Clinical relevance of pretransplant anti-HLA donor-specific antibodies: Does C1q-fixation matter?

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Abstract

Anti-HLA donor-specific antibodies (DSA) identified by single antigen bead array (SAB) are questioned for their excess in sensitivity and lack of event prediction after transplantation.

Population and methods: We retrospectively evaluated specific types of preformed DSA (class I, class II or C1q-fixing) and their impact on graft survival. Kidney transplantations performed across negative CDC-crossmatch were included (n = 355). Anti-HLA antibodies were tested using SAB to identify DSA and their capacity to fix C1q.

Results: Twenty-eight patients with pretransplant DSA+ with MFI > 2000 were selected to assess C1q fixation. DSA were C1q + in 15 patients and C1q- in 13, without significant differences in demographics, acute rejection, graft loss or renal function. The maximum MFI of DSA in patients with C1q-fixing DSA was significantly higher (p = 0.008). Patients with DSA class-I suffered more antibody-mediated rejection (AMR) and had worse graft survival than class-II. The capacity of DSA I to fix C1q did not correlate with rejection, graft function or graft loss.

Conclusions: C1q testing in pretransplant sera with DSA was unable to predict acute antibody-mediated rejection or early graft loss, but the presence of DSA class I compared to DSA only class II did. Despite non-fixing complement in vitro, pretransplant C1q-negative DSA I can mediate rejection and graft loss.

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1. Introduction

Newer solid-phase techniques based on antigen bead arrays employed to identify HLA antibodies are questioned by some groups for their excess in sensitivity and subsequent lack of prediction of clinical events after solid organ transplantation [1–4]. However, several studies have shown that pretransplant HLA donor-specific antibodies (DSA) identified by single antigen bead arrays (SAB) are associated to lower kidney graft survival and increased risk of rejection [5–9]. A recent meta-analysis – combining altogether 145 DSA positive patients of 1119 from seven studies – shows that the existence of pretransplant DSA only detected with SAB (negative with complement-dependent cytotoxicity (CDC) and flow cytometry assays), increases the risk of antibody-mediated rejection (AMR) and graft loss [10]. No data about the characteristics of DSA associated with rejection or graft loss are included in the review.

It has been suggested that pre-transplant DSA have an impact on graft survival if acute AMR happens within 90 days after transplantation [11]. Interestingly, desensitization experiences have also shown that patients with pretransplant DSA who present acute AMR are at higher risk for chronic AMR and lower kidney graft survival [12]. Means to identify which pretransplant DSA are harmful for the kidney graft and associate with early rejection are crucial in order to decide when kidney transplantation should be performed or under which immunosuppressive treatment.

New developed tests try to discriminate which HLA antibodies detected with SAB can fix complement despite not doing it in CDC assays. A novel test to detect HLA antibodies able to bind complement C4d on flow beads was developed in Vienna a decade ago, but still not commercialized [13–16]. Another test based on the capacity of HLA antibodies to fix C1q was built up later on in Stanford [17,18]. Scientific evidence to support the relevance of employing these tests in clinical transplantation comes mainly from the groups who have risen up the tests in their own laboratories. Scarce small studies have found some correlation between...
2. Objectives

We performed a retrospective study in kidney transplants to evaluate if a specific type of preformed DSA, whether class I or II or C1q fixing, has a clearer impact on graft survival.

3. Materials and methods

3.1. Population

We included 355 kidney transplants from transplant centers in Barcelona and Canary Islands (Spain): 211 had received consecutive kidney transplants between July 2006–July 2011 at Hospital del Mar (Barcelona) and 144 were transplanted in Canary Islands between February 2007–December 2011 with HLA antibody monitoring in Hospital Insular (Las Palmas). Patients signed an informed consent and the project was approved by the local internal review board. Patients received a kidney graft across a prospective negative CDC crossmatch with mixed lymphocytes without anti-human globulin.

