IMPACT OF PREFORMED AND DE NOVO ANTI-HLA DP ANTIBODIES IN RENAL ALLOGRAFT SURVIVAL

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ABSTRACT

The influence of antibodies against HLA-DP antigens detected with solid-phase assays on graft survival after kidney transplantation (KT) is uncertain. We evaluated with Luminex[®] the prevalence of pre and postransplant DP antibodies in 440 KT patients and their impact on graft survival. DP antibodies were present in 39.7% KT with pretransplant HLA antibodies and 47.7% with DSA. Graft survival of KT with pretransplant class-II DSA was worse than with non-DSA ($p=0.01$). DP antibodies did not influence graft survival. Of 346 patients monitored post-KT, 17.1% had HLA class-II antibodies, 56% with DP antibodies. Class-II DSA were detected in 39%, 60.9% of them had DP antibodies. Graft survival was worse in patients with class-II DSA ($p=0.022$). DP antibodies did not change these results. The presence of pretransplant and postransplant DSA is associated with a negative impact on graft survival. However, the presence of DP antibodies does not modify this impact significantly.

Keywords: renal transplant, HLA-DP antibodies, graft survival

1. INTRODUCTION

One of the most relevant advances in organ transplantation has been the development of more sensitive assays to detect HLA antibodies, namely solid-phase immunoassays in Luminex[®] platform [1-3]. Multiple studies have shown a correlation between the information derived from those new techniques and clinical events in renal allograft recipients [4-8]. Currently, Luminex[®] Single Antigen (LSA) studies permit the assessment of antibodies against antigens HLA A, B, C, DR, DQ and DP. Classically, HLA DP and HLA C have been considered to be less immunogenic than HLA A, B, DR and DQ molecules [9]. Two pairs of genes codify HLA DP molecules, two for region A and two for region B. Only one of these pairs of genes (DPA1 and DPB1) codify α - and β -chains in each molecule, both showing many polymorphisms. The other pair of genes (DPA2 y DPB2) show a limited polymorphism, and the codified antigens are not expressed on cell surface. Until now, 132 alleles have been located in DPB1 exon 2, whereas 27 alleles are known for the less polymorphic DPA1 (DPA1*01-04) [10,11].

The influence of HLA DP antibodies detected in Luminex[®] platform on short and long-term graft survival is not well-known. We aimed to evaluate their prevalence in renal allograft recipients before and after kidney transplantation (KT) and their impact on graft survival.

2. PATIENTS AND METHODS

2.1. Patients

From January 2008 to March 2013, 440 renal allograft recipients transplanted between 1979 and 2012 and functioning for more than 3 months were included in the study. Transplantations were performed after negative CDC crossmatch. Internal review board approved the study. A database included demographics, donor type, number of previous transplantations, initial and maintenance immunosuppression, delayed or immediate graft function and acute rejection episodes. Graft function (serum creatinine, MDRD4 estimated glomerular filtration rate (GFR) and urinary protein/creatinine ratio) was recorded at the time of HLA antibody testing.

Pretransplant serum samples were available for 291 of the 440 patients included. A total of 346 patients had postransplant serum samples studied and follow-up was completed until March 2013.

2.2. HLA antibodies determination and analyses

Serum samples were stored at -80°C until use. Anti-HLA antibodies detection was performed using Lifecodes-LifeScreen-Deluxe kits (Gen-Probe, Stanford, CT) in Luminex platform, according to manufacturer instructions. The kit comprised 7 beads with class-I glycoproteins and 5 with class-II glycoproteins. Three µl of beads were incubated with 40 µl of wash buffer and 12.5 µl of patient serum during 30 minutes and 3 washings were performed. Three µl of goat anti-human IgG conjugated to phycoerythrin or PE and 22 µl of wash buffer in each well were incubated for 30 minutes. Samples were analyzed in

Luminex 200 platform (Luminex, Austin, Tx) using Bio-Plex Manager 6.0 (software for data acquisition) and the program MatchIt!Antibody v1.1.0.2 (Gen-Probe) as software for analysis. A sample was considered to be positive for anti-HLA antibodies if: 1) at least one of the 7 beads with class-I and/or at least one of the 5 beads with class-II were positive with score 3; 2) fulfilled established criteria for the negative internal control beads (CON1, 2 & 3), and 3) showed an MFI for the positive control above 3500. Also the kit's positive and negative control sera were included in each assay.

