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Prozone phenomenon in pretransplant testing: An interesting conundrum involving solid-phase and cell-based assays

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Abstract:

BACKGROUND: Human leukocyte antigen (HLA) is a major determinant in deciding upon solid organ histocompatibility. Donor-specific anti-HLA antibodies (Donor-specific anti-HLA antibodies (DSAs)) are always a contraindication for solid organ transplantation, and identification of DSA becomes very crucial before transplantation to provide long-term graft survival. For identification of DSA, usually, either cell-based or HLA bead-based assay is being used in laboratories. However, both cell-based and bead-based assays have certain limitations. One such common limitation is "prozone effect," which can give false-negative results. Here, we would like to present a small pilot study to analyze the effect of the prozone phenomenon in the cell-based and HLA bead-based assays and its utility in histocompatibility testing.

MATERIALS AND METHODS: In a series of four experiments, cell-based assay, flow cytometric cross-match (FCXM), and HLA bead-based flow cytometric panel reactive antibodies (PRAs) were performed. Single-antigen bead (SAB) testing was conducted as a first experiment on four known positives samples for anti-HLA antibody-antibodies. In the second experiment, these four samples were pooled together (called pooled sera in the text) and tested for FCXM and PRA. In the third experiment, known commercially available positive control sera were mixed with pooled positive sera (positive control sera + pooled sera) to prepare, what we have called "positive concoction" in the text. In the fourth experiment, the positive concoction was diluted serially (1:2, 1:4, 1:8, and 1:16) and FCXM and PRA were performed again to analyze and compare the prozone effect.

RESULTS: Pooled sera did not have the expected median fluorescence intensity (MFI) values in FCXM assay, whereas the PRA was showing >90% positivity. In positive concoction, the MFI of FCXM assay was observed to be declining; however, PRA values remained almost constant. Dilutions of the pooled sera showed that MFI values of FCXM assays were increased suddenly after dilution. The highest MFI values were observed in 1:4 dilution of the sera, and then, it declined gradually, but the PRA values remained almost constant even after serial dilutions.

CONCLUSION: In our experimental findings, it was clear that cell-based assay (FCXM) was more severely affected by the prozone, whereas solid-phase (flow PRA) assay remained resistant to prozone.

Keywords:

Anti-human leukocyte antigen antibodies, flow cytometric cross-match, flow cytometry, panel reactive antibodies, prozone effect (hook effect), single-antigen bead assay, solid-phase immunoassay

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Introduction

Histocompatibility testing is broadly categorized in the cell-based assay and solid-phase immunoassay (SPI). Conventionally, a cell-based cross-match assay, called complement-dependent cytotoxicity cross-match (CDC-XM), has been used from decades for the detection of DSA in the organ-transplant recipients. Incorporation of anti-human globulin, in the CDC assay, enhanced the sensitivity several-fold.[1,2] Flow cytometric cross-match (FCXM), another cell-based assay, introduced in 1983 by Garovoy[3] is now routinely used, many times, along with CDC-XM and is known to be more sensitive assay than CDC-XM.[4,5]

The paradigm change with the advancement of technology has enabled us to use SPI assays where polystyrene microbead particles coated with human leukocyte antigen (HLA) antigens are used for the detection of anti-HLA antibodies. In this method, a pool of beads coated with HLA antigens obtained from recombinant or human cell lines are incubated with patient serum, and upon incubation, the reactivity is detected by using fluorescent-labeled anti-IgG in the flow cytometer. One of the common tests used in pretransplant testing is called panel reactive antibodies (PRAs), where the patient's serum is tested with beads coated with a panel of HLA antigens. The current "gold standard" in transplant immunology testing, single-antigen bead (single-antigen bead (SAB)) assay, is also an SPI.

Several laboratories use a combination of cell-based and HLA bead-based assays to optimize their testing algorithms.[6,7] Donor-specific anti-HLA alloantibodies (DSAs) identification is, very often, an essential test modality for completing the histocompatibility testing, when a CDC-XM or FCXM or both are positive.

