Unexpected Positive Prospective Crossmatches in Organ Transplant

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Abstract

Preformed donor-specific antibodies against human leukocyte antigen can induce antibody-mediated rejection after organ transplant. Hence, future transplant recipients undergo pretransplant screening for preformed antibodies (ie, virtual crossmatch). Subsequently, prospective (analytic) crossmatching is performed using conventional, complement-dependent cytotoxicity assays and/or flow cytometry-based methods. The present article reviews factors that must be considered when unexpected, positive, prospective crossmatches are observed. First, the prozone effect caused by the interference of complement or immunoglobulin M must be abrogated by treating the serum with moderate heat, dilution, hypotonic dialysis, EDTA, or dithiothreitol. Second, the physician must check for the presence of potentially interfering autoantibodies (in a context of autoimmune disease or human immunodeficiency virus infection) or therapeutic antibodies (such as rituximab and antithymocyte globulin). In conclusion, knowledge of each assay’s technical characteristics will enable the physician to reliably interpret any discrepancies. The reasons for an unexpected, positive, prospective crossmatch must be elucidated before transplant to ensure efficient organ allocation and optimize patient outcomes.

Key words: Crossmatch flow cytometry, Crossmatch lymphocytotoxicity, Transplantation, Virtual crossmatch

Introduction

The presence of preformed donor-specific antibodies (DSAs) against human leukocyte antigen (HLA) is a major barrier in clinical organ transplant and is one of the main reasons for antibody-mediated rejection. Hence, transplant recipients undergo pretransplant screening for preformed antibodies so that virtual crossmatch (V-XM) can be performed once a graft is proposed.1 Subsequent prospective crossmatch (P-XM) is performed by using conventional complement-dependent cytotoxicity crossmatch (CDC-XM) and/or flow cytometry crossmatch (FC-XM) methods.2 It has been consistently shown that a positive T-cell crossmatch is a marker for kidney transplant rejection. In contrast, the clinical relevance of a positive B-cell crossmatch (mostly for deceased-donor renal transplant) is subject to debate because of concerns over false-positive results.

In the V-XM technique, the donor’s HLA genotype is compared with the recipient’s anti-HLA antibody specificity, which is assessed before transplant in a single-antigen flow bead (SAFB) assay or a phenotype panel bead assay.3 Virtual crossmatching can be performed rapidly; this is especially important in heart and lung transplant procedures, where the additional time needed to perform P-XM would increase the likelihood of damage to the transplanted organ.4

Inconsistencies between V-XM and P-XM results are not uncommon. The objective of the present review was to highlight factors that must be considered when seeking to avoid or explain an unexpected positive pretransplant crossmatch. These notably include false-negative antigen bead assays and interfering autoantibodies or therapeutic antibodies.

Four different crossmatching assays may be used, depending on the transplant team’s practice and...
preferences. The characteristics of each assay are summarized in Table 1, and each is detailed below.

The complement-dependent cytotoxicity crossmatch assay

The CDC-XM assay (see Figure 1) identifies clinically significant, anti-HLA DSA-mediated responses for a given recipient. It is based on complement-dependent cytotoxicity (mediated by immunoglobulin G [IgG] and/or immunoglobulin M [IgM]), using isolated donor B and T lymphocytes previously incubated with recipient’s serum and complement. If one or more DSAs are present and bind to donor cells, activation of the complement cascade (via the classical pathway) results in lysis of the lymphocytes. Cell viability is detected by fluorescence microscopy after the addition of a vital or supravital dye (which stains viable cells green and lysed cells red), and the percentage of dead cells is rated according to the semiquantitative American Society for Histocompatibility and Immunogenetics score. Hence, American Society for Histocompatibility and Immunogenetics scores of 1 (“negative”) and 2 (“doubtful negative”) correspond to fewer than 10% and 20% of dead cells, and scores of 4, 6, and 8 correspond to increasing levels of lysis. The addition of antihuman globulin increases the sensitivity of CDC-XM because each DSA on the donor cell binds several antihuman globulins and thus increases the total number of Fc receptors available for interaction with complement. It should be noted that the CDC-XM assay detects recipient DSAs but also all types of IgG and/or IgM alloantibodies able to bind to donor cells. This assay also only detects complement-binding antibodies.

