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The ABCs (DRDQDPs) of the prozone effect in single antigen bead HLA antibody testing: Lessons from our highly sensitized patients

Anna L. Greenshields, Robert S. Liwski*

Department of Pathology, Dalhousie University, Halifax, Nova Scotia B3H 1V8, Canada

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ABSTRACT

Accurate identification of HLA antibodies using the single antigen bead (SAB) assay is critical for assessment of pre/post-transplant immunological risk and successful virtual crossmatching. Unfortunately, high titer HLA antibodies can be missed or underestimated in the SAB assay as a result of interference with the detection of IgG. This so called prozone effect has been attributed to both complement- and IgM-dependent mechanisms and can be minimized with serum dilution or treatment with heat, EDTA, or DTT. In this study we describe the frequency, nature, and degree of prozone in a cohort of highly sensitized patients (cPRA ≥ 95%), in whom accurate detection of HLA antibodies and virtual crossmatching is of paramount importance. Sera were tested by the SAB assay ± EDTA treatment, ± 1:10 dilution to identify the prozone effect. The relative contribution of complement vs IgM to prozone was assessed using anti-C3d and anti-IgM reporter antibodies, respectively. We found that prozone was very frequent in highly sensitized patients (80%), especially those with a history of previous transplantation (87%). Class I HLA specificities were more commonly affected than class II and the susceptibility to prozone was locus dependent with HLA-A(31%), -B(29%) and -DQ(26%) being affected more frequently than HLA-DP(17%), -C(16%) and -DR(5%) antigens. Interestingly, the presence of prozone could be predicted by C3d positivity (MFI ≥ 4000; sensitivity = 95.2%, specificity = 97.2%) and the degree of prozone correlated directly with the extent of C3d deposition. The role of IgM was less clear. However, serum dilution studies suggested that IgM may contribute to interference in a small subset of prozone positive specificities. Our study underscores the importance of serum treatment to inhibit complement activation and minimize prozone in the SAB assay, especially in highly sensitized patients.

1. Introduction

The presence of pre-formed donor specific HLA antibodies (DSA) in recipient serum can negatively impact graft survival, therefore, the accurate identification of these antibodies prior to transplantation is required to ensure the selection of compatible donor organs. The single antigen bead (SAB) assay has been widely adopted by laboratories around the world and has become a cornerstone of histocompatibility testing [1]. This assay uses hundreds of microparticles (beads), each coated with a recombinant HLA molecule, to characterize HLA-specific antibodies in patient sera. Patient HLA antibody reactivity patterns are used in virtual crossmatching (VXM) for the assessment of immunological risk pre-transplantation and for post-transplant monitoring [2–4].

Although the SAB assay remains an accurate and sensitive method for the identification of HLA-targeting antibodies, the test is not without limitations [5–7]. An important such limitation is the assay's susceptibility to the prozone effect, wherein the detection of high titer complement fixing antibodies is compromised, leading to falsely low or negative results for affected reactivities. Recent evidence indicates that these false negative or weak results are due to complement mediated interference with PE-conjugated anti-IgG secondary antibody binding [8,9]. Specifically, following complement activation by high titer HLA antibodies, the accumulation of complement split products (C3b/d and C4b/d) on the bead-bound antibody/antigen complexes prevents binding of the anti-IgG-PE reporter antibody [10,11]. In addition, interference by IgM antibodies targeting HLA antigens may also contribute to the prozone effect [12]. High titer complement fixing

Abbreviations: cPRA, calculated panel reactive antibody; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; HSP, highly sensitized patients; MFI, mean fluorescence intensity; PE, phycoerythrin; ROB, rapid optimized SAB assay; SAB, single antigen bead; DART, dual antibody rapid test; LWB, LABScreen wash buffer; VXM, virtual crossmatch; DSA, donor specific antibodies

* Corresponding author at: Dr. D. J. Mackenzie Building, HLA Typing Laboratory, 5788 University Avenue, Room 625, Halifax, Nova Scotia B3H 1V8, Canada.
E-mail address: robert.liwski@nshealth.ca (R.S. Liwski).

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antibodies present a significant immunologic risk in transplantation and failure to detect these antibodies can have severe clinical repercussions, which include antibody mediated rejection and graft failure. Fortunately, serum dilution [13] or treatment with dithiothreitol (DTT) [14,15], heat [16], or ethylenediaminetetraacetic acid EDTA [8,9,17] have been shown to be effective in preventing the prozone effect.

The prevalence of prozone in the general population remains unclear. In a recent publication, Guidicelli et al. identified prozone interference in 29.5% and 45.9% of patients for class I and class II, respectively [18]. Meanwhile, a separate study by Tambur and colleagues identified the prozone effect in 71% of their study population [13]. This variability is likely the result of the different study inclusion criteria. Interestingly, previous validation studies performed at our center have indicated that the highly sensitized patient (HSP) population appears to be especially prone to the prozone effect. HSP represent a unique challenge for transplant programs as they are incompatible with the vast majority of the donor population. To address this issue in Canada, a national HSP program was implemented by Canadian Blood Services to mandate inter-provincial sharing of deceased kidney donor organs to HSP recipients, thereby expanding the donor pool for this disadvantaged patient group. In this program, HSP are defined as individuals having a combined cPRA of ≥95%. Patient cPRA is calculated using the Canadian cPRA Calculator, which includes unacceptable antigens from all 11 HLA loci (A/B/C/DRB1/DRB3,4,5/DQA1/DQB1/DPA1/DPB1) [19]. As organ allocation for the HSP registry is based on a negative VXM, and involves the shipment of organs across the country, the program's success hinges on the accuracy of the HLA antibody profiles registered for each enrolled patient. Inaccurate antibody assignment as a result of the prozone effect would have significant clinical repercussions in this complex patient population and could result in the misallocation of organs to incompatible individuals. We therefore elected to center our study on the frequency of prozone in the HSP patient population.

2. Materials and methods

2.1. Reagents and sera

LABScreen class I (LS1A04 lot 10) and class II (LS2A01 lot 12) HLA single antigen bead kits (One Lambda, Canoga Park, CA) were used for HLA antibody identification. Secondary reporter antibodies included: phycoerythrin (PE)-conjugated polyclonal goat anti-human IgG (IgG-PE; One Lambda), PE-conjugated polyclonal donkey anti-human IgM (IgM-PE; One Lambda), and PE-conjugated polyclonal anti-C3d (C3d-PE; Immucor, Nocross, GA). Optimal concentration for each conjugate was determined by performing titration studies using pooled positive and negative control sera. 96-well v-bottom trays were purchased from Whatman (Pistcataway, NJ). EDTA disodium 0.5 M salt solution (E7889; Sigma-Aldrich, St Louis, MO www.sigmaaldrich.com) was used to treat sera in some experiments by adding 1 µl of 165 mM working EDTA solution per 25 µl of test sera (final EDTA concentration of 6 mM). Phosphate buffered saline (PBS; Life Technologies Inc., Burlington ON, Canada; www.lifetechnologies.com) was used in some experiments to dilute sera 1:10.