The identification of class-II specific IgG anti-HLA allo-antibodies was made with Lifecodes LSATMClass-I and/or Class-II kits (Gen-Probe), according to manufacturer instructions. The kit LSA Class-I included 93 beads with class-I HLA molecules (HLA-A , B, C), and the kit LSA Class-II included 84 beads with class-II HLA molecules (HLA-DR, DQ, DP). Data were analyzed using MatchIt! (Gen-Probe). The MFI cut-off point was set at 1000 for positivity.

The donor specificity of anti-HLA antibodies was considered with the typing of HLA A, B, DRB and in some cases for C y DQB. In case of unavailability of DQB and C typing, specificity was assigned through linkage disequilibrium.

2.3. Statistical analysis

Normal continuous variables are expressed with means and standard deviation (SD), and non-normal variables are expressed with median and interquartile range (IQR). Chi-square tests were used for comparing categorical variables. Continuous variables were assessed using non-parametric Mann-Whitney U tests or Student t tests depending on normality distribution. Survival analyses were performed using Kaplan-Meier curves with log-rank test comparisons.

The multivariate analysis was performed using the logistic and Cox regression analysis.

The studies were performed using software SPSS v.21. Significance was considered with a $p < 0.05$.

3. RESULTS

3.1. Pretransplant antibodies

3.1.1. HLA DP antibodies in patients with pretransplant HLA antibodies

Pretransplant sera were available in 291 of the 440 KT recipients included in this study. HLA class-II IgG antibody screening was positive in 68 of them (23.3%). Twenty-seven (39.7%) had HLA DP antibodies in the LSA tests. Their characteristics compared with those patients with class-II positive screening but without HLA DP antibodies (n=41) are shown in table 1. Mean age in both groups was around 50 years, and they were predominantly women. Half of the patients had previously received another KT. Patients with HLA DP antibodies did not suffer biopsy-proven acute rejection episodes compared with 9.1% in the group of patients with pretransplant HLA class-II antibodies without DP specificities.

3.1.2. HLA DP antibodies in patients with pretransplant donor-specific anti-HLA antibodies (DSA)

Among the 68 patients who screened positive for HLA class-II antibodies, 36 (52.9%) had DSA. In 47.2% patients with DSA, we found HLA DP antibodies, while this percentage was only 31.2% in those patients with HLA non-DSA.

In the group of patients with DSA no clinical or demographical differences were found among patients with HLA DP antibodies and those with HLA without DP antibodies, although the rate of delayed graft function was higher in patients with HLA DP antibodies (61,9% vs 13,3%, $p=0.005$). However, a multivariate analysis adjusted for donor age and cold ischemia time, made this significant difference in DGF to disappear ($p=0,14$; IC 95% 0,2-42,5). The incidence of biopsy-proven acute rejection according to Banff 2009 classification was numerically but not significantly higher in the group of patients with HLA DP antibodies. They were T cell-mediated (Banff 4 category), except for two acute humoral rejection episodes (Banff 2 category) (one in a patient with HLA DP antibodies and one in a recipient without HLA DP antibodies).

Available donor DNA permitted typing for DP in 7 cases with pretransplant antibodies: for 5 patients in the group with HLA non DSA, DP antibodies resulted to be non donor-specific and none of them presented acute humoral rejection (graft survival: 100%). The remaining two recipients with available donor DP typing resulted to have DP DSA, besides DQ and DR DSA; both presented antibody mediated rejection.