The flow cytometry crossmatch assay

The FC-XM assay (Figure 2) is the most sensitive cell-based method for detecting DSAs. The assay measures the extent to which alloantibodies bind to donor’s lymphocytes, which depends on antibody titer and avidity. The recipient’s serum and donor’s lymphocytes are incubated with fluorochrome-labeled secondary antibodies against human IgG. Hence, the level of fluorescence (relative to a negative control) reflects the amount of DSA bound to donor cells. This basic assay cannot discriminate between complement-binding and noncomplement-binding antibodies. Like CDC-XM, FC-XM also detects recipient DSAs but also all types of IgG and/or IgM alloantibodies able to bind to donor cells. Hence, the basic FC-XM method can be enhanced by combinations with antibody and cytotoxicity assays.
Cells and serum can be incubated with rabbit complement, and cell viability is then measured with 7-aminoactinomycin (a fluorescent intercalator that undergoes a spectral shift on binding to DNA). Furthermore, the antibody subtype can be determined by the isotype specificity of the fluorescently labeled detection antibody (i.e., IgG, IgM, or IgA). Moreover, the likelihood of complement activation can be determined by choosing a detection antibody that binds only to IgG1 and IgG3 and not to IgG2 and IgG4.

**The luminex donor-specific crossmatching assay**

The Luminex (Luminex, Austin, TX, USA) donor-specific crossmatching assay (Figure 3)2 uses beads coated with anti-HLA class I or class II antibodies that can specifically capture the donor’s HLA molecules. Beads coated with anti-HLA antibodies are incubated with a donor lymphocyte lysate. The class I and class II beads thus become coated with corresponding donor HLA antigens. The recipient’s serum is then added. After capture by the immobilized HLA, DSAs in the recipient’s serum are detected by using a secondary antihuman IgG antibody. With the use of donor antigens, the Luminex crossmatching assay combines the respective advantages of cell-based and solid-phase assays; hence, it is more sensitive and more specific than CDC-XM.10-13

**Enzyme-linked immunosorbent assay crossmatching assay**

The enzyme-linked immunosorbent assay (ELISA) crossmatching method (Figure 4) is based on the same principle as the Luminex assay method. Donor-specific antigens are detected by immobilizing detergent-extracted HLA molecules from selected donors on precoated monoclonal capture antibodies. Bound anti-HLA class I DSAs from the recipient are detected colorimetrically after incubation with alkaline phosphatase-conjugated secondary antibody.

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**Figure 2. Flow Cytometry Crossmatch**

This assay is based on measuring the binding of donor-specific antibodies (DSAs) to donor’s lymphocytes. Recipient’s serum is incubated with donor’s lymphocytes, and binding is detected with secondary fluorochrome-labeled anti-immunoglobulin G (IgG) antibodies. If no DSAs are present, no binding occurs and the result is negative. If DSAs bind to the lymphocytes, antibodies can then bind fluorescently labeled antihuman Ig antibodies. Fluorescence intensity can be measured and expressed in “channel shifts,” relative to control sample. Adapted from Mulley and Kanellis (2011).2

**Figure 3. Luminex Bead Assay Crossmatch**

This method uses beads coated with anti-HLA class I and class II antibodies to screen for HLA from a specific donor. Test beads coated with antibodies that capture donor HLA antigens are incubated with lysate prepared from donor’s lymphocytes. Class I and II beads thus become coated with HLA class I and class II molecules. Recipient’s serum is then added. If anti-HLA donor-specific antibodies (DSAs) are present, they will bind to the corresponding antigens. This reaction is tagged using fluorescent antihuman globulin, read on a dual laser flow system (Luminex), and quantified in terms of mean fluorescent intensity. Adapted from Mulley and Kanellis (2011).2
dies. Compared with this currently available ELISA crossmatch technique antibody monitoring system (AMS; GTI, Waukesha, WI, USA), which is carried out in 96-well ELISA microtiter plates, the AbCross HLA class I/II system (Biotest/BioRad, Dreieich, Germany) utilizes a 60-well CDC microtiter plate format. Owing to the lysis of donor cell-antibody immune complexes outside of the detection plate, it creates fewer background signals than the AMS system.14

Understanding the reasons for an unexpected positive crossmatch

False-negative bead assay results

The SAFB assay is considered to be the most sensitive means of detecting anti-HLA antibodies. It complements panel bead assays as a screen for anti-HLA antibodies. Together, the two-bead assays enable V-XM and provide information on the likely results of P-XM. However, the assay results may be difficult to interpret. A number of limitations can make it difficult to predict V-XM results for donor selection and thus can lead to discrepancies between antibody identification and the P-XM results.