Test sera used in this study were from 30 (18 female and 12 male) highly sensitized (Canadian cPRA ≥ 95%) renal transplant waitlist patients from the Queen Elizabeth II Health Sciences Centre in Halifax, Canada, who were also enrolled in the Canadian Highly Sensitized Patient Transplant Registry. All sera were stored at −80 °C and centrifuged at 10,000 × *g* for 2 min prior to testing. No sera were exposed to multiple freeze-thaw cycles before testing. LABScreen negative control sera (LSNC, lot 19; OneLambda) and pooled positive control sera (a pool of 20 highly sensitized patient sera) were used in all SAB assays.

2.2. Modified SAB assay testing

HLA antibody testing was performed by the LABScreen single antigen bead assay using the rapid optimized SAB (ROB) protocol as described previously [20]. Briefly, EDTA-treated or untreated sera (25 µl/test) were added to the wells of a 96-well v-bottom trays containing either Class I or Class II HLA LABScreen beads. In some experiments EDTA treated sera were diluted 1:10 in PBS before testing. Trays were incubated for 15 min at room temperature in the dark on a tray shaker. Beads were washed 3 times with LABScreen wash buffer (LWB) (200 µl/well) for 1 min at 1800 × g. Following washing, one of three reporter antibodies was added to the reaction wells. Reporter antibodies were as follows: 1) IgG-PE (1:10 dilution in LWB, 20 µl/well), 2) IgM-PE (1:10 dilution in LWB, 20 µl/well), and 3) C3d-PE (1.5:10 dilution in LWB, 15 µl/well). Trays were incubated with reporter antibodies for 5 min at room temperature, washed twice with LWB, and resuspended in 60 µl of LWB for acquisition using the Luminex FlexMAP 3D™ analyzer (Luminex).

2.3. Data analysis

Mean fluorescence intensity (MFI) values for each antibody specificity were determined using the baseline formula within Fusion 4.2 software (OneLambda). A baseline MFI threshold of 1000 was used to assign positive reactions with all reporter antibodies (IgG-PE, IgM-PE and C3d-PE). Baseline MFI data for each test serum was transferred into a Microsoft Office Excel software (Microsoft) spreadsheet for analysis and comparison between the different test conditions. The presence of prozone/interference was defined as an increase in baseline IgG-PE MFI of ≥3000 following serum treatment with EDTA for a specific reactivity compared to the MFI for the same reactivity in untreated serum (IgG-PE ΔMFI with EDTA treatment ≥3000). Prozone was further classified as: 1) mild prozone (IgG-PE ΔMFI with EDTA treatment = 3000–4999), 2) moderated prozone (IgG-PE ΔMFI with EDTA treatment = 5000–9999), and 3) marked prozone (IgG-PE ΔMFI with EDTA treatment ≥10000).

2.4. Statistical analysis

Sensitivity and specificity calculations as well as repeated measure ANOVA with the Bonferroni multiple comparison post-test were performed using GraphPad InStat (GraphPad Software Inc., San Diego, CA)

3. Results

3.1. Prevalence of the prozone effect in highly sensitized waitlist patients.

Sera from 30 highly sensitized (cPRA ≥ 95%) renal waitlist patients (18 female and 12 male) were used in this study. The degree (cPRA) and mode of sensitization are presented in Fig. 1 and Table 1, respectively. Mean cPRA values were 90.2 ± 21.8% for class I, 90.6 ± 21.3% for class II and 99.4 ± 1.3% for combined class I/II HLA. Based on the mode of sensitization patients could be categorized into two subgroups: 1) pregnancy ± transfusion (n = 6) and 2) previous transplantation ± pregnancy ± transfusion (n = 24). There were no patients with transfusion as the only mode of sensitization. There were no significant differences in cPRA between male and female patients or between patients in the pregnancy vs transplantation subgroups.

EDTA treated and untreated sera from the highly sensitized patients were tested in parallel by the LABScreen SAB assay using the IgG-PE reporter antibody (Figs. 2 and 3). Out of 5760 beads (2910 class I and 2850 class II) tested, 3518 specificities (61%; 1662 class I and 1856 class II) were classified as IgG negative (Fig. 2A; EDTA IgG-PE MFI < 1000), 1726 specificities (30%; 886 class I and 840 class II) were IgG positive/prozone negative (Fig. 2B; EDTA IgG-PE MFI ≥1000; IgG-PE ΔMFI EDTA – untreated < 3000), and 516 specificities (9%; 362 class I

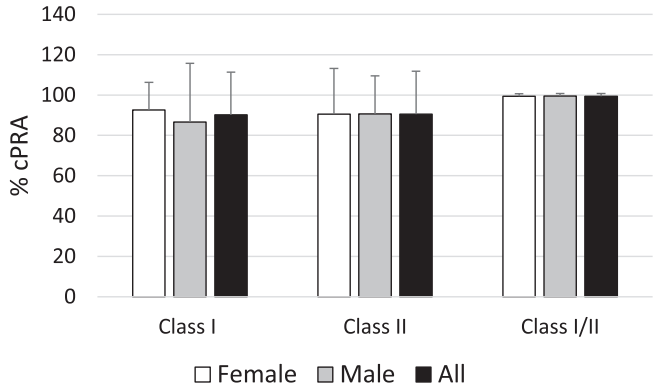


Fig. 1. cPRA demographics for the patient population used in this study. cPRA was calculated using the Canadian cPRA calculator. Mean cPRA ± SD values for class I, class II and combined class I/II are shown for female, male and all patients.

Table 1
Sensitization history demographics for the patient study population.

Sensitization	Female Patients	Male Patients	Total patients
Pregnancy	3	0	3
Pregnancy + transfusion	3	0	3
Transplantation	3	4	7
Transplantation + pregnancy	2	0	2
Transplantation + transfusion	3	8	11
Transplantation + pregnancy + transfusion	4	0	4
Total	18	12	30

and 154 class II) were IgG positive/prozone positive (Fig. 3; EDTA IgG-PE MFI ≥1000; IgG-PE ΔMFI EDTA – untreated ≥3000). Thus, 23% of all IgG positive specificities exhibited the prozone effect. Of these, 88 specificities (17%; 72 class I and 16 class II) showed mild prozone (Fig. 3A; IgG-PE ΔMFI EDTA – untreated = 3000–4999), 131 specificities (25%; 95 class I and 36 class II) showed moderate prozone (Fig. 3B; IgG-PE ΔMFI EDTA – untreated = 5000–9999), and 297 specificities (58%; 195 class I and 102 class II) showed marked prozone (Fig. 3C; IgG-PE ΔMFI EDTA – untreated ≥10,000).

