Detection and analysis of HLA class I specific alloantibodies in the sera of sensitised dialysis recipients waiting for kidney retransplantation

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Summary

Background. Almost all patients who rejected a kidney graft displayed anti-HLA antibodies (Abs), and *de novo* development of anti-HLA Abs in transplanted patients has been identified as a risk factor for both acute and chronic rejection. The aim of this study was to evaluate the specificities of anti-HLA class I Abs detected in the sera of alloimmunised patients waiting for a kidney retransplantation.

Methods. Kidney recipients (n = 62; male/female: 42/20; age: 43 ± 18 years) on waiting list for kidney retransplantation (52 for a second and 10 for a third transplant) were enrolled during 2002-2004 for HLA Abs screening. Among these kidney recipients, 50 who displayed persistently a panel reactive antibody (PRA) values >10% were selected and analysed for Abs specificity. Abs detection was performed by using complement dependent cytotoxicity technique and subsequently by ELISA to confirm or define better the % PRA and anti-HLA class I specificity. The specificities of anti-HLA Abs were classified as private, public, or multispecific.

Results. In 35 patients (70%) only Abs directed against private HLA class I specificities were found. These Abs were expressed by graft donors in 33 cases. In this group, PRA ranged from 20% to 60%. In 12 patients (24%), Abs directed against public epitopes belonging to cross reactive groups (CREG) or an association of anti-private and anti-public Abs occurred, with a PRA ranged from 25% to 90%. Three patients showed multispecific Abs with %PRA >80%.

Conclusions. The results of these study indicate that in the majority of donor-recipient pairs the immunogenic determinants were private specificities of mismatched HLA-A and B antigens, whereas in a lesser extent public CREG epitopes were found. Only in three patients no anti-HLA class I specificities were determined, as they displayed multispecific Abs. These findings may lead to improve donor-recipient matching in dialysis recipients waiting for kidney retransplantation.

Key Words: Kidney retransplantation, Sensitised dialysis patients, Anti-Human Leukocyte Antigens Antibodies, Private epitopes, Public epitopes, Cross-reactive groups.

Introduction

It is well-known that the presence of alloantibodies (Abs) against human leukocyte antigen (HLA) in the circulation of transplant recipients waiting for a subsequent kidney transplantation (KT) may increase graft rejection rates[1-3]. These antibodies can disappear after a short period or remain at elevated for different years also in the absence of further antigenic boosts[4]. It was also shown that almost all patients who rejected a kidney graft displayed anti-HLA Abs in their sera[5-7]. Moreover, development of *de novo* anti-HLA Abs in transplanted patients has been identified as a risk factor for both acute and chronic rejection[1-3]. The high polymorphism of the HLA system is not reflected by the number of different HLA class I and class II specific antibodies that can be generated following KT with mismatched HLA specificities[8]. Indeed, alloimmunised patients produce low anti-HLA Abs with highly poly-

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Table I. Demographic characteristics of patients in waiting list for kidney retransplantation.

	Total (%)	*Female (%)*	*Male (%)*	*P value**
Patients	62 (100)	20 (32.3) **	42 (67.7)	0.0144
Age (years; mean ± SD)	43 ± 18	43 ± 17	43 ± 15	1.000
Donor for the first kidney transplantation				
Cadaveric donor	58 (93.5)	18 (29.0)	40 (69.0)	0.0062
Living donor	4 (6.5)	2 (3.25)	2 (3.25)	1.000
Pre-transplant antibody status				
Positive	7 (11.3) ***	1 (1.6)	6 (9.7)	0.3679
Negative	55 (88.7)	19 (30.6)	36 (58.1)	0.0534
Causes of graft loss				
Acute irreversible rejection	4 (6.4)	1 (25.0)	3 (75.0)	0.0994
Chronic allograft nephropathy	44 (70.9)	16 (36.4)	28 (63.6)	0.0803
Vascular thrombosis	2 (3.2)	1 (50.0)	1 (50.0)	1.000
PLTD	1 (1.6)	-	1 (100.0)	-
Internal iliac artery stenosis	1 (1.6)	-	1 (100.0)	-
Recurrent primary disease	2 (3.2)	1 (50.0)	1 (50.0)	1.000
Surgical complications	5 (8.1)	2 (40.0)	3 (60.0)	0.6907
Viral infections	2 (3.2)	-	2 (100.0)	-
No compliance	1 (1.6)	1 (100.0)	-	-

