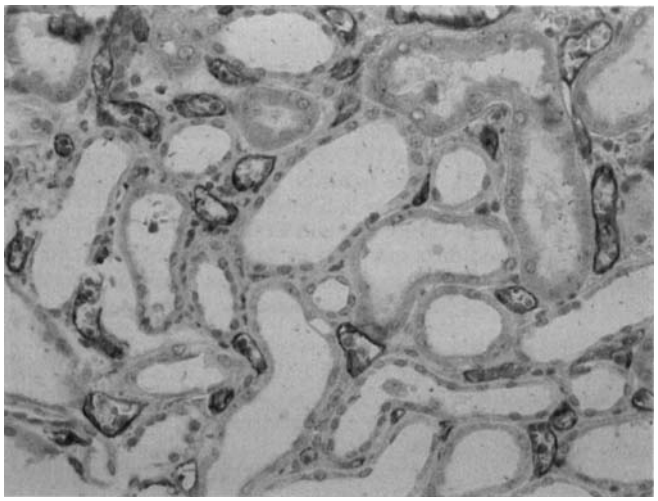

Fig. 1 Formalin-fixed, paraffin-embedded kidney biopsy stained with polyclonal anti-C4d. Intense staining is seen in the peritubular capillaries, which also contain mononuclear cells

All patient sera collected immediately before transplantation were negative with the respective donor cells in the complement-dependent cytotoxicity (CDC) test. However, testing of these sera by FCXM revealed the presence of non-complement dependent IgG antibodies to donor cells before transplantation in 9 patients (8%) (true positive (TP) plus false positive (FP), see Materials and methods) and after transplantation in 17 patients (14%) (Table 3). Two FCXM-positive pre-transplant patients turned FCXM-negative post-transplant. Pre-transplant FCXM test negativity was associated with a lower incidence of acute rejection episodes in the first year after transplantation ($P < 0.01$), while no correlation was seen between the result of the FCXM test performed 1 and 2 weeks post-transplant, and the occurrence of rejection episodes (Table 3). There was no statistically significant difference in kidney graft function, as expressed by median serum creatinine levels between the FCXM-positive and FCXM-negative patient groups 6 months (results not shown) and 1 year after transplantation (Table 4).

Analysis of the presence or absence of anti-HLA class I or II antibodies in all sera by the ELISA-LATM tests showed anti-HLA class I antibodies in 21 (18%) and 27 patients (23%) before and 2 weeks after transplantation, respectively (Table 3). No correlation was observed between the presence of anti-HLA class I antibodies (as detected by the ELISA-LATM test) and the incidence of acute rejection episodes. However, a significant association between anti-HLA class II antibodies after transplantation with the incidence of rejection episodes in the first post-transplant year was demonstrated ($P < 0.05$) (Table 3). In analogy with FXCM data, we found no statistically significant difference between the median serum creatinine levels of the ELISA-LATM-positive and negative patient groups at 6 months (results not shown) and at 1 year after transplantation (Table 4).

Table 2 Correlation (expressed as coefficient of agreement) between the FCXM test performed after transplantation and C4d staining

Test[a]		C4d	
		Positive (+)	Negative (−)
FCXM	Positive (+)	5	1
	Negative (-)	1	36
χ^b		0.81	

[a]Forty-three patients tested by both FCXM and C4d staining were evaluated

[b]Coefficient of agreement kappa (χ)

Table 3 Predictive value of the FCXM and ELISA tests performed before and after transplantation in relation to the incidence of acute rejection (*NS* not significant)

Acute rejection	Test	FCXM		ELISA			
				LATM class I		LATM class II	
		Before Tx[a]	After Tx	Before Tx	After Tx	Before Tx	After Tx
Yes (+)	True positive (TP)	6 (5%)	5 (4%)	7 (6%)	9 (8%)	7 (6%)	6 (5%)
	False positive (FP)	4 (3%)	12 (10%)	14 (12%)	18 (15%)	10 (8%)	6 (5%)
No (−)	True negative (TN)	87 (72%)	77 (64%)	78 (64%)	74 (61%)	82 (68%)	86 (71%)
	False negative (FN)	21 (18%)	21 (18%)	19 (16%)	18 (15%)	19 (16%)	21 (18%)
	Not performed (ND)	2 (2%)	5 (4%)	2 (2%)	1 (1%)	2 (2%)	1 (1%)
	Sensitivity	22%	27%	27%	33%	27%	22%
	Specificity	96%	85%	85%	80%	89%	93%
	P value	<0.01	NS	NS	NS	<0.05	<0.05

[a]Transplantation

Table 4 Median, minimal and maximal (shown in brackets) serum creatinine levels of FCXM /or ELISA-positive and negative patients 1 year after transplantation (*NS* not significant)