3.1.3. Characteristics of DP antibodies

Median MFI of HLA DPB1* antibodies was similar in those with DSA and without DSA (3623 [IQR: 2067-15.736] vs 3672 [IQR: 1683-7279]). The most frequently

detected DPB1* specificity was DPB1*05:01, in 18.5% patients. In the subgroup with DSA, the most frequent specificity was DPB1*04:02 and in those with HLA non-DSA, it was DPB1*02:01. The distribution of specificities is depicted in Figure 1A. Analysis of DP epitopes in patients with pretransplant DP antibodies showed that 84DEAV was the most frequent (19,5%), followed by 35LV (14,6%); and 127L, 55DE and 84 VG were present in 9,8% of the patients.

All 27 patients with pretransplant anti HLA DP antibodies had prior sensitizing events (51.8 % prior transplant, 26% blood transfusion and 24% pregnancy). We could not find any correlation between a specific sensitizing event and different epitopes.

In addition to HLA DP antibodies, other specificities were detected in 90% of patients (Figure 2B). Only 3 patients had isolated HLA DP antibodies before KT. None of them had a sensitizing event (male, no previous transplantation and no blood transfusions). The median (IQR) MFI was 3059 (1937-3590). After KT, they did not show these DP antibodies. At follow up, graft survival was 100%, and none of them suffered an acute rejection episode.

3.1.4. Impact of pretransplant HLA DP antibodies on graft survival

Patients with pretransplant DSA showed worse censored-for-death graft survival than those with anti-HLA non-DSA ($p=0.01$) after a median follow-up of 76.5 months (IQR 35-149.7). The presence or absence of HLA DP antibodies did not affect graft survival in patients with DSA ($p=0.54$) and with HLA non-DSA (Figure 2).

A Cox regression analysis adjusted for retransplantation, recipient gender and pretransplant CDC PRA showed similar survival in patients with DSA despite the presence of HLA DP antibodies before kidney transplantation (Table 3).

3.2. Postransplant antibodies

3.2.1. HLA DP antibodies in patients with postransplant HLA antibodies

The analysis of postransplant antibodies in 346 patients showed that 59 (17.1%) had a positive screening for HLA class-II IgG antibodies. Thirty-three of them (56%) with HLA DP antibodies. When we compared patients with HLA DP antibodies (n=33) and those without (n=26), there were no differences in age, gender, type of donor, retransplantation, HLA mismatches and pretransplant sensitization (table 2). Biopsy-proven acute rejection rate was similar in both groups, being all T cell-mediated. No significant differences were detected in the proportion of patients with pretransplant DSA between those with postransplant HLA DP antibodies and patients without HLA DP antibodies (42.4 vs 35.6%, $p=0,81$)).

3.2.2. HLA DP antibodies in patients with postransplant DSA

Postransplant assessment evidenced DSA in 23 patients (39%). The presence of HLA DP antibodies in this group was 60.9% vs 52,8% in patients with anti-HLA non-DSA.

3.2.3. Characteristics of postransplant DP antibodies

Postransplant HLA DP antibodies showed a median MFI of 3116 (1814-4322). The most frequent DPB1* specificities were DPB1*17:01 (21,2%) followed by DPB1*13:01 and DPB1*18:01, present in 18.2% of patients. Similarly to pretransplantation, in most patients (91%) the presence of HLA DP antibodies was associated with other anti-HLA class-II DR and/or DQ specificities (Figura 3A). Only 3 patients had isolated HLA DP antibodies. In 2 cases, they appeared *de novo* after transplantation. At the end of follow-up, those 3 patients had a functioning graft and had not suffered rejection episodes. Median anti-HLA DP MFI values was similar in patients with DSA and patients with anti-HLA non-DSA [2435 (1718-3869) vs 2650 (1654-4289), respectively]. *De novo* postransplant HLA DP antibodies were detected in 5 patients with DSA and 8 patients without DSA. In 6 patients we could not confirm the *de novo* appearance, as pretransplant sera were not available (Figure 3B). Analysis of DP epitopes revealed that 84DEAV was the most common target postransplantation being present in 19% of patients with DP antibodies, same as before kidney transplantation. Epitopes 8V, 76I, 55DE and 35FV were detected in 14,3% patients respectively.