* t-test for Female *vs.* Male. ** Five females (25%) had pregnancies (one or more). *** Patients in waiting list for a third kidney transplant and with antibodies against HLA-A and/or B antigen of the first donor.

morphic regions and relatively predictable specificity[9]. Since reactivity or non reactivity of recipients immune system depends on which epitope is presented to alloreactive T cells, it exists a hierarchy of HLA epitopes relevant to transplantation[9]. Patients may produce hierarchically induced-Abs against all mismatched epitopes, including intra-cross reactive groups (CREG) epitopes[10]. Patients with panel reactive antibody (PRA) activity more than 80% are considered to be highly sensitised, although this percentage might simply reflect the presence of anti-public epitope or anti-BW4 Abs[10]. Thus, a careful screening of patient sera to define the specificity of anti-HLA Abs might improve the chance of successful transplant, through the selection of a suitable kidney not carrying the HLA antigens corresponding to the antibody aspecificity[11]. In this study, the antigenic specificity of the anti-HLA class I Abs found in the serum of sensitised dialysis patients (SDP) waiting for kidney retransplantation was evaluated.

Patients and Methods

Patients

Blood sera from 62 kidney recipients (males: 42; females: 20; age: 43 ± 18 years) on waiting list for kidney retransplantation (52 for a second and 10 for a third transplant) from 2002 to 2004 at Department of Transplantation in San Martino University Hospital were enrolled for HLA antibody screening. These patients underwent a previous KT between 1983 and 2001 (Table I). Causes of return to dialysis after graft failure are shown in Table I. Among these kidney recipients, 50 that displayed persistent PRA values ≥ 10% were selected and included in this study for antibody specificity analysis. Serum samples (2 ml) were collected every three months from these patients during their waiting period on the list and stored at -40°C until analysis. All recipients and their specific donors were typed for class I and DRB1* HLA Antigens by standard lymphocytotoxicity and PCR-SSP technique, respectively. No patients had developed HLA class I specific Abs before first kidney graft, whereas 6/10 patients who were waiting for a third transplant showed Abs specific for HLA class I antigens (A or B) of the first donor (Table I). In these six patients no *de novo* antibody responses against the second failed graft antigens were found. Allograft biopsies for the detection of the fragment C4d were not available.

Serological analysis

Lymphocyte cell panels used for antibody screening were obtained locally from 75 HLA-class I (A, B, C) and class II (DR, D, Q) phenotype donors. Only Abs directed against HLA-A and B antigens were analysed in this study. Patients were considered to have anti-HLA class I Abs if they manifested 20% or higher PRA in three consecutive serum samples[12]. Sera were routinely treated with DTT (0.0025M - 0.005 M; pH 7.0 - 8.0) to establish that their reactivity was not due to IgM[13]. Patient blood sera was screened by complement dependent cytotoxicity (CDC) at different incubation temperatures, before and after treatment with the reducing agent DTT both against autologous and

Table II. Serological analysis of dialysis patients in waiting list for kidney retransplantation.