All study patients were followed after transplantation. A transplant data base including demographics, type of donor, number of transplant, pregnancies, initial immunosuppression, delayed graft function (need of dialysis within the first week post-transplantation), biopsy-proven acute rejection, serum creatinine and protein to creatinine urinary ratio was employed for analysis. Graft loss was considered when the patient returned to dialysis or died with a functioning graft. No deaths before graft loss were registered during follow-up in the group of patients with pretransplant DSA. Immunological data were also collected (CDC mixed lymphocyte panel reactive antibodies [PRA], HLA typing and SAB results).

3.2. SAB antibody testing

3.2.1. Anti-HLA antibody screening and characterization

Serum samples were collected from the patients and stored at –80 °C until use. Screening for HLA antibodies was performed using the Luminex Lifecodes LifeScreen Deluxe assay (Gen-probe, Stanford, CT, USA), according to manufacturer’s instructions. The kit was composed of seven beads coated with HLA class I molecules and five beads coated with HLA class II molecules. Briefly, 5 μL of multiplexed microbeads were incubated with 12.5 μL of patient’s serum for 30 min and washed 3 times to remove unbound antibody. Fifty μL of anti-human IgG antibody conjugated to phycoerythrin was added for 30 min. Samples were analyzed on a Luminex 100 instrument (Luminex, Austin, TX, USA) using Luminex 100 IS v 2.3 as software for data acquisition, and MatchIt program as analysis software. To determine if a bead is positive, three adjusted MFI ratios were calculated dividing the individual bead median MFI by the median MFI of three negative control beads. A positive value for 3 of the calculations indicated a positive bead reaction. A sample was considered positive for HLA antibodies if at least one of seven class I and/or five class II beads was positive. Positive and negative control sera were included in each test. To discard an eventual prozone effect, before the screening test 30 samples were pre-treated with dithiothreitol (incubation for 30 min at 37 °C with 0.01 M dithiothreitol) and other 30 samples were heat inactivated for 30 min at 56 °C as described by Schnaidt M et al. in 2011. We found no discordances in the screening results.

Donor-specific antibodies (DSA) on single antigen beads were tested using Lifecodes LSA class I and/or class II assays (Gen-probe), according to manufacturer’s instructions. The LSA class I kit was composed of 93 beads coated with HLA class I molecules (HLA-A, B, C), and LSA class II had 69 beads with HLA class II molecules (HLA-DR, DQ, DP). Data were analyzed using MatchIT software and the cut-off for a positive reaction was set in MFI raw value > 2000. Antibodies against HLA molecules of the donor were assigned as DSA considering low resolution HLA typing data. Since no data for donor’s HLA-DP were available, anti-DP antibodies weren’t considered in the analysis. One Lambda commercial kits were used to confirm DSA detected with Gen-probe kits before C1q testing in all patients with DSA.

3.2.2. C1q single antigen bead assay

Detection of antibodies capable of fixing complement was performed using SAB and C1q screen kits (One Lambda Inc., Canoga Park, CA, USA) according to manufacturer’s instructions. Samples were analyzed on a Luminex 100 instrument using Luminex 100 IS v 2.3 as software for data acquisition, and Fusion 2.0 program (One Lambda) as analysis software. Sera were inactivated by heating 30 min at 56 °C, spiked with the complement component C1q and incubated with 5 μL of antigen-coated beads for 20 min at room temperature. Later, the samples were incubated with 5 μL of phycoerythrin labeled anti-C1q antibody for 20 min at room temperature, washed twice with 80 μL of wash buffer and measured on the Luminex. Data were analyzed using raw MFI values, and the cut-off for a positive reaction was set in MFI raw value > 500. Positive and negative control sera were included in each test. This technique detects IgM antibodies and the subset of IgG antibodies able to bind C1q.