Test	FCXM		ELISA			
			LATM class I		LATM class II	
	Before Tx[a] Cr[b] (μmol/l)	After Tx Cr (μmol/l)	Before Tx Cr (μmol/l)	After Tx Cr (μmol/l)	Before Tx Cr (μmol/l)	After Tx Cr (μmol/l)
Positive (+)	146 (83–361)	123 (83–259)	146 (82–361)	135 (82–293)	135 (82–214)	122 (83–195)
Negative (−)	131 (72–554)	139 (72–554)	124 (72–554)	135 (72–554)	133 (72–554)	135 (72–554)
P values	NS	NS	NS	NS	NS	NS

[a]Transplantation
[b]Serum creatinine levels

Table 5 Results of analysis of FCXM-positive (+/+) patient sera before and after transplantation (*PRA* panel reactive antibodies)

Patient	HLA mismatch with donor	PRA		Specificity in ELISA-LAT			
				Before Tx		After Tx	
		Last	Maximal	Class I	Class II	Class I	Class II
1.	A24, A11, B56, DR14	2%	34%	A36[a] (A11), Cw9	DR53, DQ6	A36[a]	DQ2, DQ4
2.	A3, B8, DR14	6%	14%	B39[a] (B8)	DR4, DR8	B57	DR4
3.	A24, B51	24%	24%	B27	Negative	A69[a] (A24)	Negative
4.	A3, A23, B44	28%	62%	B49[a] (B44)	Negative	B49[a], B38, Cw4	Negative
5.	B15, B51, DR11	78%	78%	A32, B15 (anti-donor)	DR15, DQ4	A32, Cw1, Cw7, Cw8	DR8
6.	A1, B8, DR9	34%	34%	B38[a] (B8)	DQ4	B8 (anti-donor)	DR7
7.	A2, A32, B62, DR16	0%	22%	A19 (anti-donor)	Negative	A19 (anti-donor), B38	Negative

[a]Antibody to a CREG antigen with the mismatched donor antigen indicated in brackets

Results of the fine anti-HLA specificity analysis of FCXM-positive sera before and after transplantation using the ELISA-LAT test are shown in Tables 5 and 6. In the pre- and post- transplant FCXM-positive group (+/+), the ELISA-LAT test revealed donor-specific anti-HLA class I antibodies in only 2 of 7 patients (29%) (5 and 7, Table 5). Four patients (1, 2, 4 and 6) had antibodies reacting with antigens belonging to the same cross-reacting group (CREG) as the respective mismatched donor HLA class I antigens (Table 5). One patient (3) had specificity to HLA-B27, i.e., even though positive in FCXM, the specificity of the anti-HLA antibody was different from that of the respective mismatched donor HLA antigen. Donor-specific antibodies / or antibodies to CREG antigens with the respective mismatched donor HLA antigens were also found in this group (+/+) shortly after transplantation. One patient (6, Table 4) who developed anti-donor antibody

(HLA-B8) shortly after transplantation experienced irreversible rejection and lost his graft. The anti-donor antibody (HLA-B15) of patient 5 could not be detected after transplantation. The results of the specificity analysis of antibodies in the pre-transplant negative but post-transplant positive FCXM (−/+) group are shown in Table 6. The majority of patients were ELISA-negative before transplantation. With exception of patient 9, in the FCXM (−/+) group no donor-specific HLA class I antibodies could be detected after transplantation. Four patients (1, 3, 5, and 8, Table 6), although FCXM-positive after transplantation, had "irrelevant" HLA specificity of antibodies, while 3 patients were ELISA-negative. No donor-specific HLA class II antibodies could be detected by the ELISA-LAT test in the FCXM (+/+ and −/+) groups before and after transplantation.

Graft and patient survival after transplantation

Graft survival was 96% at 1 year after transplantation in our patient cohort. Five patients experienced graft failure, with two (1 and 4) (40%) due to immunological reasons (Table 7). One patient, (4 Table 7), who irreversibly rejected his graft, had antibodies strongly positive in both FCXM and ELISA-LAT tests before and after transplantation. No statistically significant correlation was demonstrated between graft survival at 1 year after transplantation and any of the tests performed (FCXM, ELISA-LAT). Patient survival was 97%; 4 patients died, 3 with a functioning graft, and 1 patient after graftectomy due to septic and hemorrhagic complications (patient 4, Table 7).

Discussion

The role of anti-HLA antibodies undetectable by classical methods in acute and chronic rejection is still not satisfactorily elucidated. There is prevailing consensus in earlier [13, 14, 15, 16] and more recent studies [17, 18] that a lack of anti-donor antibodies in the FCXM test before a second kidney transplantation is associated with a lower incidence of immunological complications and a better graft prognosis. Nevertheless, data concerning the predictive value of the FCXM test

Table 6 Results of analysis of FCXM-positive (−/+) patient sera before and after transplantation (*ND* Anti-HLA specificity not determined)