Study condition	*Patient Number (%)*
a) *PRA definition*	
Tested patients	62 (100)
PRA <10% *	12 (19.3)
PRA from 11% to 40%	30 (48.4)
PRA from 41% to 60%	11 (17.8)
PRA >60%	9 (14.5)
b) *Antibody specificity identification* **	
defined HLA class I Antibodies	47 (94)
one anti-private Antibody	30 (60)
two anti-private Antibody	5 (10)
Anti-CREGs epitopes	9 (18)
Anti-CREGs+ anti-private	3 (6)
SDP with undefined HLA class I Antibodies (PRA ≥80%)	3 (6)

* These patients were considered as non immunized as they did not display any even single anti-HLA antibody. ** In patients with PRA >10%. PRA, panel reactive antibody; CREG, cross reactive groups; SDP, sensitized dialysis patients.

panel lymphocytes. Blood sera that became unreactive to panel lymphocytes following the DTT treatment were assumed to contain allo-IgM, whereas those reactive sera to autologous lymphocytes were considered to display IgM against autologous target cells. DTT was obtained from Sigma (St. Louis, MO, U.S.A.). All blood sera were further analysed by a commercial enzyme-linked immunosorbent assay (ELISA) Lambda Antigen Trays (LAT™) and LAT™ Single Antigen (One Lambda Inc., CA, U.S.A.) in order to confirm or better define the % PRA as well as anti-HLA class I specificity. Anti-HLA class I positive patient sera were also screened for HLA anti-class II antibodies by LABScreen class II PRA and LABScreen class II Single Antigen (One Lambda Inc., CA, USA) technology that uses a panel of color-coded microspheres coated with HLA antigens and the LABScreen 100 flow analyzer for data acquisition and analysis. Abs were considered to be directed against the private specificity of the HLA molecules when reactivity against single HLA antigens and absence of reactivity against any other member of the same CTRG cluster was established. Abs were considered to be directed against public specificities when reactivity against a portion of a CREG reflecting or not a discrete epitope of all HLA antigens[14]. Abs were considered to be multispecific when a very broad reactivity against the lymphocyte cell panels were found.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). The Shapiro-Wilk W test was used in testing for normality. If the W statistic is significant, then the hypothesis that the respective distribution is normal should be rejected[15]. Continuous variables were compared by unpaired t test. Antibody specificity assignments was analyzed in contingency tables by χ^2 test to determine significant correlations χ^2 >3.84) between serum reactivity patterns and the presence of specific HLA marker in the lymphocyte panel. Antibody specificity was also assigned by using a correlation coefficient R (R values greater than 0.6 with P value <0.05 to indicate a relationship). Statistical significance was assumed for P value less than 0.05.

Results

The demographic characteristics and clinical information for the patients waiting for a kidney retransplantation are listed in Table I. The mean age of overall patients waiting list for kidney retransplantation was 43±18 years, with no statistical difference (P<0.998) between female (43±17) and male (43±15). Fifty patients among a total of 62 were found to show a PRA >10% (range 20%-100%) that reflected the presence of single or multiple Abs directed against different HLA-A and/or B specificities. The group of SDP was followed-up during a two years period for presence and specificity of anti-HLA-class I Abs. Collectively, 57 recognizable HLA private and/or public specificities were defined. The first kidney transplant was performed by using graft procured from cadaveric donors in 58 cases and living donors in 4 cases (P<0.0001). Pre-transplant antibody status was negative in 55 cases and positive in 7 cases for patients waiting for a second or third kidney transplantation, respectively.

Results of the serologic analysis are shown in Table II. Our finding showed that PRA ranged from 20% to 40% in 30 SDP (48.3%), from 41% to 60% in 11 SDP (17.8%), and from 70% to 100% in remaining 9 SDP (14.5%) (Table II). HLA antibody specificity and definition of the CREG clusters are shown in Tables III, IV and V. Sensitised patients were classified in three groups, according to the type of antibody (i.e. private, private and/or CREGs, multispecific). The first group

Table III. HLA class I phenotype and panel reactive antibody (PRA) in 35 patients with a failed kidney graft showing antibodies against private HLA class I (A, B) specificities.