3.3. Statistical analysis

Continuous variables were analyzed by Student’s t-test or non-parametrical Mann–Whitney U test. Categorical data were analyzed with square Chi or Fisher’s exact test. Graft survival in kidney transplant recipients with pretransplant HLA class I and II DSA, and between patients with C1q positive and negative DSA were compared using the Kaplan–Meier method and the log-rank tests. Data are expressed as means ± standard deviation or medians with interquartile range (IQR) as appropriate. Statistical analyses were performed using SPSS version 20 (SPSS Inc, Chicago, IL, USA).

4. Results

A flow chart of the study population is shown in Fig. 1. Sixty-six patients of 355 (18.6%) had HLA antibodies positive screening tests before transplantation and were tested for donor specificity. In our experience pretransplant DSA with MFI over 2000 but not between 1000 and 2000 has a significant impact on kidney graft survival (Fig. 2). Therefore, we selected 28 patients with pretransplant DSA with raw mean fluorescence intensity (MFI) > 2000 against HLA donor antigens to assess the capacity to fix C1q via SAB. Table 1 shows the characteristics of both groups of patients with and without pretransplant DSA.

4.1. Patients with preformed DSA

The characteristics of the 28 patients with pretransplant DSA are detailed in Table 1. All received initial immunosuppression with steroids, tacrolimus (except one with rapamycin) and mycophenolic acid. They suffered a high rate of acute AMR (28.6%) and early graft loss (14.3%) compared to patients with no DSA pretransplantation. Seven of 24 patients whose grafts survived >6 months underwent biopsies; 5 showed chronic transplant glomerulopathy. Surviving grafts (76%) maintain good function with last median creatinine = 1.27 mg/dl and urine protein/creatinine = 232 mg/g.

Of 28 DSA positive patients, five showed HLA class-I DSA, 18 HLA class-II and five had combined DSA class-I and II. All 10 DSA class-I and 13 patients with pretransplant DSA class-II were tested with One Lambda kits and DSA were confirmed. Fifteen patients had DSA C1q fixing antibodies. Two patients had C1q positive DSA class-I and 13 C1q DSA II. At least one C1q DSA I patient had not shown that specificity in the IgG SAB assay.

4.2. Comparing patients with DSA class-I and patients with DSA class-II (Table 2)

As HLA class-I DSA are more frequently involved in acute AMR leading to early graft loss, we grouped 10 patients with DSA class-I irrespective of their class-II and 18 patients with only class-II DSA to compare the impact of DSA on graft survival (Fig. 3A). DSA class-I were directed against HLA-A (n = 7 2 A2, 2 A11, 2 A29, 1 A24) or HLA-B antigens (n = 3); five of these patients had also class II directed against DRB1 (n = 3) or DQB1 (n = 2). Class II only DSA were against DRB1 (n = 7), DQB1 (n = 4), DRB3 (n = 1), DRB4 (n = 1), combined DRB1–DRB4 (n = 3) or combined DRB1–DQB1 (n = 2).
Patients with DSA I suffered more frequent acute AMR than those with DSA II (40% vs 16.7%, p = 0.06) and worse graft survival (p = 0.01) Fig. 2B. All four early graft losses (<3 months after transplant) occurred in the DSA I group. Follow-up was shorter in DSA I group due to the concentration of graft losses in that group. More patients with DSA class I showed positive peak CDC-PRA (p = 0.055) than patients with DSA II, as systematic PRA on the waiting list are performed with a panel of mixed lymphocytes, consisting of HLA class I and class II antigens.

4.3. Comparing patients with C1q positive and patients with C1q negative DSA (Table 3)

Thirty-three C1q tests were performed for 5 patients with DSA I, 5 patients with DSA I and II (10 C1q tests) and 18 with DSA II. Sera from 10 patients included in the study were biopsied early after transplantation for clinical reasons. A similar proportion of C1q DSA positive and C1q negative patients showed C4d deposits in peritubular capillaries (67 vs 80%). Half C1q positive DSA II were directed against DQB1 antigens compared to C1q negative DSA II (7/14 vs 1/9, p = 0.2). The MFI of the DSA with the maximum MFI was clearly higher in the C1q positive group than in the negative one (p = 0.001). Most patients with C1q positive pretransplant DSA II showed persistence of DSA II when monitored after transplantation (9/11 vs 3/7) but this difference did not reach statistical significance.