3.2.4. Impact of postransplant HLA DP antibodies on graft survival

Death-censored graft survival was better in patients without DSA after a median follow-up of 48 (35-50,7) months ($p=0.022$) after HLA antibody testing. The presence of HLA DP antibodies did not modify the impact on graft-survival. Patients with DSA with or without HLA DP antibodies showed comparable graft survival ($p=0.34$). Similarly, among those patients with non-DSA HLA antibodies, patients with and without HLA DP antibodies did not

show differences in graft survival at the end of follow-up ($p=0.50$) (Figure 4). A Cox regression analysis adjusted for retransplantation, receptor gender and pretransplant CDC PRA showed no differences in graft survival between patients with DSA with or without HLA DP after the transplant (Table 3).

4. DISCUSSION

In our experience, the prevalence of Luminex[®] detected anti-HLA DP antibodies is similar before and after KT (9.2% vs 9.5% respectively). However, the prevalence of isolated anti-HLA DP is very low. Only 3 patients before and 3 patients after KT showed those antibodies without any other class-II specificities (1% vs 0.9% respectively). In our study, 53% of patients with pretransplant anti-HLA antibodies had DSA, and almost half of them anti-HLA DP. In the postransplant study, 39% of patients showed DSA, 60% of them being anti-HLA DP. We cannot provide information of whether these anti-HLA DP antibodies are against the donor. Our results suggest that these antibodies do not impact graft survival, neither in patients with DSA nor in those without DSA. Consequently, it is possible that the assessment of anti-HLA DP specificities would only be necessary in selected cases, for instance in those with suspicion of humoral rejection and apparently negative DSA.

Before LSA assessments became common practice, clinical studies including the detection of HLA DP antibodies were scarcely reported. Technical complexity for the detection of such antibodies with lymphocytotoxicity probably explains this fact. In the most relevant pre-Luminex study, the presence of HLA DP antibodies in sera from 505 patients included in a waiting

list for KT showed a prevalence of 7.3% [12].

Despite the fact that solid-phase immunoassays allow precise detection of HLA antibodies, only few studies have reported their data on Luminex detected HLA DP antibodies. One of the first studies assessed 738 waitlisted patients with FlowPRA[®] beads. Anti-HLA class-II antibodies were detected in 23.1% of patients, a similar proportion to ours (23.3%) [13]. In this series of KT candidates, 12% of patients showed HLA DP antibodies. Two additional studies using Luminex[®] beads showed similar pretransplant prevalences [11,14].

The postransplant impact of the presence of HLA DP antibodies has been even less frequently reported. Before the development of solid-phase assays, a large study in more than 3600 patients receiving a deceased-donor primary KT evaluated the influence of mismatches in HLA DPB locus in one-year graft survival. No differences were observed when comparing all patients with 0, 1 or 2 DPB mismatches [15]. The increased number of DPB mismatches only showed a negative influence in graft survival in the subgroup of 1305 retransplantations: the 345 KT recipients without DPB mismatches, showed one-year graft survival of 83%, significantly better than those with 1 or 2 mismatches. In a later study, undertaken with Luminex platform, the presence of HLA DP antibodies was more frequent in patients with graft rejection (19,5% vs 5,1%, $p < 0,001$), though the impact on survival was not reported [16].