The prozone effect was more commonly seen with class I (362 specificities; 70%) than class II (154 specificities; 30%) beads (Fig. 4A). When adjusted for the number of total positive reactions with class I (n = 1278) vs class II (9 9 4) beads, the proportion of prozone positive/total positive specificities was overall higher for class I (28%) than for class II (15%). Among class I beads, 62% of specificities affected by prozone were HLA-B, 32% were HLA-A, and 6% were HLA-C (Fig. 4B). After adjusting for the total number of positive reactions per locus, the proportion of prozone positive/total positive specificities was 31% for HLA-A, 29% for HLA-B, and 16% for HLA-C (Fig. 4D). Among class II specificities affected by prozone, 68% were HLA-DQ, 18% were HLA-DP and 14% were HLA-DR (Fig. 4C). When adjusted for the total number of positive reactions per locus, the proportion of prozone positive/total positive specificities was 26% for HLA-DQ, 17% for HLA-DP, and 5% for HLA-DR (Fig. 4D).

Overall, 24 out of 30 patients (80%) in the highly sensitized cohort had at least one prozone positive specificity (Fig. 5; 7% mild and 73% marked and moderate). Within the subgroup of patients with pregnancy as a sensitizing event (n = 6), 50% were affected by prozone (Fig. 5; 17% mild; 33% marked and moderate). In contrast, in the subgroup of patients with a previous transplant (± pregnancy, ± transfusion) as a sensitizing event (n = 24), 87% of patients were affected by prozone (Fig. 5; 4% mild; 83% marked and moderate). The number of specificities showing either marked or moderate prozone was higher in the previous transplant patient subgroup (mean = 19.2, median = 13) compared to the pregnancy subgroup (mean = 5.7; median = 0; $p < 0.001$). The total number of specificities that would be falsely assigned as negative due to the prozone effect was: 25, 72, and 106 using an IgG-PE MFI threshold of 1000, 2000 and 3000, respectively. Using a threshold of 2000 IgG-PE MFI, 8 out of 30 patients (27%) had at least one specificity that would be falsely assigned as negative without treatment with EDTA (mean = 9; median = 6, min = 1, max = 33). All 8 of the affected patients were from the previous transplant subgroup. All 72 affected specificities (40 class I and 32 class II) in this group (Fig. 6) fixed high levels of complement (C3d-PE MFI mean = 18,119.8 ± 2303.9, median = 18,406.2, min = 10,752.0, max = 22,489.9), and most had EDTA IgG-PE MFI values > 20,000 (EDTA IgG-PE MFI mean = 21,894.6 ± 3912.1, median = 22,507.1, min = 5067.5, max = 26,055.5). IgM positivity was prevalent in this group, with 68/72 (94.4%) specificities showing IgM-PE MFI > 1000. However, the IgM-PE MFI values were significantly lower compared to C3d-PE MFI in this group of specificities (IgM-PE MFI mean = 5407.9 ± 2794, median = 5502, min = 35.3, max = 12,169; $p < 0.001$).

3.2. Detailed analysis of prozone negative IgG specificities.

Fig. 2A shows the IgG negative specificities based on EDTA IgG-PE MFI threshold < 1000. Most of these specificities (3475 out of 3518; 98.8%) were also IgG negative (IgG-PE MFI < 1000) when untreated sera were tested (Fig. 2A), with only 43 beads (1.2%; 25 class I and 18 class II) exhibiting MFI values slightly above 1000 (mean = 1194.6 ± 172.3; min = 1000.9; max = 1725.8; mean IgG-PE ΔMFI untreated vs EDTA = 423.4 ± 262.7). Overall, the IgG-PE MFI values for IgG negative specificities were similar when sera were tested with or without EDTA (mean MFI 341.2 ± 225.8 vs 316.5 ± 224.0; $p = 0.6$). As expected, the majority of IgG negative specificities did not fix complement and were C3d negative (Fig. 2A; C3d-PE MFI < 1000), with only 6 of 3518 (0.17%) specificities showing C3d positivity. Three of the six C3d positive specificities (C3d-PE MFI values of 1969.8, 4250.1 and 8432.1) were also positive for IgM (IgM-PE MFI values of 6078.7, 7479.8 and 4061.2, respectively), which may explain their complement activating potential. However, the three remaining C3d positive specificities (C3d-PE MFI values of 1006.9, 1179.4 and 1852.2) were IgM negative (IgM-PE MFI < 150) and were likely a result of false positive reactivity. Overall, weak IgM positivity was noted in 164 out of 3518 (4.7%) IgG negative specificities, with a mean IgM-PE MFI value of 2406 ± 1564.9 (Fig. 2A).

Fig. 2B depicts IgG positive/prozone negative specificities based on an EDTA IgG-PE MFI threshold ≥1000 and ΔMFI EDTA – untreated < 3000. Overall, the number of C3d positive specificities within this group was 183 (10.6%) with a relatively low average C3d-PE MFI of 3152 ± 1848 (Fig. 2B). Interestingly, the frequency of C3d positivity was directly proportional to EDTA IgG-PE MFI with 0.7% (EDTA IgG-PE MFI = 1000–1999), 2.6% (EDTA IgG-PE MFI = 2000–4999), 8.1% (EDTA IgG-PE MFI = 5000–9999), 29.8% (EDTA IgG-PE MFI = 10,000–14,999), and 67.7% (EDTA IgG-PE MFI ≥ 15,000) of specificities being able to fix complement (C3d-PE MFI ≥1000). However, C3d-PE MFI values remained low regardless of the EDTA IgG-PE MFI, with an approximate C3d-PE MFI mean ranging between 3000 and 3600. The number of IgM positive specificities in the IgG positive/prozone negative group was 130 (7.5%) with a mean IgM-PE MFI of 2327 ± 1796.9 (Fig. 2B). The frequency of IgM positivity was as follows: 6.8% (EDTA IgG-PE MFI = 1000–1999), 6.7% (EDTA IgG-PE MFI = 2000–4999), 4.8% (EDTA IgG-PE MFI = 5000–9999), 10.1% (EDTA IgG-PE MFI = 10,000–14,999) and 20.8% (EDTA IgG-PE MFI ≥ 15,000), with an approximate mean IgM-PE MFI ranging from 1800 and 4000 (Fig. 2B).