				Class I HLA Antigen			*Antibody specificity*	
Patients	*Sex*	*%PRA*	*Tx*	*Recipient*	*1st Donor*	*2nd Donor*	*DS*	*NDS*
1	M	25	II	A2,3; B18,35	A2,**24**; B18,**44°**	A2,3; B18,35	A24	
2	M	25	I	A1,2;B51,63	A1,2; **B57,41**		B57,B41	
3	F	30	I	A24,11;B51,35	A24,11;B51,**7**			A1
4	M	30	I	A2,26;B51,38	A2,**3**; **B7**,39		B7	
5	F	30	I	A24,x;B51,8	**A1,29**;B51,8		A1	
6	M	50	II	A11,29;B35,44	**A2**,**28**; **B62**,44°	A29,x;B44,x	A2	
7	M	30	I	A2,24;B7,39	**A1**,2; B38,**51**		A1	
8	F	60	I	A32,x;B27,35	**A1**,**2**;B27,**49**		A1,A2	
9	F	50	II	A3,33;B35,x	A3,29;B35,**7**	**A2,**3; B35**,57&**	A2	
10	M	45	I	A1,11;B8,35	**A2**,11; B35,x		A2	
11	M	25	I	A2,29;B35,44	**A23**,30; B35,**49**		A23	
12	M	35	I	A28,30;B44,53	A29,x; B44,**51**		B51	
13	F	25	I	A1,2;B39,65	**A29**,x; B39,**44**		B44	
14	F	20	I	A2,31;B38,44	**A3,26**; **B13,**44		A26	
15	F#	20	I	A1,x;B8,63	A1,**24**; **B55**,63		A24	
16	F	40	I	A30,32; B35,58	**A2**,29; **B44**,x		A2	
17	F	50	I	A23,30; B39,49	**A2**,24; **B7,**39		A2	
18	M	50	I	A29,x; B7,51	**A2**,**25**; B7,51		B13	
19	M	20	I	A2,24 B35,70	**A1**,2; **B51**,**13**		A2	
20	M	40	I	A3,24;B51,x	**A2,32**; **B7**,**8**		A2	
21	M	25	II	A3,30; B13,35	**A2**,3; **B7**,**37**	**A11**,30; B13,35**&**	A11	
22	M	50	I	A30,x;B13,38	**A2**,30; B13,**18**		A2	
23	M	25	I	A34,68;B35,41	**A24**,x; B35,**61**		A24	
24	M	35	II	A2,x;B44,50	**A1**,**11**; B49,**57°**	2,x; B44**,41**	A1	
25	M	50	II	A24,29; B7,18	**A3**,30; B18,x**°**	A24,x; B18,x	A3	A2
26	M	30	II	A2,23; B8,35	A2,**3**; **B7**,8°	A2,x; **B51**,35	A3	
27	M	20	II	A23,30; B18,57	**A2,**x; **B51**,**44°**	A3,**23**; **B44**,**49**	B51	
28	M	40	I	A2,24; B35,37	**A1**,24; **B44**,**63**		A1, B44	
29	M	25	I	A2,3; B39,51	A2,3; **B49**,51		B49	
30	M	40	I	A3,24; B7,x	**A1**,3; B7,**63**		A1, B63	
31	F	30	II	A2,24; B14,22	**A3**,24; **B38**,55°	A24,**33**; **B62**,14	A3	
32	F	30	I	A2,28; B57,41	**A1**,2; **B38**,**60**		A1	
33	M	50	I	A1,34; B8,49	**A2**,26; B8,**51**		A2	
34	M	30	I	A30,33; B13,65	**A26**,30; **B27**,**37**		A26	
35	M	20	I	A2,x; B52,63	A2,**24**; B52,**49**		A24	

The HLA-A and HLA-B mismatches between recipient-donor pairs are shown in bold. The HLA typing of the first donor is shown. # Female patient with two pregnancies before renal disease and first admission to dialysis. ° Patients in waiting list for a third transplantation and with antibodies against HLA class I Antigens of the first donor but not against the second failed graft. & The HLA typing of the second donor is shown; indeed these two patients developed antibodies only against HLA class I Antigens of the second donor. DS, Donor specific; NDS, Non donor specific.

(Table II and Table III) included 35 patients (70%) showing only Abs directed against private HLA class I specificities that in 33 cases were those expressed by previous graft donors (first or second transplant) (94%). Only 2 patients showed Abs to specificities not related to the first donor (6%). In addition, only 3 patients out of 10 that were waiting for a third kidney transplant showed antibodies directed against HLA- class I Antigens of the second donor (Table III). Forty single anti-HLA and/or B private Abs were defined (Table III).