5. Discussion

C1q fixation in pretransplant sera with DSA was not able to predict acute antibody-mediated rejection or graft loss in our experience (30 vs 46% and 20 vs 23% in C1q positive DSA and C1q negative DSA). Our sample size may have been a limitation to detect the impact of C1q positive DSA. Nevertheless the same number of patients showed significant differences in clinical outcome related to HLA class of DSA. The capacity to fix complement may change after transplantation when cells synthesizing those DSA produce a switching in antibody isotype, so they become able to fix complement.

Two studies have reported a higher incidence of acute AMR in pediatric population who received heart transplantation with positive C1q DSA before transplantation based on very low number of C1q positive patients [19,21]. The first report focused on a cohort of 18 children with endomyocardial biopsy within one month of heart transplantation. Only three of eight patients whose pretransplant samples were tested for C1q were positive and two developed acute AMR [19]. The authors found that five patients with AMR had post-transplant C1q positive DSA, but not all the C1q positive post-transplant children had AMR. The second study by Zeevi et al. found three of 13 heart transplant recipients who had preformed C1q positive DSA and suffered rejection; accordingly all three had positive CDC pretransplant crossmatches, so C1q did not add information [21]. Both studies suggest that the presence of
C1q positive DSA early after heart transplantation, whether they were negative or positive pretransplant, correlate better with acute AMR than pretransplant C1q+ DSA. In renal transplantation, scarce published information exists about the impact of pretransplant C1q+ DSA. The widest experience comes from a Dutch group who found a significant association between pretransplant DSA I and/or II and graft survival but absence of significant correlation between having C1q positive preformed DSA and graft survival based on 30 patients with C1q positive preformed DSA and 290 with C1q negative DSA[22]. No data about rejection was provided in this study. Another study by Ata et al. found that nine of 33 desensitized renal transplant recipients with pretransplant weak flow crossmatches had C1q DSA. They do not provide information about acute rejection, but found that C1q positive patients had longer delayed graft function[23]. Differently, two recent and well designed studies employing a different way to identify complement involvement with SAB techniques have shown that C4d activating pretransplant DSA correlate with AMR and graft survival[16,24].

