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Brief communication

Positive virtual crossmatch with negative flow crossmatch results in two cases

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ABSTRACT

Pre-transplant (Tx) presence of HLA antibodies (HLA-Ab) especially donor specific antibodies (DSA) has been correlated with post-Tx rejection. While crossmatch (XM) is the specific method to identify DSA, logistical reasons prevent performing a prospective XM in all transplants. In such cases DSA as identified by solid-phase assay (SPA) are being used to perform a virtual crossmatch (VXM). We present two cases, a heart-lung transplant and a kidney transplant, for which testing detected a presumptive DSA with discordant results: a negative flow cytometric crossmatch (FXM) and a positive VXM using SPA. The subsequent investigation determined the antibody, in both cases, was presumably directed against an epitope of a HLA-B*44 antigen found on the single antigen beads (SAB) used in the SPA but not against the native form on the donor lymphocytes used in the FXM. Manufacturing of SAB beads results in denaturation of epitopes, majority of which are removed from the final product, but residual amount is present on the final product. Denaturation of majority of antigen epitopes on single antigen beads did not remove the activity of the recipient's antibodies but it did diminish the activity of positive control serum. This indicates denaturation of some of the HLA-B*44 antigen during manufacturing of the SAB may have lead to the reactivity. Antibody mediated rejection does not appear to be associated with the titer of this antibody to denatured antigen in the first case and so clinical relevance of such antibodies is unclear. Subsequently a second case of discordant FXM and VXM was identified in a potential kidney transplant patient who went on to an uneventful transplant. In this case, lymphocytes from the donor were positively shown to express HLA-B*44:02 using known anti- HLA-B*44:02 control serum. Platelets identified as HLA-B*44:02 could adsorb the anti-HLA-B*44:02 from the control serum activity but not from that of the recipient's anti- HLA-B 44 antibody adding evidence that this antibody should best be classified as a false positive finding. The presence of such an antibody if misidentified may result in unnecessary therapy being instituted or the inappropriate denial of an organ for transplantation.

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Solid organ transplantation has become an important and viable method of treatment in a variety of illness of the kidney, liver, heart and lung. The number of potential recipients however far outstrips the available donors for various well-discussed reasons. The need to accurately allocate donor organs to recipients, allowing for the best outcome, is essential whether the outcome measure is survival of the recipient, graft survival or graft function. Various techniques have been developed to assess proper matching. Some of these techniques require a physical interaction with donor and recipient samples while others are done virtually. As adaptation of these techniques becomes more accepted, the potential causes of false positive and false negative reactions become even more vital.

Antibody mediated rejection is a well-accepted mechanism of rejection in renal transplants [1]. More recently, acute and chronic humoral rejection has started to gain more acceptance in heart transplants [2–4]. The specificity of the offending antibodies is, not surprisingly, usually directed against the mismatched donor major histocompatibility complex (Human Leukocyte Antigen system-HLA) [5]. As reviewed by Reed et al, other antigens such as major histocompatability class I-related chain A, vimentin, heat shock proteins, skeletal muscle and cardiac myosin are also potential targets in cardiac transplants [4]. These antibodies may be formed prior to transplantation or formed due to exposure to the transplanted organ itself.

While any HLA mismatch offers the potential for antibody formation and rejection, preformed antibodies offer both peril and warning. Peril since the pre-transplant (Tx) presence of donor specific HLA antibodies (HLA-Ab) has been correlated with post-Tx rejection in various solid organ transplants [4,6]. Warning because the recipient immune system has already demonstrated the ability to react against a donor antigen; thus allowing the transplant team to avoid potential rejection prior to the transplant by not selecting a particular organ. HLA-Abs in the recipient serum are detected by a variety of methods:

Abbreviations: CDC-PRA, Cytotoxicity based Panel Reactive Antibodies; DSA, Donor Specific Antibody; MFI, Mean Fluorescence Intensity; HLA, Human Leukocyte Antigen; HLA-Ab, HLA Antibody; SPA, Solid Phases Assay; Tx, Transplant; FXM, Flow Cytometric Crossmatch; VXM, Virtual Crossmatch; XM, Crossmatch.

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cytotoxicity based panel reactive antibodies (CDC-PRA), flow cytometric crossmatching (FXM) using donor lymphocytes and antibodies in the recipient serum, and by antigens on a solid phase (SPA). While FXM is a useful method to identify donor/recipient incompatibility, it may not be feasible to perform a prospective FXM in all transplants due to the lack of appropriate donor or recipient material. In such cases, DSA as identified by solid phase antigen (SPA) testing, are being used to perform a virtual crossmatch (VXM). VXM has been shown to correlate with FXM in the majority of the cases albeit with controversy surrounding how one determines clinically significant cutoff points [6]. In this report, we present two cases of discordant testing results: a negative FXM in the presence of DSA detected by SPA testing. The first case is that of a heart lung transplant recipient and the second case a potential living related kidney transplant recipient.