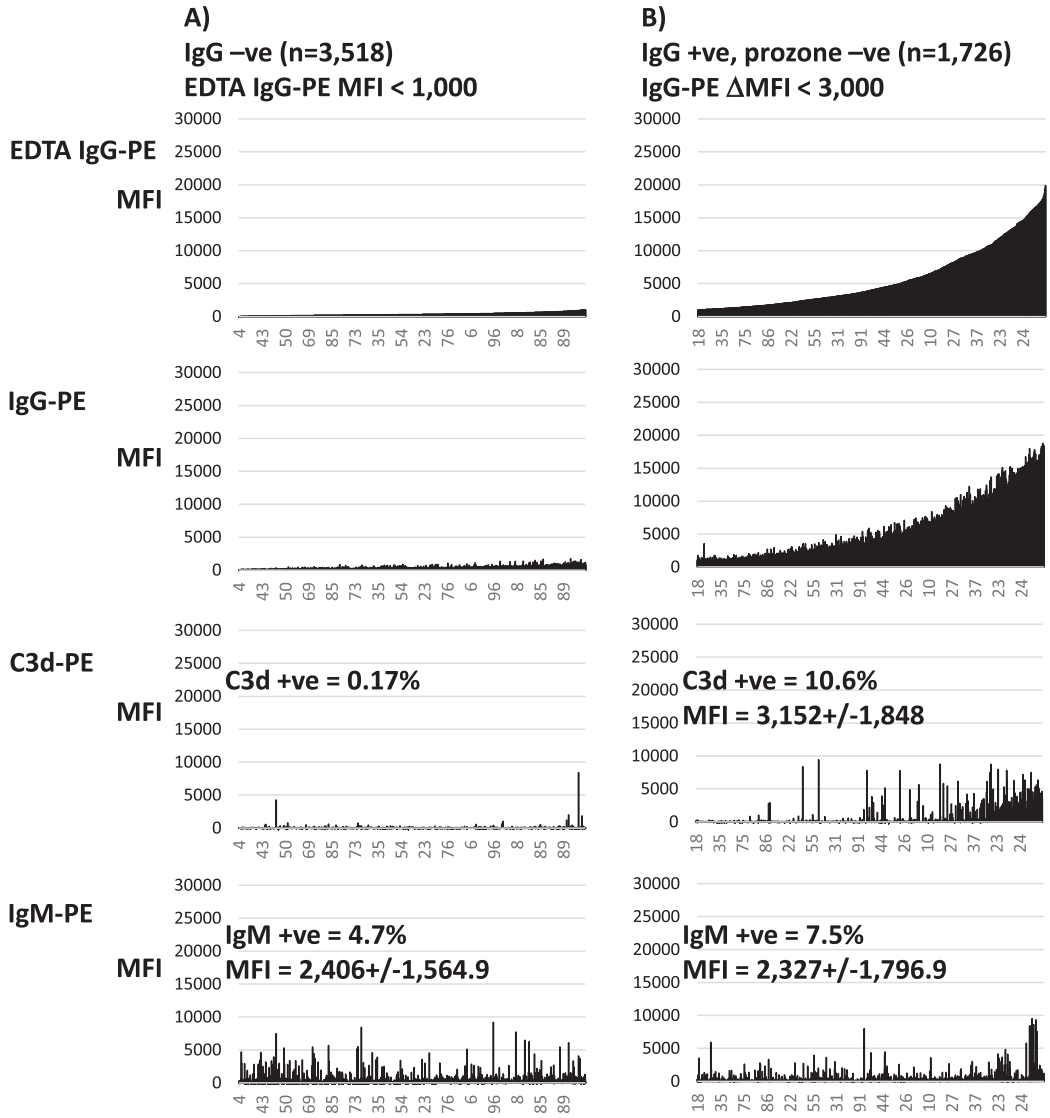


Fig. 2. Reactivity patterns of IgG negative and prozone negative specificities. SAB reactivity of 30 highly sensitized patient sera tested with IgG-PE ± EDTA, IgM-PE, or C3d-PE, are shown. Data shown compares IgG negative (panel A; IgG-PE EDTA MFI < 1000) and IgG positive, prozone negative (panel B; ΔMFI < 3000) specificities. ΔMFI = IgG-PE EDTA MFI – IgG-PE MFI. All specificities are sorted by IgG-PE EDTA MFI.

3.3. Detailed analysis of prozone positive IgG specificities.

Fig. 3 shows IgG positive specificities (EDTA IgG-PE MFI ≥ 1000) that were affected by mild (IgG-PE ΔMFI EDTA – untreated = 3000–4999; Fig. 3A), moderate (IgG-PE ΔMFI EDTA – untreated = 5000–9999; Fig. 3B), and marked (IgG-PE ΔMFI EDTA – untreated ≥ 10,000; Fig. 3C) prozone. The number of C3d positive specificities was 77/88 (87.5%) for mild, 131/131 (100%) for moderate, and 297/297 (100%) for marked prozone (Fig. 7A). Thus, the majority (97.9% overall; 100% in marked/moderate prozone groups) of prozone positive specificities fixed complement (C3d-PE MFI ≥ 1000). The 11 C3d negative specificities that were categorized in the mild prozone group are shown in Fig. 8. All 11 specificities were identified in a single patient serum and with the exception of one specificity (HLA-B81, IgM-PE MFI = 4028.0) were also IgM negative. Thus, there was no clear explanation for the increased IgG-PE MFI (3000–4999) reactivity upon treatment with EDTA for these specificities. Importantly, C3d-PE MFI was directly proportional to the degree of prozone (mean C3d-PE MFI of 5878.2 ± 2462.7 for mild, 9593.2 ± 1972.3 for moderate, and 15,572.6 ± 2945.0 for marked; Fig. 7B) and there was a good correlation between the C3d-PE MFI and the IgG-PE ΔMFI EDTA – untreated (3848.0 ± 571.9 for mild, 7329.4 ± 1471.8 for moderate and 16,656.9 ± 3991.5 for marked; Fig. 7B). Finally, C3d positivity predicted the presence of prozone with a high degree of accuracy, especially when using a C3d-PE MFI threshold of 4000 (sensitivity = 95.2%, specificity = 97.2%).

The percentage of IgM positive specificities also increased with the degree of prozone (13.6% for mild, 29.8% for moderate and 64.0% for marked; Fig. 7A). However, overall the majority of prozone positive specificities (53.3%) were IgM negative. In addition, the mean IgM-PE MFI remained relatively low regardless of the degree of prozone (IgM-PE MFI = 2847.7 ± 1515.8 for mild, 2898.6 ± 1330.4 for moderate, and 4794.3 ± 2965.6 for marked; Fig. 7B) and did not correlate with the IgG-PE ΔMFI EDTA – untreated (Fig. 7B). Finally the presence of IgM positivity was not a good predictor of prozone due to poor sensitivity (sensitivity = 46.7%, 35.6%, and 27.3% with IgM-PE MFI thresholds of 1000, 2000, and 3000, respectively), although the specificity was high (specificity = 92.5%, 97.3% and 98.6%, for the same IgM-PE MFI thresholds, respectively).