Table 2

<table>
<thead>
<tr>
<th>Demographics and clinical data</th>
<th>DSA HLA I (n = 10)</th>
<th>DSA HLA II (n = 18)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female recipients</td>
<td>70%</td>
<td>77.8%</td>
<td>0.67</td>
</tr>
<tr>
<td>Recipient age at transplant</td>
<td>48.9 ± 11.6</td>
<td>54.8 ± 12.4</td>
<td>0.23</td>
</tr>
<tr>
<td>(years, mean ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deceased donor kidney</td>
<td>100%</td>
<td>88.9%</td>
<td>0.52</td>
</tr>
<tr>
<td>Previous transplants</td>
<td>60%</td>
<td>66.7%</td>
<td>1</td>
</tr>
<tr>
<td>Induction treatment</td>
<td>10%</td>
<td>11.1%</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10%</td>
<td>11.1%</td>
<td></td>
</tr>
<tr>
<td>AntiCD25</td>
<td>30%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Thymoglobulin</td>
<td>60%</td>
<td>38.9%</td>
<td></td>
</tr>
<tr>
<td>Delayed graft function</td>
<td>60%</td>
<td>50%</td>
<td>0.7</td>
</tr>
<tr>
<td>Days of delayed graft function</td>
<td>11 (5, 60)</td>
<td>13 (12, 16)</td>
<td>0.68</td>
</tr>
<tr>
<td>Biopsy proven acute rejection</td>
<td>50%</td>
<td>33.3%</td>
<td>1</td>
</tr>
<tr>
<td>Acute cellular rejection</td>
<td>0%</td>
<td>16.7%</td>
<td>0.53</td>
</tr>
<tr>
<td>Acute antibody-mediated rejection</td>
<td>50%</td>
<td>16.7%</td>
<td>0.06</td>
</tr>
<tr>
<td>C4d in early biopsy (~3 months), n = 11</td>
<td>50%</td>
<td>16.7%</td>
<td>0.1</td>
</tr>
<tr>
<td>6-month creatinine (mg/dl, median IQR), n = 24</td>
<td>1.4 (1.3, 1.8)</td>
<td>1.48 (1.16, 1.77)</td>
<td>0.58</td>
</tr>
<tr>
<td>Last creatinine (mg/dl, median IQR), n = 22</td>
<td>1.23 (1.08, 1.39)</td>
<td>2.23 (167, 989)</td>
<td>0.97</td>
</tr>
<tr>
<td>Last urine protein/creatinine (median IQR), n = 22</td>
<td>247 (120, 660)</td>
<td>1.16 (1.3, 1.8)</td>
<td></td>
</tr>
<tr>
<td>3-month graft loss</td>
<td>40%</td>
<td>0%</td>
<td>0.01</td>
</tr>
<tr>
<td>Global graft loss</td>
<td>40%</td>
<td>23.1%</td>
<td>0.14</td>
</tr>
<tr>
<td>Follow-up (months, median IQR), n = 15</td>
<td>1.39 (14, 51)</td>
<td>24 (167, 989)</td>
<td>0.09</td>
</tr>
<tr>
<td>Immunological data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak PRA &gt; 5%</td>
<td>80%</td>
<td>38.9%</td>
<td>0.055</td>
</tr>
<tr>
<td>Pretransplant PRA &gt; 5%</td>
<td>30%</td>
<td>27.7%</td>
<td>1</td>
</tr>
<tr>
<td>Pretransplant HLA I screening (% positive)</td>
<td>100%</td>
<td>50%</td>
<td>0.01</td>
</tr>
<tr>
<td>Pretransplant HLA II screening (% positive)</td>
<td>100%</td>
<td>100%</td>
<td>0.5</td>
</tr>
<tr>
<td>AB mismatches</td>
<td>0%</td>
<td>16.7%</td>
<td></td>
</tr>
<tr>
<td>1–4</td>
<td>100%</td>
<td>83.3%</td>
<td></td>
</tr>
<tr>
<td>DR mismatches</td>
<td>0%</td>
<td>0%</td>
<td>0.14</td>
</tr>
<tr>
<td>0</td>
<td>20%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>&gt;0</td>
<td>80%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Maximum MFI of DSA pretransplant</td>
<td>44069 ± 1844</td>
<td>11963 ± 7083</td>
<td>0.0001</td>
</tr>
<tr>
<td>C1q positive DSA</td>
<td>20%</td>
<td>61.1%</td>
<td>0.19</td>
</tr>
<tr>
<td>Last original DSA status</td>
<td>16.7%</td>
<td>66.7%</td>
<td>0.06</td>
</tr>
</tbody>
</table>


Fig. 3. A) Graft survival comparing patients with isolated DSA I, isolated DSA II or combined DSA I and II. B) Graft survival depending on the existence of pretransplant DSA I (with or without DSA II), isolated DSA II or no DSA. C) Graft survival depending on pretransplant C1q positive DSA, C1q negative DSA or no DSA.
more specific for clinical events, as few publications from Stanford suggest. In pediatric renal transplantation, they found a correlation between post-transplant C1q + DSA and graft loss comparing 15 patients with C1q positive and 20 C1q negative post-transplant DSA [20]. In a different report, they observed some correlation with chronic transplant glomerulopathy in 15 patients selected for having graft biopsies and serum samples: only 2 of them had preformed C1q+ DSA [17].