Patient	HLA mismatch with donor	PRA		Specificity in ELISA-LAT			
				Before Tx		After Tx	
		last	maximal	Class I	Class II	Class I	Class II
1.	A1, A66, B8, B41, DR3	0%	10%	Negative	Negative	B52	Negative
2.	A1, A3, B7, B8	2%	2%	ND	ND	B27[a] (B7)	Negative
3.	A2, B49, DR13	0%	0%	A23[a] (A2)	DQ7	B54	Negative
4.	A2, B18, DR16	0%	4%	Negative	Negative	B54[a] (B18)	Negative
5.	A24, A29	0%	4%	Negative	Negative	B48	DR7
6.	A3, A28, B60, B44	4%	4%	Negative	Negative	Negative	Negative
7.	A32, B44, B55, Cw2, Cw7	8%	8%	Negative	Negative	Negative	Negative
8.	A23, B44, DR7	14%	42%	Negative	Negative	B54	Negative
9.	A19, B60, B18, DR11	2%	2%	Negative	DR17	A19 (anti-donor), B13[a](B60)	Negative
10.	B18	0%	68%	Negative	Negative	Negative	Negative

[a]Antibody to a CREG antigen with the mismatched donor antigen indicated in brackets

Table 7 Causes for graft failure and results of the FCXM and ELISA-LAT tests

Patient	Causes of graft failure	FCXM		Specificity in ELISA-LAT			
				Before Tx		After Tx	
		Before Tx	After Tx	Class I	Class II	Class I	Class II
1	Afunctional graft, – rejection grade IIA, then grade III	Negative	Negative	Negative	Negative	Negative	Negative
2	Graft rupture	Negative	Negative	Negative	Negative	Negative	Negative
3	Thrombosis of renal vein	Negative	Negative	Negative	Negative	Negative	Negative
4[a]	Rejection grade IIB, then grade III	Positive	Positive	B38	DQ4	B8	DR7
5	Acute tubular necrosis, intragraft thrombosis	Negative	Negative	Negative	Negative	Negative	Negative

[a]The same patient as patient 6 indicated in Table 5

performed before first transplantation are still contradictory [19, 20, 21]. In our patient cohort, pre-transplant (anti-donor) FCXM test negativity was strongly associated with a lower incidence of acute rejection episodes in the first post-transplant year. This finding implies the usefulness of performing the FCXM test in (at least) high-risk patients before transplantation and is in agreement with several recent studies from other centers [22, 23, 24]. There is increasing evidence suggesting that the presence of donor-specific antibodies before and after transplantation as detected by FCXM (or ELISA) is associated with acute rejection episodes and a poor graft prognosis [4, 5, 25, 26, 27, 28, 29]. Analysis of the HLA specificity of pre- and post-transplant FXCM-positive (+/+) sera in our study revealed that the majority of patients in this group had either donor-specific antibodies /or antibodies reacting with crossreacting antigens (CREG) with the mismatched class I donor antigens. The reactivity with CREG antigens to the mismatched donor antigens could be explained by the assumption that these antibodies react with public epitopes and not with unique epitopes expressed by single HLA antigens [30]. With the exception of one patient, all patients in this group had either anti-donor antibodies or "anti-CREG" antibodies also shortly after transplantation. Surprisingly, the predictive value of the FCXM test with respect to rejection was not significant when the test was performed shortly after transplantation. Analysis of the specificity of the newly developed FCXM-positive antibodies (−/+) by ELISA showed that only 3 (33%) of the 10 patients had anti-donor /or cross-reacting antibodies with the respective mismatched donor antigens, while the remaining patients (Table 6) showed either "irrelevant" specificity or were ELISA negative. The FCXM-positivity in these cases might be due either to the presence of non-HLA antibodies, false positivity of the FCXM test, or because ELISA has a lower sensitivity than FCXM [31].

Although extensive studies have been performed, the specific role of anti-HLA class I and/or class II antibodies in graft rejection and failure has not yet been clearly defined [32, 33]. An advantage of the ELISA test over the classical CDC test is the capacity of the former to discriminate readily between antibodies to HLA class I and class II antigens [34]. The results of our study suggest the presence of anti-HLA class II antibodies before and after transplantation might be a risk factor for the occurrence of immunological complications after transplantation. This observation is in contrast with the study of Christiaans et al. [35] showing no correlation between the presence of class II antibodies and rejection; however it is in agreement with the study of Schönemann et al. [25] and that of Itescu et al. [36] supporting the detrimental role played by class II antibodies in kidney and heart graft rejection and failure. Interestingly, no donor-specific HLA class II antibodies could be detected in any case where specificity analysis was performed using the ELISA-LAT test. An explanation for this finding might be that either donor-specific class II antibodies are not produced or they are bound to the graft so they cannot be found in the serum. Further investigation of the role played by HLA class II antibodies in kidney graft rejection is needed.

Conclusion

Our study supports the findings of other centers of the detrimental role to the kidney graft played by anti-HLA antibodies undetectable by the classical CDC test. Analysis of these antibodies before and after transplantation by both flow cytometry and ELISA may help predict the onset of immunological complications. The presence of C4d, a component of the classical complement pathway, in PTCs is a sensitive diagnostic marker of acute humoral rejection. In summary, these diagnostic tests may serve as a basis for the improvement of therapeutic management and consequently help increase the rate of graft survival after kidney transplantation.

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