Both kinds of assays, cell-based assay and HLA bead-based assay, are sometimes affected by prozone effect which leads to false negativity.[8] Here, we present a small experimental pilot study performed at our center, where we found that the prozone phenomenon is more likely to affect cell-based assays (FCXM) in comparison to solid-phase HLA bead-assay (PRA).

Materials and Methods

Patient samples

Clinical samples of four renal-transplant recipients, who had a definite history of sensitization (previous transfusion/pregnancy/solid-organ transplant) and were strongly positive in FCXM, were selected. Sera from all these four recipients were collected and tested with SAB-based assay individually.

Pooled sera

Patient's samples from four known positive patients (1 ml of each) were mixed in a falcon tube to make what we have called "pooled sera" (PSs) in this manuscript.

Positive concoction (PC)

Positive control sera (anti-HLA antibodies-positive control, One Lambda, USA) were mixed with PS in a ratio of 1:1 to experimentally create what has been called"positive concoction" (PC) in this manuscript.

Pooled donor mononuclear cells

Five healthy voluntary donors were selected and 10 ml of the peripheral blood was collected from each; in heparin tubes, the peripheral blood mononuclear cells (donor mononuclear cells [DMNCs]) were extracted from the donors' samples, using the density gradient centrifugation. Individual DMNCs were pooled together and dissolved in 1 ml of the McCoy's media in a 15 ml Falcon tube.

Single-antigen bead assay

SAB-based assay (LIFECODES LSA Class I and Class II kits, using Luminex®, Immucor Transplant Diagnostics, Inc., Stamford, CT, USA) was performed following the standard manufacturer's recommended procedure for the detection of anti-HLA antibodies. In this method, 10 µl of the recipient's sera was allowed to incubate with 40 µl SAB coated with recombinant HLA antigens for 30 min. After incubation, the beads were washed with wash buffer to remove the unbound antibodies, followed by addition and incubation of 30 min with anti-human IgG phycoerythrin (PE) conjugated to detect the bound IgG human anti-HLA antibodies. The reaction was acquired on the Luminex 200 platform, and the data were analyzed using the Match IT antibody software (LIFECODES, Stamford, CT, USA).

Flow panel reactive antibody assay

All these sera were pooled together and tested for flow PRA screening assay (One Lambda Inc., Canoga Park, CA, USA). In this assay, 20 µl of pooled sera was allowed to incubate with 5 µl Class I and Class II beads for 30 min. Followed by washing was performed with 1X buffer to remove unbound antibodies. After this, 1 × 100 µl goat anti-human IgG (Fcγ)-FITC (secondary antibodies) was added and incubated for 30 min. The beads were washed again and suspended in 800 µl of wash buffer. After the completion of the assay, the acquisition was done at logarithmic scale on forward scatter (FSC) versus side scatter (SSC) dot plot to identify the population of interest (class I and II beads). Then, a new dot plot was created as PE versus SSC and beads gating was applied here to discriminate the class I and II beads. To check the positivity of beads for anti-human IgG antibodies, the FL1 (FITC) histograms

were used and class I and class II beads were applied on the histogram; the events >10% beyond the negative control were considered positive for class I and/or class II.

Flow cytometric cross match assay

The same pooled sera tested on flow PRA above were also tested for FCXM to check for the presence of donor-specific HLA antibodies. In this method, 250,000 peripheral blood mononuclear cells of the pooled sample above (DMNCs) were incubated with 50 µl of sera at 4°C for 30 min and then washed with prechilled McCoy's media thrice, followed by addition of 1x, 50 µL of goat-anti human IgG FITC (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). Followed by 1x, 50 µL of goat-anti human IgG FITC (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) and 10 µl of anti-human CD22-Phycoerythrin (Beckton Dickinson, Franklin Lakes, NJ, USA) were added together and incubated again for 30 more min at 4°C. The cells were then washed and re-suspended in 1000 µL of phosphate-buffered saline (PBS). This was followed by the acquisition step on the flow cytometer. The lymphocytes were gated on FSC versus SSC dot plot, and then, these gated lymphocytes were discriminated into B cells using CD22 marker. Each of these gated B cells was now evaluated for anti-human IgG in FL1 channel histograms.