Numerous studies have demonstrated that the presence of DSA, both pre- and postransplantation is associated with a negative impact on renal allograft survival [17-20]. Our results confirm this negative impact, both for pre- and

postransplant DSA. Donor HLA DP typing is not usually performed, and consequently, it is difficult to know whether or not the IgG antibodies detected with single antigen studies are directed specifically against the donor antigens. As a result, the potential clinical relevance, the relationship with humoral rejection, and the impact on graft survival are not easy to assess, as it has more easily been analyzed for class-II anti-HLA DR and DQ [21-23]. Only one report evaluated the impact of the presence of DSA against HLA DP compared with anti-HLA DP non-DSA. Three of 6 patients with pretransplant donor-specific anti-HLA DP, without any other DSA, developed antibody-mediated rejection, compared with only 1 of 15 patients with non donor specific anti-HLA DP ($p=0,02$). The main concern of this latter study, in addition to the low number of patients, is that no data was reported on the impact of humoral rejection on graft survival or graft loss [24]. Other authors have described isolated cases of antibody-mediated rejection in patients with donor specific HLA DP antibodies without other DSA [25-27].

The main limitation of our study is the absence of information regarding donor HLA-DP typing, resulting in the impossibility of identifying true donor-specificity of anti-HLA DP antibodies. Besides, patients were included in the study only if their kidney grafts survived over three months. LSA tests were not performed for all patients included in the study but only for those who had a positive HLA class-II screening result before or after transplantation, thus assuming sera which screened negative should not show DP antibodies.

5. CONCLUSIONS

In our population, approximately 10% of KT recipients show anti-HLA DP antibodies in single antigen studies, both pre- and postransplantation. The presence of pretransplant and postransplant DSA are both associated with a negative impact on graft survival. However, the presence of HLA DP antibodies does not modify this impact significantly. Nevertheless, DP antibodies have been identified in cases of antibody-mediated rejection. In selected cases of humoral rejection with undetected HLA A, B, DR or DQ DSA, HLA DP antibodies, as well as other non-HLA antibodies, should be assessed. Larger studies with KT donors well typed for HLA DP are needed to confirm our results.

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Authorship: DRP, JP and MC designed research the study, performed the study, analyzed data and wrote the paper; CG and JJH performed the antibody tests; JG did the pathology studies; MJPS and MM collected data. All authors reviewed the manuscript draft and approved the final version.

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Table 1. Demographic and clinical characteristics of studied patients distributed according to the presence or absence of donor-specific antibodies (DSA) and the presence or absence of HLA DP antibodies before kidney transplantation.

	No antibodies (n=223)	Anti-HLA no DSA (n=32)		p	DSA (n=36)		p
		HLA DP antibodies (n=10)	No HLA DP antibodies (n=22)		HLA DP antibodies (n=17)	No HLA DP antibodies (n=19)	
Recipient age (years, mean $\pm$ SD)	52,5 ($\pm$ 14,5)	49,2 ($\pm$ 15,2)	48,8 ($\pm$ 13,3)	0,86	52,3 ($\pm$ 14,03)	46,4 ($\pm$ 10,2)	0,95
Female recipient (%)	32,1%	42,9%	64,7%	0,19	66,7%	64,7%	1
Deceased donor (%)	91,1%	92,9%	100%	0,45	95,2%	93,3%	0,34
Retransplantation (%)	8%	20%	13,6%	0,67	71,4%	80%	0,70
Peak PRA CDC>5% (%)	11,7%	28,6%	17,6%	0,61	42,9%	40%	0,86
Pretransplant PRA CDC>5% (%)	1%	0	0	-	26,7%	23,5%	0,57
HLA A/B/DR mismatch (mean $\pm$ SD)	4,1 ($\pm$ 1,2)	4 ($\pm$ 1,1)	3,9 ($\pm$ 1,2)	0,89	4,4 ($\pm$ 0,9)	3,6 ($\pm$ 1,6)	0,19
Delayed graft function (%)	37,9%	42,9%	23,5%	0,45	61,9%	13,3%	0,005
Acute rejection (%) (ACR/AHR/Borderline)	7,2% (8/2/6)	0%	9,1% (2/0/0)	-	23,5% (2/1/1)	5,2% (0/1/0)	0,29
DSA class I (n, %)							
Only class I	4 (1,7%)	0	0	-	0	0	0,99
Class I and II	0	0	0		4 (23,5%)	2 (10,5%)	
Follow-up after KT [months: median (IQR)]	45 (26-66)	56 (28-97)	80 (52-103)	0,22	66 (40-86)	47 (23-82,5)	0,21

Table 2. Demographic and clinical characteristics of studied patients distributed according to the presence or absence of donor-specific antibodies (DSA) and the presence or absence of HLA DP antibodies after kidney transplantation.