While C3d positivity was highly predictive and C3d-PE MFI correlated with the degree of the prozone effect uncovered by EDTA treatment, it was important to determine if additional factors such as IgM-

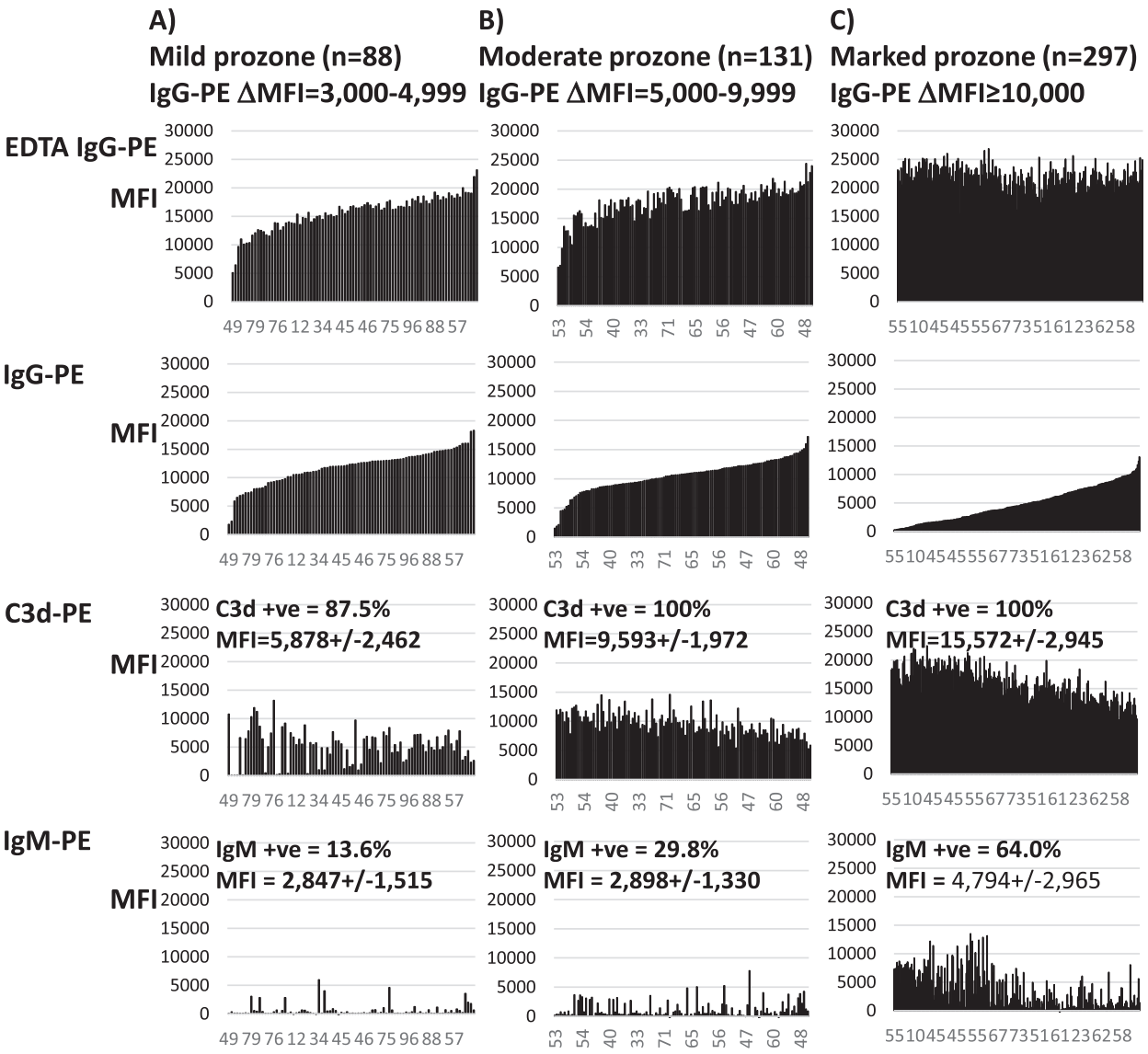


Fig. 3. Reactivity patterns of prozone positive specificities. SAB reactivity of 30 highly sensitized sera tested with the IgG-PE ± EDTA, IgM-PE, or C3d-PE, are shown. Data depicts specificities exhibiting either slight (panel A; ΔMFI: 3000–4999), moderate (panel B; ΔMFI: 5000–9999) or marked (panel C; ΔMFI ≥ 10,000) prozone. ΔMFI = IgG-PE EDTA MFI – IgG-PE MFI for each specificity. All specificities are sorted by IgG-PE MFI.

mediated interference also played a role. To this end, we performed parallel SAB assay studies using EDTA treated sera tested neat vs 1:10 diluted. Fig. 9A shows that no significant prozone was uncovered by diluting sera 1:10 and that the great majority of specificities displayed lower MFI values following serum dilution. There were only 41/5760 (0.73%; 30 class I and 11 class II) specificities (Fig. 9B), which exhibited higher (> 1000) MFI values upon 1:10 dilution compared to undiluted sera (EDTA IgG-PE ΔMFI 1:10 diluted – neat mean = 2512 ± 1363.7, median = 2216.4, min = 1073.7, max = 7430.5). 39 of these specificities were from the marked, 1 from the moderate, and 1 from the mild prozone groups, and were therefore already identified as prozone positive with the EDTA treatment alone. All 41 specificities in this group fixed high levels of complement (C3d-PE MFI mean = 18,392.9 ± 3298.1, median = 19,709.6, min = 6428.7, max = 22,618.3) and had EDTA IgG-PE neat MFI values > 15,000, therefore, uncovering additional interference by diluting sera 1:10 would be of limited value for these specificities (Fig. 9B). Interestingly, the frequency of IgM positivity (40/41, 97.6%) was higher in this group than in the overall marked prozone group (64%). Similarly, the IgM-PE MFI values (mean = 7791.5 ± 3221.1, median = 7758.2, min = 456.1, max = 13,612.0) were higher in this group compared to the overall marked prozone group (IgM-PE MFI = 4794 ± 2965, $p < 0.001$). Consequently, it is possible that IgM interference played a role, in addition to complement, in modulating IgG-PE MFI values in this group of specificities. Importantly, 1:10 dilution did not uncover any additional interference in the IgG negative or IgG positive/prozone negative groups.