Quality control and calibration

Respective negative and positive controls were tested along with these samples and performed satisfactorily. Both the instruments, flow cytometer and Luminex, were quality control tested before performing these assays as per the manufacturer's recommendations.

Experiment design

In a series of four experiments, cell-based assay, FCXM, and an HLA bead-based flow cytometric PRAs were performed Table 1. The first experiment comprised of SAB assay conducted on four known anti-HLA antibody positive samples. In the second experiment, these four samples were pooled together (called pooled sera in the text) and tested for FCXM and PRA. In the third experiment, known commercially available positive control sera were mixed with pooled positive sera (positive control sera + pooled sera) to prepare, what we have called "positive concoction" in the text. In the fourth experiment, the positive concoction was diluted serially (1:2, 1:4, 1:8, and 1:16) using a PBS, and then, each dilution was tested for PRA and FCXM using the same extracted DMNC to check for the presence (or absence) of prozone phenomenon.

Statistical analysis

The analysis included median fluorescence intensity (MFI) for the analysis of FCXM and SAB assay and percentage of PRA (%PRA) was used for the evaluation of flow PRA. All the expressed values are expressed in a different format, so the direct comparison of values was not possible.

Ethical clearance

Ethical clearance was not required since no personal identifiers were used and only anonymized samples were used to generate experimental data, which was analyzed, thereafter.

Results

SAB results of the four individual patient serum samples

The result of SAB assays performed on the four individual patient serum samples was analyzed with "Match IT antibody" software (Immucor), and class I results are represented in Figure 1. Class I results shows that out of four sera, two were >50% positive for anti-HLA antibodies, another was 20% positive, and the last showed <10% positivity for class I anti-HLA antibodies.

Class II results showed that out of four, three were >30% positive for anti-HLA antibodies and one was 15% positive for Class II antibodies Figure 2.

Flow panel reactive antibodies and flow cytometric cross-match result

Flow PRA and FCXM results were evaluated through "FCS Express 6 software" (De Novo Software 400N., Brand Blvd., Suite 850 Glendale, CA 91203, USA), and class I and class II PRA results were recorded as percentage of the positive beads in FITC (FL1) channel, and the value beyond 10% of the negative control is marked as positive. All the values of flow PRA are mentioned in Table 2.

In FCXM, an MFI of anti-human IgG (FITC) in FL1 channel is recorded Figure 3 and mentioned in Table 2. The control sera's (negative and positive) results were within range as expected in both the assays of FCXM (128 and 1472) and PRA (9% and 63%); however, the pooled sera results are not up to the expectation in the FCXM assay (593), whereas PRA results were satisfactory (93% and 99%).

The addition of positive control (PC) with the pooled sera decreases the MFI of FCXM from 593 to 439, whereas PRA value does not get much affected (90% and 94%).

Dilution results showed that there was a consecutive increase in MFI values of FCXM; 1:4 dilution of the serum gave the highest MFI values, which was 3261.