Table IV. HLA class I phenotype and panel reactive antibody (PRA %) in 12 patients with a failed kidney graft showing antibodies against private and/or public HLA class I (A, B) specificities.

				Class I HLA Antigen		*Antibody specificity*		
Patients	*Sex*	*%PRA*	*Tx*	*Recipient*	*Donor*	*DS*		*NDS*
						Private	*CREGs*	
1	M	80	I	A24,29; B35,44	**A1**,29; B44,**51**		1C	
2	F	60	I	A29,30; B13,57	**A3**,32; **B18**,**44**		Partially 12C(12,21)	A2
3	M	90	I	A3,11; B35,x	A3,**24**; **B13**,35		1C	
4	M	80	I	A1,24; B18,41	**A11**,24; **B35**,41		1C	
5	M	35	I	A11,31; B35,63	**A3**,30; **B61**,63		partially 7C (7,47,40 - 27p epitope**)	
6	M	80	I	A29,31; B44,x	**A2,**29; **B51**,44	A2	partially 5C(51,35)	
7	M	30	I	A24,x; B18,35	**A30**,**32**; **B44**,x	B44	partially 12C(12,21)	
8	M	30	I	A1,24; B14,49	**A26**,x; **B40**,14		partially10C (25,32 - 4c epitope**)	
9	M	80	I	A1,33; B8,64	**A2**,32; **B44**,**51**	Bw4	2C	
10	F	25	I	A2,24; B49,x	**A1**,2; **B18**,49		partially 8C (18,14 - 6c epitope**)	
11	M	25	I	A24,11; B35,51	A24,11; **B7**,51		partially 7C(7,27)	
12	M	70	I	A31,33; B64,50	**A28**,33; B64,**35**		partially 2C(2,28) 5C partially (18,35,5 - 5 p epitope**)	

The HLA-A,B mismatches between recipient-donor pairs are shown in bold. ** Intra CREG epitopes were defined according to Rodey[14]. DS, Donor specific; NDS, Non donor specific.

In this group PRA ranged from 20% to 60%. The second group (Table II and Table IV) included 12 patients (24%) showing Abs directed against public epitopes belonging to CREGs or an association of anti-private and anti-public Abs. Reactivity against public epitopes comprised: 1) epitopes shared by all class I molecules within a defined CREG; 2) discrete epitopes expressed by a more limited number of molecules within the CREG; 3) a partial reaction against only two or three molecules within a CREG, not reflecting the entire extension of the corresponding discrete epitope. In this group PRA ranged from 25% to 90%. The third group (Table II and Table V) was represented by three patients (6%) displaying multispecific antibodies with a PRA >80%. In these patients no HLA class I specificities could be determined.

Among HLA class I specific antibodies, 75% belonged to the HLA-A locus and only 25% of the defined antibodies were directed against HLA-B locus, although the number of incompatibilities towards both loci were almost identical (37 for HLA antigens of the HLA-A locus and 39 for those of HLA-B locus (Table III). In Figure 1 are showed the antibody specificities that occurred with a major frequency for both HLA-A locus (A2, A1, A24, A3, A26, A11, A23) and HLA-B locus (B7, B51, B44, B49, B13, B41, B57, B63). The Shapiro-Wilk test for normal distribution reached statistical significance (P = 0.0004).

