Case Report

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Implication of a positive virtual crossmatch with negative flow crossmatch: A mind-boggler

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Abstract:

There are occasions when tests performed before considering a patient for transplant are ambiguous and require further workup. One such condition is the presence of a positive virtual crossmatch (VXm) (anti-human leukocyte antigen [HLA-A]*26:01 antibody in this case) with a negative complement-dependent cytotoxicity, Luminex, and flow crossmatch. To ascertain the nature of the antibody, the beads used in single-antigen bead assay (SAB) were treated by acid to denature the antigens and retested with the control and test sample. The mean fluorescence intensities (MFIs) from the patient sera with acid-treated beads increased considerably as compared to the regularly untreated SAB indicating additional antigen epitopes become available by the denaturation process. The MFIs of the antibodies from that of the control sera were reduced to half on testing with the acid-treated SAB assay, indicating that HLA antigen HLA-A*26 was susceptible to acid treatment. Therefore, results of VXm should be interpreted with caution.

Keywords:

False-positive single antigen bead assay, renal transplant, transplant immunology

Introduction

With the rise in affordability, accessibility, and availability of good patient and organ donor management protocols, solid organ transplantation is on the rising trend. Opposed to a limited pool of renal organ donors, the number of patients awaiting renal transplant is on the rise owing to a better quality of life.[1] For the optimum utilization of the grafts, a panel of investigations was done using various techniques of donor matching to obtain the best outcome of transplant. These investigations are mainly centered on the detection of preformed antibodies, which can cause hyperacute, acute, and chronic rejection. There are various methods to detect these antibodies, and thereby decrease the occurrence of antibody-mediated rejection. These techniques include Luminex antibody screening, complement-dependent cytotoxicity (CDC) using anti-human globulin (AHG) crossmatch (CDC-AHG Xm), Luminex Crossmatch (LumXm) (Lifecodes, LMD, Immucor), flow crossmatch (FlowXm), and single-antigen bead assay (SAB). SAB assay along with the donor human leukocyte antigen (HLA) typing determines the virtual crossmatch (VXm).[2]

VXm and FlowXm/LumXm correlate well if proper cutoffs are maintained in the SAB assays.[3] However, there are a few occasions when the tests are either ambiguous or require further workup before "ruling in" or "ruling out" the patient for transplant. One such condition is the presence of a positive VXm with a negative CDC, Luminex, and FlowXm which we present here.

Case Report

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A 39-year-old female diagnosed with chronic kidney disease-V for 5 years and on hemodialysis for 1 year, was planned for a renal allograft transplant. She is a hypertensive and nondiabetic female with a baseline serum creatinine value of 4.76 mg/dL. She was sent to our department of transfusion medicine and transplant immunology for the pretransplant immunological workup. As a part of routine investigation, the HLA typing was performed for the patient and the prospective donor using sequence-specific primer method (Invitrogen, USA). The mother of the patient was the prospective organ donor. The patient and donor typing to establish relationship are shown in Table 1.

As mandated by the Transplant of Human Organs Rules, 1995[4] and the amendments thereof, CDC-AHG was performed which turned out to be negative LumXm, and FlowXm was also negative for the patient. HLA antibody screening (Lifecodes, LMX, Immucor, USA) was performed, which showed high mean fluorescence intensity (MFI) for HLA Class I (MFI 22,659) and II (MFI 19,191).

The cutoff for a positive result is more than or equal to 1000 MFI in our laboratory.

To detect the presence of donor-specific antibody (DSA) against HLA-Class I and II, SAB testing (Lifecodes, LSA Class I and II, Immucor, USA) was performed as a part of the routine workup in transplant patients. There was no DSA detected against HLA Class II antigens. DSA was detected against HLA Class I: A *26:01 (MFI 3826), that is, the VXm was positive in this case.

LumXm was repeated using a fresh sample after heat inactivation at 56° temperature for 30 min and 1:10 dilution to overcome the prozone effect if any. The results of crossmatch were still negative for both HLA Class I and II after heat treatment and/or dilution studies.

SAB was repeated with a fresh sample and with the different lot numbers of the same manufacturer for HLA Class I, but the results did not differ from the previous one.

To summarize, the VXm was positive repeatedly with the negative flow and LumXm. After discussion and determining that proper cutoffs are maintained in all assays, it was hypothesized that these findings were as a result of denatured HLA antigens, and further advanced workup is needed. To test this hypothesis, acid treatment of the beads to denature the antigens was initiated.

Denaturation of Proteins (Epitopes) on Single-Antigen Beads

There have been few case reports in the literature,[5] commenting on the presence of antibodies against cryptic/altered antigens present on the beads of single-antigen assay. To ascertain the nature of the antibody found against anti-HLA-A*26:01 in our case, the beads used in SAB assay were treated by acid to denature the associated antigens. Beads were mixed with ×10 volume of 0.1 M NaAc pH 2.7 (e.g., 10 µl of beads + 100 µl of 0.1 M NaAc) and incubated at room temperature for 30 min. The mixture was then washed two times with basic wash buffer and suspended in phosphate buffer solution.[5]

SAB assay was repeated using these acid-treated beads with the patient's serum. Control sera with known anti-HLA-A*26 specificity were also run using the same acid-treated beads. The MFIs of the antibody from the patient sera with acid-treated beads increased considerably as compared to the regularly untreated SAB. This indicated that additional antigen epitopes became available by the denaturation process. The MFIs of the antibodies from that of the control sera with known anti-HLA-A*26 specificity were reduced to half on testing with the acid-treated SAB assay indicating that the HLA antigen HLA-A*26 on the SAB assay was susceptible to acid treatment [Figures 1 and 2]. This provided evidence in favor of the presence of antibody against cryptic epitopes present on the beads.[5]