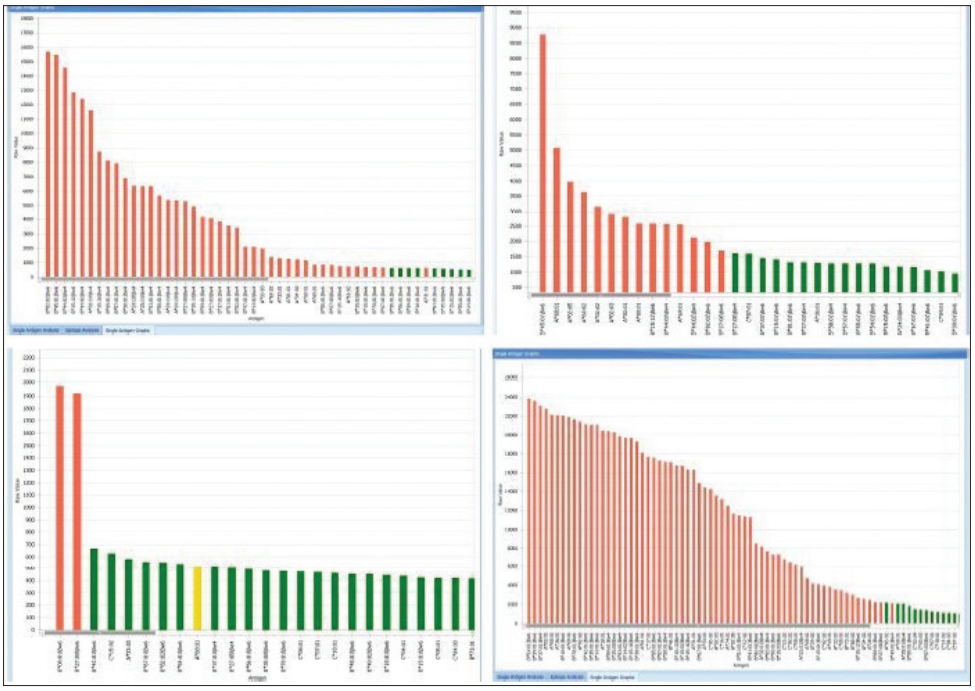

Figure 1: Class I-SAB assays, X-axis represents HLA antigens in panel and Y-axis represents median fluorescent intensity (MFI) of anti-HLA antibodies (IgG)

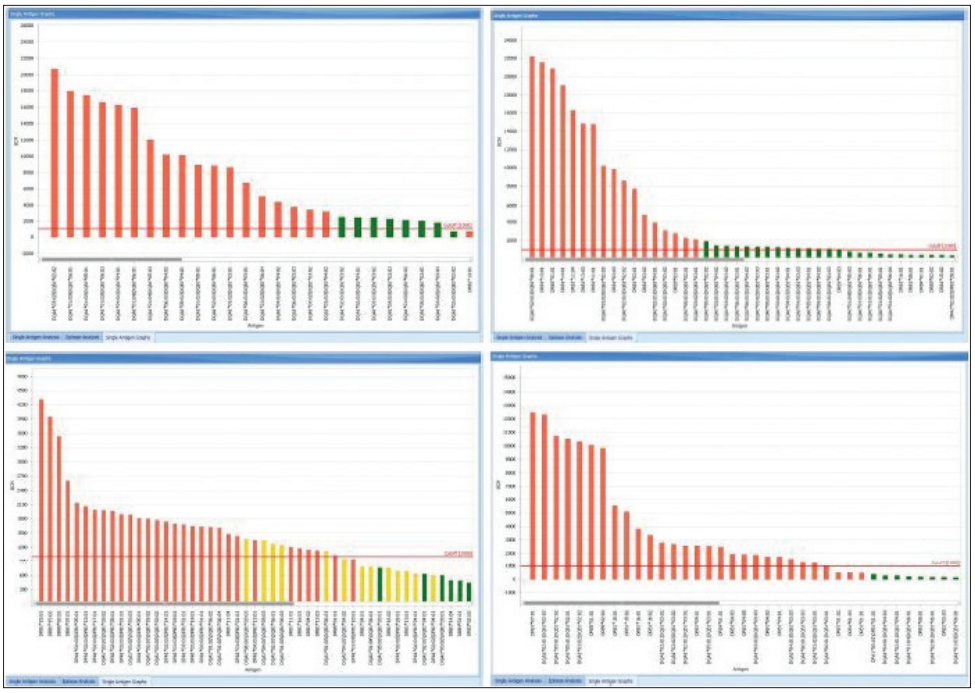

Figure 2: Class II-SAB assays, X-axis represents HLA antigens in panel and Y-axis represents median fluorescent intensity (MFI) of anti-HLA antibodies (IgG)