4. Discussion

Our study demonstrates that the prozone effect is very common (80%) among highly sensitized patients (cPRA ≥ 95%). Even when only marked prozone (ΔMFI ≥ 10,000 with EDTA) was considered, the frequency of prozone was still high (73%) in this cohort of patients. A history of previous transplantation was strongly associated with the prozone effect (87% overall and 83% marked prozone), while prozone was observed less frequently in patients sensitized by pregnancy alone or in combination with transfusion (50% overall and 33% marked prozone). Therefore, a history of previous transplantation appears to be an important factor in determining the risk of prozone. In fact, all

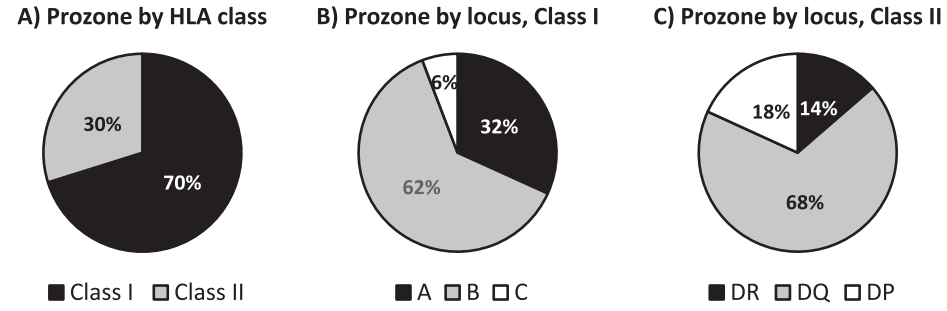
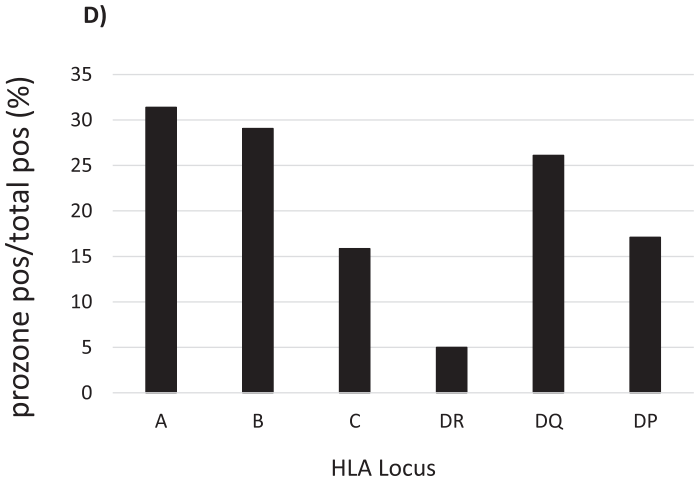


Fig. 4. Prozone frequency and susceptibility across HLA loci following SAB assay testing of 30 highly sensitized patients. Data depicts the distribution of the prozone effect across class I and class II specificities (panel A), HLA class I loci (panel B), and HLA class II loci (panel C). Percentage of prozone positive reactions per loci, adjusted for the total number of positive reactions per locus is also shown (panel D). Reactivities were considered prozone positive in cases where the ΔMFI ≥ 3000.



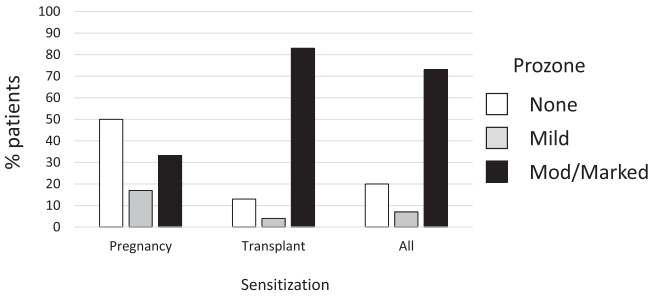


Fig. 5. Prozone frequency across two sensitization subgroups following SAB assay testing of 30 highly sensitized patients. Data depicts the percentage of patients with different levels of prozone (no prozone, mild prozone, or moderate (mod)/marked prozone) with a sensitization history of pregnancy ± transfusions (pregnancy) or transplantation ± pregnancy ± transfusions (transplant). Reactivities were considered prozone positive in cases where the ΔMFI ≥ 3000.

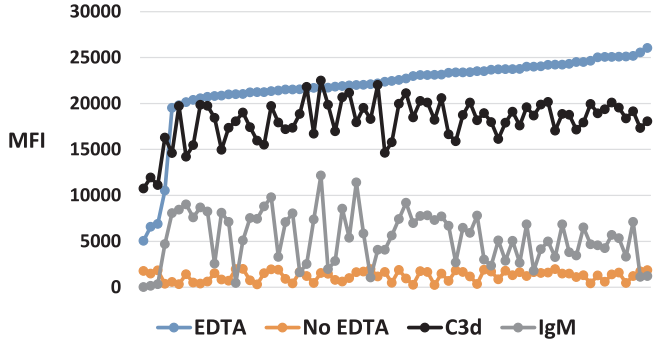


Fig. 6. Characterization of HLA specificities that would have been falsely assigned as negative without serum EDTA treatment, using a positive IgG-PE MFI threshold of 2000. Sera from 30 highly sensitized patients were tested by the SAB assay with IgG-PE ± EDTA, IgM-PE, or C3d-PE. Data depicts the levels of IgG-PE EDTA, IgG-PE, C3d-PE, and IgM-PE for 72 specificities (class I and class II) from 8 different highly sensitized patients that would have been falsely assigned as negative without serum EDTA treatment, based on a 2000 IgG-PE MFI positive cutoff.

patients with at least one specificity falsely assigned as negative (using a 2000 MFI cutoff) without EDTA treatment, as a result of prozone, had a history of previous transplantation. Taken together, these findings underscore the importance of serum treatment in HLA antibody identification and virtual crossmatching, especially in highly sensitized patients with a history of transplantation.

The frequency of prozone in our study was similar (albeit slightly higher) to the report by Tambur et al. (71%), who tested sera from sensitized patients selected for HLA antibody titration based on clinical indications, suspected prozone, and/or presence of strong HLA antibodies (> 10,000) [13]. While the authors did not report patient cPRA values or sensitizing events, we suspect based on the inclusion criteria that the degree of patient sensitization in their study would be comparable to that observed in our patients. In contrast, the rate of prozone reported by Guidicelli and colleagues was much lower (29.5% for class I and 45.9% for class II) [18]. This is not surprising given that the authors studied unselected patient sera (both pre- and post-transplant) that were tested in their laboratory during a one month period. Thus, the proportion of highly sensitized patients in this group would be predicted to be relatively low.