Discussion

The SDP have few possibilities of receiving a new KT, because the presence of anti-HLA Abs induce positive cross-matches against most potential donors. In addition, the shortage of cadaveric donors increases the difficulty of avoiding repeated mismatches, thus adding further limitations for these patients. Thus, for a good graft outcome is crucial to determine the specificity of HLA-class I and class II antibodies in the patient's sera. Differently from anti-HLA class I Abs[14,16,17], until now the negative role of pre-transplant donor specific (DS) anti-HLA class II Abs on kidney graft outcome has not been well established[18-22]. Indeed, as reported by previous studies[18-20], their presence may not be deleterious unless associated with the simultaneous detection of DS anti-HLA class I Abs. For this reason, in the present study only anti-HLA class I specific Abs were evaluated. In our series 50/62 patients (80.6%) was sensitised from a previous failed kidney graft, although only in 47 it was possible to establish a clear correlation between antibody specificities (private or public) and mismatched HLA-A and B antigens of the previous graft. HLA-A and B specific stable Abs belonged to IgG class and no auto- or allo-IgM Abs were detected in our series. In the majo-

Table V. HLA class I phenotype and panel reactive antibody (PRA) in patients with a failed kidney graft showing multiple antibodies with no HLA class I specificities.

				Class I HLA Antigen			*Antibody specificity*
Patients	*Sex*	*%PRA*	*Tx*	*Recipient*	*1st Donor*	*2nd Donor*	
1	F	100	I	A2,x; B60,55	**A24**,**26**; **B65**,x		Multispecific
2	M	80	II	A2,32; B57,39	A2,**11**; **B35**,**60**	A2,33; B39,57	Multispecific
3	F	80	I	A1,32; B8,18	**A2**,**26**; **B44**,**49**		Multispecific

The HLA mismatches between recipient-donor pairs are shown in bold.

rity of patients (70%) Abs were directed against the unique epitope configuration of the mismatched HLA specificities (private epitopes), whereas in about a fourth (24%) they appeared specific for public epitopes related to donor mismatched A and B antigens. Only in three cases (6%) the Abs were classified as multispecific, as they showed a very broad reactivity from which no public or private specificities could be identified.

Our data indicate that in a large proportion of donor-recipient pairs the immunogenic epitopes involved in humoral allostimulation were mainly the private epitopes of the mismatched HLA determinants. However, reactivity to public epitopes was still responsible for allo-immunization in about a quarter of patients and generally determined an high PRA. Indeed, the presence in the majority of patients of only anti-private Abs may make easier to transplant them simply by avoiding donors bearing HLA-A and B specificities. Such as approach, in addition to the availability of a wide array of potent immunosuppressive agents for both rejection prevention and treatment, will allow a successful graft outcome for this category of immunised recipients, until now considered at increased risk for graft loss. The same strategy remains still valid also for remaining portion of immunised patients displaying well defined Abs directed to public HLA class I epitopes, although in these cases finding of suitable donors appears more difficult Only for a minority of highly immunised patients showing multispecific Abs (6% in our study) other protocols aimed to patient desensitisation (pre-transplant conditioning either using plasmapheresis followed by intravenous Immunoglobulins (IVIG), or high dose IVIG alone) was needed to successfully receive a kidney transplant[23-26].

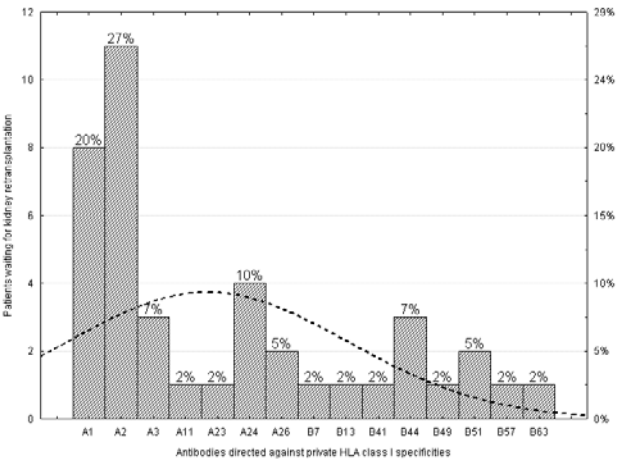

Figure 1. Main frequencies (%) of antibodies directed against private HLA class I specificities in 33 patients in the waiting list for kidney retransplantation.

The results of this study may lead to improve donor-recipient matching in dialysis recipients waiting for kidney retransplantation.

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