The prozone effect is one of the main limitations of bead assays; interference by complements can result in the underestimation of high-titer antibodies. With the SAFB One Lambda assay kit (One Lambda, Canoga Park, CA, USA), the prozone phenomenon affects 2.1% and 1.1% of assays for HLA class I and II molecules.15 Activation of the classical complement pathway through component 1 (C1) prevents the fluorescent antibody conjugate used in the SAFB assay from binding to an IgG alloantibody.16 This C1-mediated prozone phenomenon is calcium dependent because it can be observed in serum but not in EDTA-treated plasma. Similarly, HLA-specific IgMs can also compete in the SAFB assay with IgG alloantibodies.18,19 When the prozone phenomenon occurs, an antibody may even become transiently undetectable if its mean fluorescence intensity falls below the positivity threshold. However, it has been found that the treatment of serum with moderate heat, dilution, hypotonic dialysis, EDTA, or dithiothreitol abrogates the prozone effect.18-20 By way of an example, serum can be pretreated for 10 minutes with a 0.1 M solution of pH 7.4 disodium EDTA diluted 1:10 prior to the SAFB assay.12

The complement-dependent cytotoxicity crossmatch assay can be positive with immunoglobulin M only. In this case, we can use flow cytometry crossmatch and antigen bead assays with an anti-immunoglobulin M as the secondary antibody. An anti-IgG antibody is usually used as the secondary antibody in antigen bead assays. If IgM CDC-XM is positive and IgG FC-XM is negative, the antigen bead test and FC-XM must be performed by using anti-IgM antibody as the secondary antibody. This may demonstrate that the positive CDC-XM was due to the presence of anti-HLA IgM antibodies against one of the donor-specific antigens.21

Interfering antibodies

Non-anti-HLA antibodies can target antigens expressed on lymphocytes and thus induce positive crossmatching; these notably include antibodies against vimentin, MHC class I polypeptide-related sequences A and B, and the angiotensin A1 receptor.22 Furthermore, other substances in the recipient’s serum may bind to Fc receptors (eg, autoantibodies) or...
may target lymphocytes (eg, therapeutic antibodies). Autocrossmatching (using recipient’s lymphocytes and serum) can show these phenomena.

**Autoimmune disease**
False-positive CDC-XM may be caused by autoimmune disease. A study of women with systemic lupus erythematosus described 2 positive CDC crossmatches (for B cells in the first case and for both T and B cells in the second), even though DSAs were not detected in an SAFB assay. The lack of HLA specificity in these positive crossmatches was confirmed by (1) a negative ELISA-XM using HLA antigen from a donor cell lysate and (2) the favorable outcome of transplant at 2 years. It is noteworthy that the autoantibodies leading to a false-positive CDC-XM during autoimmune disease may correspond to complement-binding IgGs rather than IgMs.

**Human immunodeficiency virus-positive cases**
In human immunodeficiency virus (HIV)-positive recipients, false-positive Fc crossmatches have been shown to occur after the treatment of lymphocytes with pronase (a nonspecific protease that cleaves Fc receptors from the surfaces of T and B cells and can expose cryptic epitopes). In a population of 28 HIV-positive men and 72 HIV-negative men, CDC-XM and FC-XM were used to screen for autoantibodies against autologous T and B cells. The T-cell CDC-XM was negative in all HIV-positive and HIV-negative individuals. In contrast, the proportions of positive B-cell CDC crossmatches and FC crossmatches were higher in HIV-positive individuals than in HIV-negative individuals (71% vs 4% for CDC-XM and 45% vs 2% for FC-XM). Moreover, the proportion of positive FC-XMs was greater in patients with detectable circulating HIV RNA (57%) than in those without (25%). The investigators considered that these positivity profiles might also be due to autoantibody production, antiretroviral therapy, and the activation of complement pathways by HIV disease. In contrast, another study found that HIV-infected patients produced positive T-cell FC-XM results with both pronase-treated CD4-positive and CD8-positive T cells but not with B cells. A significant reduction in T-cell FC-XM reactivity was observed for HIV-positive sera preadsorbed with pronase-treated T cells but not with untreated T cells. This strongly suggested that FC-XM reactivity was due to specific autoantibodies recognizing cryptic epitopes exposed by the pronase treatment of T cells.