After discussion with the clinician, a consensus was obtained that the patient could be taken up for the transplant, and that these antibodies were actually against the cryptic epitopes, as hypothesized. The patient underwent renal

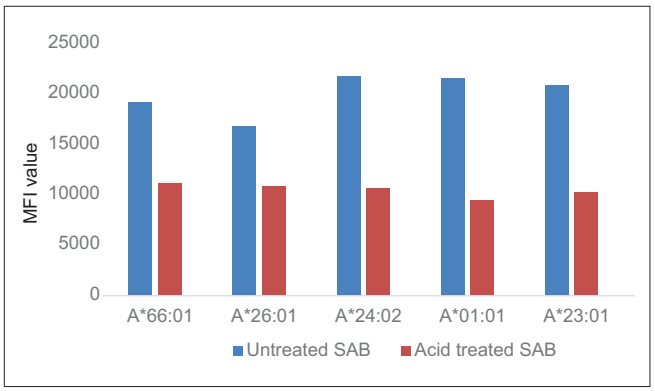

Figure 1: Mean fluorescence intensity values of untreated and acid-treated single antigen bead assay from a control sample

Table 1: Human leukocyte antigen typing results of patient and donor

	HLA-A*	HLA-B*	HLA-C*	HLA-DRB1*	HLA-DQB1*	DRB3*/4*/5*
Patient	02, 32	40, 55	01, 12	13, 15	05, 06	DRB3*, DRB5*
Donor	26, 32	40, 55	01, 15	13, 15	06, --	DRB3*, DRB5*

HLA=Human leukocyte antigen

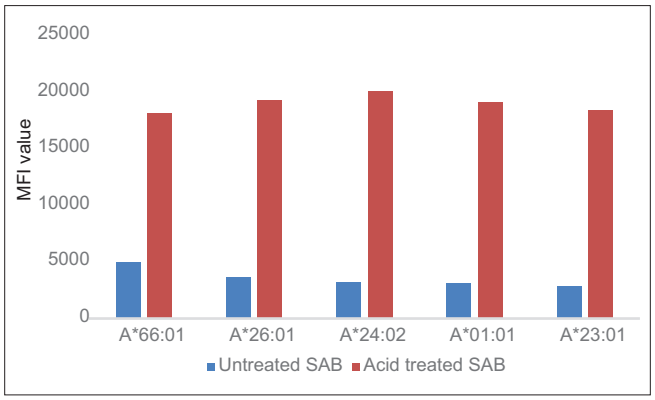

Figure 2: Mean fluorescence intensity values of untreated and acid-treated single antigen bead assay from the test sample

transplant with triple immunosuppression (tacrolimus, prednisolone, and mycophenolate mofetil). Her serum creatinine at discharge on day 7 was 0.8 mg/dL, and the urine output was 4.8 L/day. DJ stent was removed after 4 weeks. Although the follow-up period is too short for evaluation, 6-month posttransplant, the creatinine value continues to be within the normal range (0.76 mg/dL) with no complaints.

The clinical significance of DSA has been enumerated in various studies.[6] One of the biggest advances in the field of histocompatibility testing has been the implementation of VXm based on Luminex-based assays. The SAB assay has enabled the precise analysis of the DSA without the actual "wet crossmatch." The antigens against which these DSAs are detected are considered unacceptable to proceed for a transplant as such. This augments the need of desensitization in these patients to reduce the DSAs. A negative VXm with no prior history of sensitization such as blood transfusion, pregnancy, or previous transplant is generally safe to proceed for transplant. However, controversies over the implication of a positive VXm in the presence of a negative FlowXm has been elucidated in the literature to a lesser extent. The antibody detected could either by a low-titer antibody not detected in FlowXm but detected in a VXm. Furthermore, the antibody found in a VXm could be against any cryptic antigen/epitope. The implication of such an antibody is unclear. However, it is clear that in the organ allocation criteria, the presence of DSA by the SAB assay would lead to an organ being denied to a patient, which is actually due to the false positivity seen with the denatured antigen. Jacob *et al*.[5] also support the same theory in their publication, wherein they state that MFI values will increase after acid treatment of beads if additional epitopes are exposed. Ferris *et al*.[7] and Nishida *et al*.[8] quote that the human immune system can react to denatured antigens. Alternatively, Morales-Buenrostro *et al*.[9] have theorized that this may represent the specificity of some "natural" HLA antigen antibodies. However, despite the hypothesis of various origins of these antibodies, the clinical significance still remains unclear.

As described by Otten *et al*.[10] that a newer generation of beads is available known as iBeads or clean beads, which are devoid of such denatured antigen and can overcome the concerns pertaining to antibodies against denatured antigens. They studied the clinical implication of such antibodies and concluded that only antibodies against intact HLA determine the risk for graft loss, whereas those against denatured epitopes do not. This has further been supported by Visentin *et al*.[11] In our case, the graft function was maintained until the latest follow-up with normal serum creatinine values supporting the above conclusion.

Conclusion

SAB assays are many a times considered to be superior in antibody detection assays, but should be interpreted in the light of other useful tests such as FlowXm and LumXm which actually detect the actual DSA, more so in case of discordance between the Vxm and Flow/LumXm. In our case, antibodies were directed against denatured form of antigen but not its native form. This antibody did not cause any rejection in the patient (till follow-up). Therefore, we conclude that results of VXm should be interpreted with caution taking into consideration, the clinical history and results of other supplemental tests, as no single test is the ideal one or provides a perfect scenario.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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