From the technical point of view, we found that MFI of C1q positive DSA were higher than the MFI of C1q negative DSA, mainly due to DSA HLA II. The proportion of C1q + DSA I was lower than C1q + DSA II, as most DSA I able to fix complement were probably avoided when patients were transplanted with negative pretransplant CDC crossmatches. The group who developed the assay and another report have claimed that C1q is independent of MFI [18,25], but we and others have found that MFI of immunodominant DSA from C1q positive patients are significantly higher [22]. Though small amounts of an IgG3 antibody can fix complement, we saw raw MFI > 10,500 in 12/15 C1q+ patients compared to only 4/13 C1q- patients. If such is the case, the MFI level could be accepted as a surrogate for C1q fixation taking into account the elevated cost to perform C1q tests. One C1q+ DSA class I had not been detected by IgG SAB suggesting these could be IgM antibodies, as other authors have suggested [25].

Our study has limitations. First, the sample size may not have been enough to show the impact of preformed C1q DSA in kidney transplantation. Our results should be confirmed with a larger study. Second, we did not retest post-transplant samples for C1q DSA, as our aim was focused on the possible prediction of post-transplant major events with pretransplant information. We have not evaluated the impact of C1q negative DSA that become C1q positive after transplantation or vice versa, probably due to isotype switching. However, the strength of our study is that it is able to show the clear impact of preformed DSA class I on acute AMR and graft loss early after kidney transplantation despite the apparent inability to fix C1q in SAB tests. Early graft loss in this subgroup is unacceptable (40%), despite all four patients received specific treatment for AMR at diagnosis, but that might have been administered too late. Most of our pretransplant positive DSA I samples were tested either at the time of acute rejection or retrospectively for the study. DSA had not been detected by the prospective pretransplant CDC crossmatches, indicating that other strategies using flow cytometry or AHG enhanced CDC crossmatches in the absence of recent SAB tests should be adopted. No specific therapy to modify antibody impact apart from usual immunosuppression was implemented in this population before the rejection episode was diagnosed. Early studies in the nineties which described acute AMR highlighted the fact that these episodes were mainly, but not only, caused by DSA I [26,27]. In the modern era of SAB, most studies have evaluated the impact of DSA class I and II altogether in AMR or survival [5,7].

Interestingly, except for one, the six patients with preformed DSA I and long-term functioning grafts have cleared their DSA I and show good renal function. Recent publications on liver-kidney transplantation but also on single kidney transplantation show similar results [28,29]. Neither the coexistence of DSA I and II nor the capacity to fix C1q predicted acute rejection or graft loss in the subgroup of patients with pretransplant DSA-I. On the other hand, three of 18 patients with only preformed DSA II developed AMR and responded to treatment. No correlation was observed between C1q positivity or MFI and acute AMR in this group. DSA II persisted in 66.5% cases. Post-transplant DSA II are suggested to be involved in late dysfunction via chronic AMR [29–32]. In our study, at least five of those pretransplant DSA II positive patients have developed chronic transplant glomerulopathy, particularly suggestive of chronic AMR. Four of them had persistent DSA II and three were C1q+ pretransplant.

### 5.1. Conclusion

In conclusion, our study shows that preformed SAB DSA HLA class-I antibodies but not C1q positive DSA predict acute AMR and graft loss. Despite lack of capacity to fix complement in vitro, pretransplant C1q-negative DSA HLA class-I or II can mediate rejection and graft loss. When the presence of DSA HLA class-I is not followed by early graft loss, the DSA tend to disappear from recipients' sera. On the other hand, a high burden of pretransplant DSA HLA class-II persist after kidney transplantation and may play a role in post-transplant outcome.

### Disclosure

No disclosures.

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