Discussion

Prozone phenomenon is a known cause of false negatives and is due to an excess amount of anti-HLA antibodies. In the present study, we observed that, when we increased the amount of anti-HLA antibodies in pooled sera by adding commercially available positive control (PC), the MFI of FCXM decreased. Followed by upon serial dilutions of positive concoction showed, a sudden increase in the MFI of FCXM. Surprisingly, the flow PRA had almost the same positivity against the pooled sera as well as 1:16 diluted sera. Hence, in these experimental assays, we observed that with an increasing amount of anti-HLA antibodies in the sera,

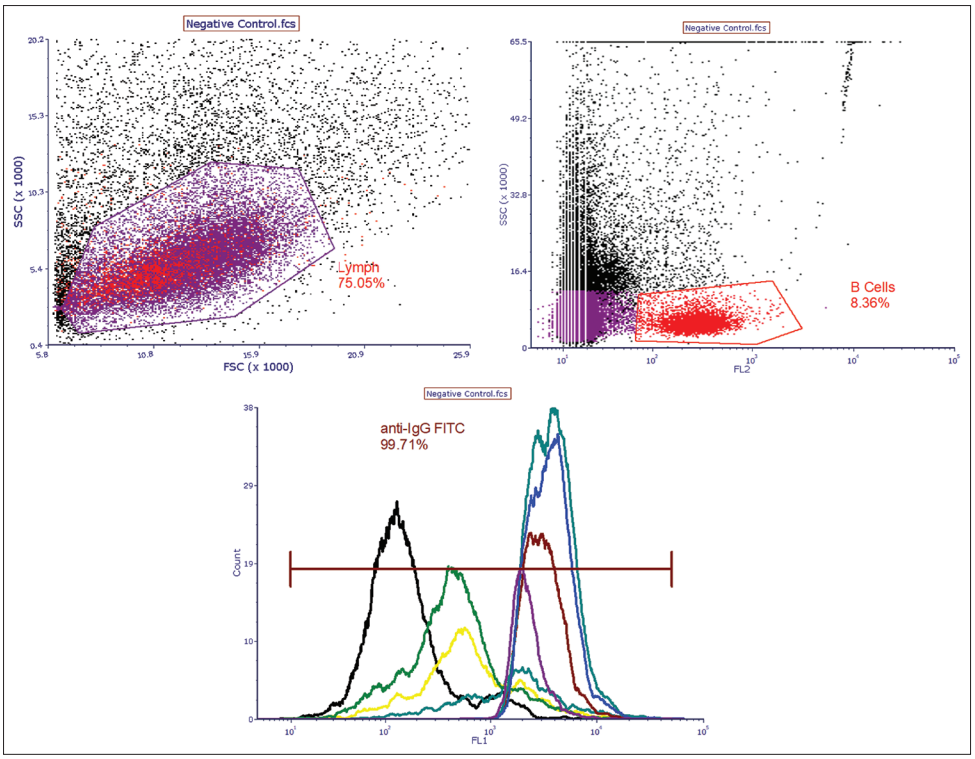

Figure 3: Flowcytometric cross match (FCXM) representing median fluorescent intensity (MFI) of anti-human IgG in FITC channel; Lymph's were gated at FSC vs SSC dot plot, then CD22+ B cells were discriminated from total lymph's in second dot plot as FL2 vs SSC. Followed by MFI of anti-human IgG were recorded in FITC channel histogram and layover was applied from rest of the experiments

there was a resultant decrease in MFI of cell-based assay (FCXM).