Another factor that may contribute to variability in the frequency of prozone observed in different studies is the way in which prozone is defined. In our study we defined prozone as the increase in SAB MFI of ≥3000 upon serum treatment with EDTA. We further classified the prozone effect based on the magnitude of MFI increase with EDTA into mild (ΔMFI 3000–4999), moderate (ΔMFI 5000–9999) and marked

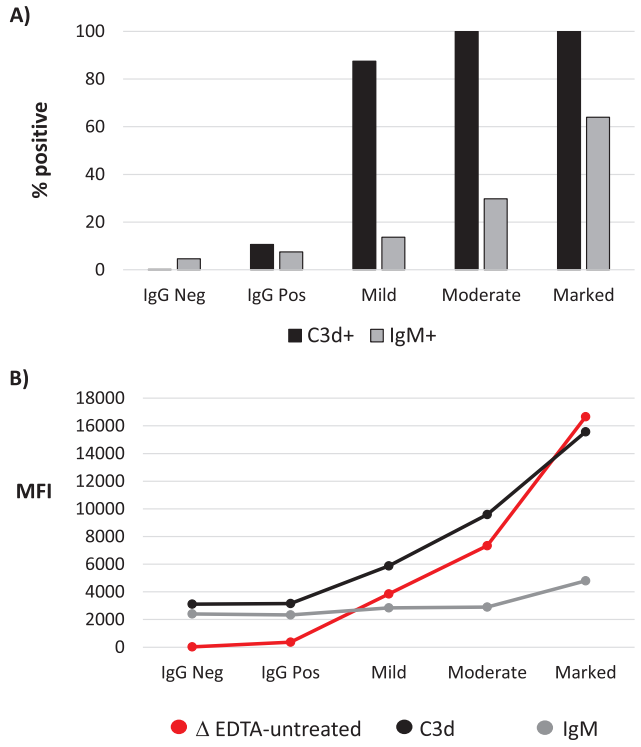


Fig. 7. Investigation of the association between C3d and IgM positivity and prozone level. Sera from 30 highly sensitized patients were tested by the SAB assay with IgG-PE ± EDTA, IgM-PE, or C3d-PE. Data depicts the percentage of reactivities that were positive for C3d-PE or IgM-PE in IgG negative sera (IgG neg), IgG positive, prozone negative sera (IgG pos), and each of the prozone categories (mild, moderate, and marked; panel A). Graphical representation of the correlation between mean C3d-PE MFI, IgM-PE MFI and the level of prozone (ΔMFI; ΔMFI = mean IgG-PE EDTA values – mean untreated IgG-PE values) is shown (panel B).

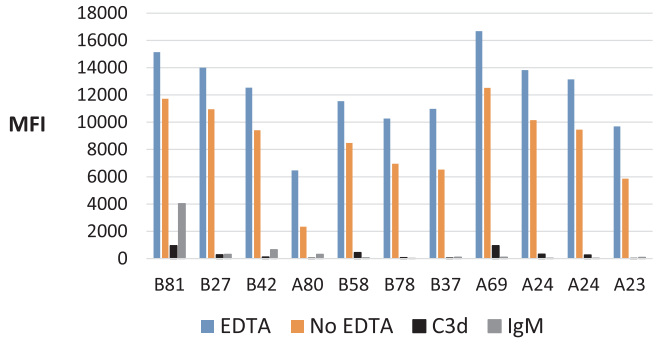


Fig. 8. Characterization of prozone positive, C3d negative specificities. Sera from 30 highly sensitized patients were tested by the SAB assay with IgG-PE ± EDTA, IgM-PE, or C3d-PE. Data depicts the MFI values for IgG-PE ± EDTA, IgM-PE, and C3d-PE from 11 C3d negative specificities that were categorized in the mild prozone group.

(ΔMFI ≥ 10,000) prozone categories. While our definition of prozone was arbitrary, the application of this classification schema in our study effectively categorized all the antibody specificities into distinct groups with varying potential for complement fixation (based on C3d positivity): absent/low (prozone negative), intermediate (mild and moderate prozone), and high (marked prozone), thereby validating our prozone classification approach.

The study by Tambur et al. defined prozone as ≥100% increase in MFI upon serum dilution, however, the authors did not provide justification or data to support this choice. When we applied this definition of prozone to our data set, virtually all mild and moderate prozone

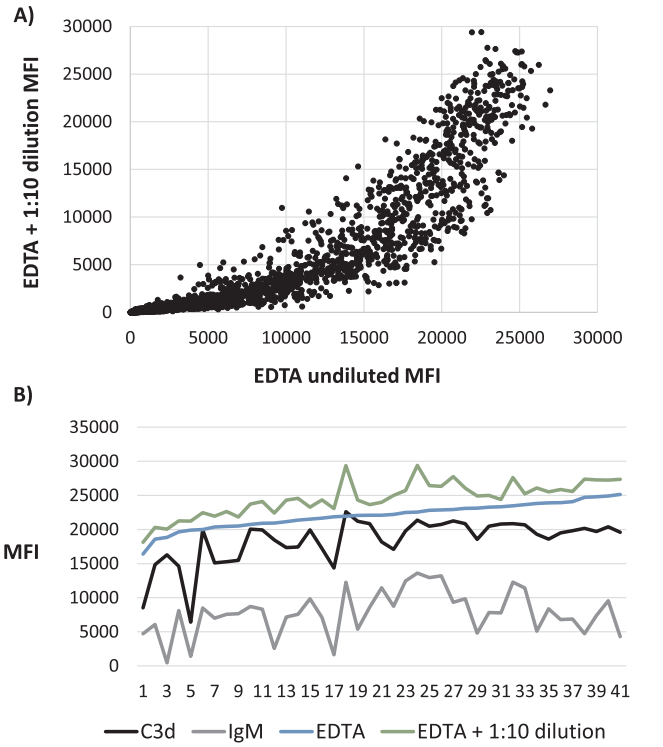


Fig. 9. Comparison of SAB assay reactivities for EDTA treated sera tested neat or diluted 1:10 in PBS. Sera from 30 highly sensitized patients were tested by the SAB assay with IgG-PE + EDTA ± 1:10 dilution, IgM-PE, or C3d-PE. Data depicts the correlation between SAB assay MFI for EDTA treated sera tested neat or diluted 1:10 in PBS (panel A). Characterization of the 41 specificities identified to exhibit higher (> 1000 MFI) values upon dilution, compared to undiluted serum is shown in respect to IgG-PE, C3d-PE, and IgM-PE MFI levels (panel B).

specificities (197/219, 90.0%) were re-classified to the prozone negative category, while the majority of marked prozone specificities (291/297, 98.0%) remained prozone positive. As a result, the prozone positive category assigned by Tambur and colleagues underestimated the true extent of complement mediated interference in the SAB assay and their prozone positive classification appeared to coincide with the marked prozone group in our study. When we applied the ≥100% increase in MFI rule to our patients, the rate of prozone was estimated to be 75%, which is remarkably similar to the frequency reported by Tambur et al. (71%). In contrast, Guidicelli and colleagues, used a novel, data driven approach to define the prozone effect. Specifically, they assigned bead specific prozone cutoffs using the mean and 3SD MFI information obtained by running positive control sera on multiple SAB assay runs. While this approach appears scientifically sound, it was not validated against the complement fixing potential of HLA antibodies and had several limitations. Firstly, prozone assignment using this approach was not based on objective MFI differences associated with serum treatment, and thus may not be representative of the changes seen with the prozone effect. Second, the prozone cutoff varied for each specificity, making the prozone classification complex and difficult. Third, the prozone positive cutoffs assigned with this approach would likely be specific to a particular positive control serum, making intra- and inter-laboratory comparisons virtually impossible. As a result, the frequency of prozone reported by Guidicelli et al. is difficult to compare with other studies.