**Interference by therapeutic antibodies and other treatments**
The therapeutic antibodies used to treat acute rejection or to desensitize patients (such as rituximab [an anti-CD20 antibody], daclizumab [anti-CD25], and alemtuzumab [anti-CD52]) can interfere with crossmatching assays. In a recent study, CDC-XM was performed after the addition of various concentrations of therapeutic antibodies (intravenous immunoglobulins, rituximab, basiliximab, eculizumab, and antithymocyte globulin) to negative and positive control sera. Rituximab and antithymocyte globulin were respectively associated with false-positive B-cell CDC crossmatches and false-positive T- and B-cell CDC crossmatches. The other 3 therapeutic antibodies were not associated with false-positive results (Table 2).

An unexpectedly positive B-cell CDC crossmatch (which was not due to the presence of rituximab in the recipient’s serum) was attributed to the presence of this monoclonal antibody at the surface of the donor’s B cells. Shortly before organ retrieval, the donor had been treated with rituximab because of severe idiopathic thrombocytopenic purpura.

In the context of rituximab therapy, CDC-XM positivity is restricted to B cells. False-positive CDC-XMs may be produced at low serum rituximab levels.

| Table 2. Interference By Therapeutic Antibodies During Crossmatching (XM) Procedures |
|---------------------------------|---------------------------------|-----------------|-----------------|
| **CDC-XM**                      | **FC-XM**                       | **Luminex-XM**  |
| Rituximab (anti-CD20)28         | Yes (B-cells)                   |                  |
| Daclizumab (anti-CD25)27        | Yes (a high dose of daclizumab  |                  |
|                                 | produces low-level interference |                  |
| Basiliximab (anti-CD25)        | No                             | No              |
| Alectuzumab (anti-CD52)27      | Yes (T- and B-cells)           | No              |
| Eculizumab (anti-C5)28         | No                             | No              |
| Antithymocyte globulin28       | Yes (T- and B-cells)           | No              |

Abbreviations: CDC, complement-dependent cytotoxicity; FC, flow cytometry; XM, crossmatching
concentrations (0.02 μg/mL), which can still be observed several months after the last infusion. Moreover, serum pretreatment with DTT reduced the rituximab-associated lysis.

The Fab fragment in rituximab is able to bind to the CD20 receptor found on the surface of most B cells. This rituximab-CD20 complex is not internalized; therefore, rituximab’s Fc fragment can generate functional immune effects, such as B-cell lysis after binding to a C1q fragment. Furthermore, rituximab is eliminated slowly from the circulation, with a half-life of between 20 and 30 days.31

It has also been shown that rituximab interferes with FC-XM, although the false-positive crossmatches disappear after pronase treatment of B cells.32 Several studies have shown that the sensitivity and specificity of B-cell crossmatches increase when nonspecific IgG binding to lymphocytes is reduced by pronase pretreatment. It has also been shown that rituximab’s CD20 target (which is structurally homologous to Fc receptors) is removed by pronase treatment. However, pronase treatment can occasionally induce a false-positive B-cell FC crossmatch33 or interfere with donor HLA expression; this will yield unreliable results.34

Before transplant, various pretreatments (such as Staphylococcus aureus protein A [SPA] immunoadsorption) are used to significantly reduce the blood level of DSAs in immunized patients. In 7 immunized patients initially presenting with negative T-cell CDC crossmatches, it was suspected that SPA having leached during the immunoadsorption step might cause a weak, positive T-cell CDC crossmatch before transplant.35 Indeed, the investigators detected leached SPA in one of the patient samples.

Conclusions

Virtual crossmatching enables transplant physicians to stratify risk in sensitized patients and thus a more accurate risk-benefit analysis for each prospective transplant procedure. The SAFB assay is a highly sensitive screening technique for anti-HLA antibodies, and there is usually a high level of agreement between V-XM and P-XM. However, physicians must understand the technical characteristics of each test if they are to correctly interpret any discrepancies. The reasons for unexpectedly positive P-XM results must be elucidated before transplant, to ensure efficient organ allocation and thus optimize patient outcomes.

References