Literature suggests that complement component deposition mainly C3 leads to activation of key mechanism for prozone phenomenon.[9,10] It has also been suggested that IgM antibodies can compete with the IgG antibodies and inhibit the binding of anti-IgG antibodies (secondary antibodies), resulting in undetectable IgG antibodies. Further, it has been recommended that, in such cases, pretreatment with dithiothreitol (DTT) leads to the destruction of IgM molecules and prozone phenomenon is inhibited.[11] It has also been found that complement component C1 binds to two nearby molecules of IgG and block the Fc receptor of approaching secondary antibody.[12]

To avoid prozone phenomena, proper dilutions are needed to resolve all the antibodies into 1:2, 1:4, 1:8, and 1:16 in the FCXM. MFI of 1:4 dilution FL1 (FITC) channel was highest in comparison to others, and flow PRA showed >75% positivity on neat sera as well as 1:16 diluted sera. This shows that FCXM (cell-based assay) is more prone to prozone phenomenon in comparison to flow PRA (solid-phase assay).

As per the published literature, HLA antigens are not fixed on the cells surface, and when anti-HLA antibodies approach to bind with HLA antigen, they drag the antigen to pole[13] resulting in the reduction in the distance between HLA antigens, which in turn facilitates the binding and occupying both the antigen-binding site of anti-HLA antibodies to different antigens (divalent binding).

Our study supports the hypothesis mentioned by Schnaidt *et al*.[12,14] that the antigen density plays a crucial role in the prozone phenomenon, and also, for the binding and activation of complement-mediated prozone, two adjacent closely related IgG molecules are required to initiate the prozone.

Through this observation, we concluded that the HLA antigens of the flow PRA beads are bound on the solid surface and are immobilized. This results in a decrease in divalent binding of approaching anti-HLA antibodies, and thus, the complement molecules cannot find two adjacent closely related IgG molecules, failing to initiate prozone cascade. In the case of FCXM, cell surface membrane motility of HLA molecules helps in an increased divalent binding with approaching anti-HLA antibodies; thus, the complement molecule finds the rich amount of closely related IgG molecule, and then, complement-mediated prozone is initiated.

Serial dilutions are the better option to resolve the prozone phenomenon as compared to the addition

Table 1: showing the experiment design, type of sera used in each assay, and the test performed on each serum

Experiment number	Type of sera used	Test performed
1	Individual patient serum (four)	LSA class I and II (individually)
2	PS	PRA, FCXM
3	PC	PRA, FCXM
4	PC in dilution of 1:2, 1:4, 1:8, and 1:16	PRA, FCXM

PS=Pooled sera, PRA=Panel reactive antibodies, FCXM=Flowcytometric cross-match, PC=Pooled concoction

Table 2: Panel reactive antibodies and flowcytometric cross-match results comparison

Sera tested	PRA results		Sera tested, MFI (B-cells)
	Class I results (%)	Class II results (%)	
Negative control	9	9	128
Positive control	93	63	1472
PS	93	99	593
PC	90	94	439
PC 1:2 dilution	91	93	2616
PC 1:4 dilution	91	92	3261
PC 1:8 dilution	89	90	3191
PC 1:16 dilution	78	89	1714

MFI=Median fluorescence intensity, PRA=Panel reactive antibodies, PS=Pooled sera, PC=Pooled concoction

of ethylene diamine tetraacetic acid, which chelates the calcium which was required for the activation of complement molecule C1 and helps in the minimizing the prozone effects.[15] Some authors also suggest DTT treatment of the serum beaks the pentameric structure of IgM antibodies, leading to unmasking of the clinically relevant antibodies.[10]

A case report published by Sağıroğlu *et al*.[16] found the weak positive (T cell 6% and for B cell 40%) sera on FCXM which after serially diluting revealed strong positivity (48% and 72%, respectively), supporting the role of prozone effect on FCXM assay.

Conclusion

Prozone phenomena are one of the major causes for the false negativity, and hence, we recommend to use both cell-based assay and solid-phase assay to overcome this effect, resulting in reduced false negative reporting of results. As per our findings, it is clear that cell-based assay (FCXM) is affected more by the prozone, whereas solid-phase (flow PRA) assay does not have such effect.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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