	No antibodies (n=287)	Anti-HLA no DSA (n=36)		p	DSA (n=23)		p
		HLA DP antibodies (n=19)	No HLA DP antibodies (n=17)		HLA DP antibodies (n=14)	No HLA DP antibodies (n=9)	
Recipient age (years, mean $\pm$ SD)	48,8 ($\pm$ 14,2)	50,1 ($\pm$ 14,9)	48,9 ($\pm$ 15,6)	0,98	48,1 ($\pm$ 12,2)	48,3 ($\pm$ 12,4)	0,96
Female recipient (%)	34,1%	66,9%	56,5%	0,29	60%	61,9%	0,94
Deceased donor (%)	91,6%	100%	100%	-	100%	84,6%	0,43
Retransplantation (%)	9,4%	23,1%	47,8%	0,14	60%	61,5%	0,92
Peak PRA CDC>5% (%)	12%	23,1%	26,1%	0,84	30%	61,5%	0,21
Pretransplant PRA CDC>5% (%)	1,8%	0	0	-	0	13,3%	0,07
HLA A/B/DR mismatch (mean $\pm$ SD)	3,7 ($\pm$ 1,4)	4,1 ($\pm$ 0,98)	3,04 ($\pm$ 1,5)	0,02	3,9 ($\pm$ 1,1)	3,9 ($\pm$ 1,2)	0,19
Delayed graft function (%)	41,4%	53,8%	47,8%	0,83	50,1%	53,6%	0,85
Acute rejection (%) (ACR/AHR/Borderline)	5,9% (9/2/6)	0	23,5% (3/0/1)	-	14,3% (0/2/0)	22,2% (2/0/0)	0,59
Pretransplant DSA (%)	6,8%	44,1%	46,6%	0,91	83,3%	85,7%	0,97
Postrasplant DSA class I (n, %)							
Only class I	2 (0,7%)	0	1 (5,9%)	-	0	0	-
Class I and II	0	0	0		0	0	
Follow-up after HLA test [months: median (IQR)]	48 (40-51)	48 (29-52)	47 (44-51)	0,61	45,5 (30-48)	29 (26-34)	0,87

Table 3. Cox regression analysis before and after transplantation comparing DSA patients with and without HLA DP antibodies

	Pre-transplantation		
	HR	IC 95%	p
Retransplantation	1,59	0,18 13,8	0,67
Female recipient	1,05	0,097 11,39	0,96
Pretransplant PRC CDC	0,99	0,93 1,05	0,81

	Post-transplantation		
	HR	IC 95%	p
Retransplantation	1.14	0,16 7,81	0,892
Female recipient	1,26	0,12 12,66	0,842
Pretransplant PRC CDC	1,04	0,95 1,061	0,890

Figure legends

Figure 1 (A) Pretransplant HLA-DP antibodies specificities (B) Detection of DP antibodies and other HLA clase II antibodies (DR, DQ).

Figure 2 Kidney Allograft Survival acording to donor specific antibodies (DSA) and antiHLA DP antibodies detected pretransplantation.

Figure 3 (A) Detection of DP antibodies and other HLA clase II antibodies (DR, DQ). (B) Preformed and *de novo* antiHLA-DP antibodies in patients with donor specific antibodies (DSA) and non-donor specific antibodies (HLA non-DSA).

Figure 4 Kidney Allograft Survival acording to donor specific antibodies (DSA) and antiHLA DP antibodies detected postransplantation.

Figure 1

Figure 2

Figure 3

Figure 4