In the current study, the prozone effect was seen more frequently in class I (70%) reactivities than those of HLA class II (30%). This is in contrast to previous reports where class II antigens were more commonly affected [13,18]. This disparity may simply be the result of the

differences in the degree of sensitization and antibody composition between the study populations, and may have been further impacted by how prozone was defined in each study. Among class I beads, HLA-A and HLA-B specificities appeared to be equally susceptible to prozone (31% and 29%, respectively), while HLA-C antigens were affected less often (16%). As described previously, the lower rate of prozone seen with antibodies targeting HLA-C may result from the higher density of denatured HLA-C molecules on the SABs and/or weaker sensitization possibly due to lower levels of HLA-C expression in tissues [18]. In fact, the average EDTA MFI for HLA-C locus antibodies was significantly lower compared to that of HLA-A/B (7791 vs 10,114; $p < 0.001$). Interestingly, in agreement with previous reports [13,18], our study showed that HLA-DQ locus antibodies were affected by prozone more often (26%) than HLA-DR (5%) or HLA-DP (17%). It is possible that the contribution of both alpha and beta chain specific antibodies often seen with HLA-DQ locus specific reactivity led to a higher density of bead-bound antibody. This could have resulted in a higher likelihood of complement fixation and prozone at this locus in comparison to cases with HLA-DR and HLA-DP specificities, where antibodies targeting the alpha chain are rarely found. As seen with the class I HLA, there were locus specific MFI differences for class II HLA antigens, with HLA-DQ exhibiting significantly higher MFI values (10,506; $p < 0.001$) compared to either HLA-DR (MFI = 6466) or HLA-DP (MFI = 5423). The relatively high susceptibility to prozone of HLA-DP antigens (when compared to HLA-DR) may be overestimated in our study, as all prozone positive HLA-DP specificities were identified in a single patient who had strong antibodies targeting the 84DEAV epitope.

Previous reports identified complement mediated interference as the main mechanism for the prozone effect [11]. Our observation that the majority of prozone positive specificities (97.9%) fix complement (C3d-PE staining) is consistent with this mechanism of action. In fact, C3d positivity (C3d-PE MFI ≥ 4000) in our study predicted the susceptibility of individual specificities to the prozone effect (sensitivity = 95.2%, specificity = 97.2%), while prozone correlation with IgM level was poor. Only 11 out of 516 prozone positive specificities, all in the mild prozone category, were C3d negative. We believe that the increase in MFI seen with serum treatment for these specificities may represent an off-target effect of EDTA as there was no significant IgM component to explain the interference. Interestingly, we also found that C3d-PE MFI values were proportional to the degree of prozone, which indicated that the amount of complement deposition on the beads is directly related to the extent of interference with HLA antibody detection in the SAB assay. Collectively this data confirms our previous findings and supports the usefulness of the dual antibody rapid test (DART) SAB assay protocol that was recently developed by our laboratory in order to prevent prozone [21,22]. The DART protocol is resistant to prozone as it uses a cocktail of IgG-PE and C3d-PE antibodies to simultaneously detect both prozone negative (mainly with IgG-PE) and prozone positive (C3d-PE ± IgG-PE) specificities. This obviates the necessity for serum treatment and avoids any potential off target effects associated with treating sera [21,22].

Our study is in agreement with the recent report by Zhang and Reinsmoen who showed that serum treatment with EDTA is very effective at minimizing prozone, and that serum dilution (1:8) is not likely to reveal additional specificities at risk for prozone [23]. However, serum dilution in our study did identify a small subset (41/516) of prozone positive specificities which exhibited a further increase in MFI upon serum dilution (EDTA vs EDTA + 1:10 dilution). This indicated that additional interference that was independent of Ca^{2+} and complement was responsible for the change in MFI. While the choice of 1:10 dilution in our study was based on the protocol published by Schnaidt et al. [8] and is similar to the 1:8 dilution used by Zhang and Reinsmoen [23], it is possible that using higher dilutions such as 1:50 or 1:100 may have been more effective in uncovering additional prozone. Interestingly, the majority of the specificities in this group (40/41) had an IgM component with high IgM-PE MFI values suggesting that IgM may indeed contribute to the interference. As recently demonstrated in an elegant study by Visentin et al., in which two patients exhibiting IgM dependent interference were identified, the mechanisms of IgM mediated prozone may include direct interference with IgG binding due to shared epitope specificity, steric interference with IgG or IgG-PE reporter binding, or contribution to complement activation and deposition [12]. Therefore, while IgM interference does not appear to be a common phenomenon and is usually present in addition to complement mediated blockade, it is prudent to consider this mode of interference in cases where donor specific HLA antibodies are not identified with EDTA treated sera but are suspected to be present based on crossmatch results or clinical findings. In such cases the presence of IgM can be identified using IgM-PE reporter antibodies and the interference can be revealed by treating sera with DTT.

In summary, the prozone effect is ubiquitous in highly sensitized patients, especially those with a history of previous transplantation. Antibodies against any HLA locus can be affected, however HLA-A, B, and DQ are most susceptible to prozone. A significant number of highly sensitized patients exhibit potent interference with HLA antibody detection whereby many strong complement fixing antibodies go undetected or are severely underestimated when untreated sera are used in the SAB assay. The chance for misinterpretation of SAB results in such cases is high and may compromise virtual crossmatching with the potential for serious clinical consequences. This is completely preventable when effective serum treatment and/or serum dilution are used to inhibit the prozone effect. Therefore, it is our opinion that all HLA laboratories should implement strategies to prevent/minimize prozone in their antibody testing workflow. While our study did not compare different treatment modalities, results reported by others suggest that EDTA and heat treatment are equivalent, while treatment with DTT may be less effective at overcoming complement mediated interference [17,23]. Therefore, DTT may not be the best option for routine treatment of sera but rather should be reserved for cases where IgM mediated interference is suspected. In our experience, treatment with EDTA is a simple and very effective way of preventing complement mediated interference. While we observed some off target effects of EDTA resulting in a slight change in MFI value, the overall impact does not appear to be significant in regards to HLA antibody assignment.

Conflicts of interest

The authors declare they have no conflicts of interest.

Acknowledgments

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