The methods used have been previously discussed and are briefly described here [6]. Flow Crossmatch: In this assay, donor cells are incubated with recipient sera. Recipient DSA adhere to donor cells and are detected by flow cytometry using a fluorochrome labeled secondary antibody against human IgG. A positive reaction at our institution is defined as an increase in the mean channel shift (MCS) of fluorescence above what is seen in the negative control which is MCS >52 for T cell-FXM and MCS>106 for B cell-FXM on a 1024 scale at our institution. Single Antigen Bead Assay: Purified HLA antigen coated color-coded microspheres (LABScreen ™ Single Antigen Beads, One Lambda, Canoga Park, CA/Single Antigen Beads, Tepnel Lifecodes/ Lifematch) are incubated with patient sera and reacted with a fluorescent tagged monoclonal anti-human secondary antibody. If present, anti- HLA antibodies are detected based on intensity of fluorescent emission from the bead and the secondary antibody using the Luminex® platform (Luminex Corporation, Austin, TX). Mean fluorescence intensity (MFI) defined as bead intensity − control bead intensity allows the SAB to be used as a semiquantitative test.

Complement dependent cytotoxicity panel reactive antibodies (CDC-PRA): PRA was determined by a CDC-AHG assay using a 60 well commercial T-lymphocyte frozen cell tray (SeraScreen FCT 60, Gentrak Inc, Liberty NC). This test is used for identifying HLA class I antibodies based on antibody specific and complement dependent disruption of the lymphocyte cell membrane. Denaturation of Proteins (Epitopes) on Single Antigen Beads (SAB): SAB were treated by acid treatment to denature the associated antigens using a technique suggested by the manufacturer. 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 2× with basic wash buffer and resuspend in PBS (One Lambda, Canoga Park, CA).

Adsorption using platelets: An equal volume of platelet rich plasma from several donors was pooled and centrifuged to produce a packed platelet button. After removal of the donor plasma, the pellet was reconstituted with an equal volume of patient serum. This tube was incubated for 30 min at room temperature using a tube rotator. The tube was then centrifuged at 4000g. for 1 min. The supernatant was taken off the tube which is the absorbed patient serum.

1. Case one

A 29 year-old multiparous female patient with a history of primary pulmonary hypertension and secondary right ventricular heart failure was listed for a combined heart and lung transplantation. After being at Status 1A for sixty days, she received an ABO blood group compatible, 3/6 mismatched HLA-A, B and DR combined heart and bilateral transplant from a 19 year old donor (Table 1). Her retrospective FXM was negative (T-cell FXM: MCS = 18, *positive cutoff: 52* and B-cell FXM: MCS = 56, *positive cutoff: 106*). The patient had undergone screening for HLA-Abs starting six years prior to the transplant by CDC-PRA which had always been negative. SAB testing showed various HLA class I and class II antibodies. SAB analysis demonstrated the presence of DSA to HLA-B44 with mean fluorescence intensity (MFI) of 6503 from the historical sample and an MFI = 13,486 from the current pretransplant serum. This testing was performed retrospectively after the transplant. Therefore, this situation represents a negative FXM with a positive VXM. While unusual, this contradictory scenario is possible and we conducted a detailed investigation of the following possibilities.

The SAB demonstrated antibodies to various HLA including to HLA-B44.

To rule out the possibility of errors during testing, FXM was repeated using the historical samples and also using splenic derived cells instead of peripheral blood; all results were negative. In addition, historical SAB profile was similar to the current SAB results.

To rule out a non-HLA antibody that is product or manufacturer specific, the SPA was performed using a different batch of SAB from the same manufacturer and using a product from a different manufacturer (Tepnel Single Antigen Bead, currently LSA Single Antigen, Gen-Probe Incorp, San Diego, CA) with similar results. Specifically, reactivity to HLA-B44 is still present.

After a discussion with the manufacturer, it was hypothesized these findings may result from antibodies to denatured HLA antigens. To test this hypothesis, we performed acid treatment of the beads to denature the majority of the antigens.

Acid treated SAB testing with positive control serum demonstrated a significant decrease in MFI values as compared to the regular untreated SAB for nearly all HLA specificities (Fig. 1). Control serum with anti-B44:02 specificity was also tested and showed loss of reactivity when the SAB were acid treated indicating that the HLA B44:02 antigen on the SAB was susceptible to acid treatment. Both the untreated SAB and the acid treated (denatured) SAB were tested with the patient's serum and demonstrated similar antibody profiles (Fig. 2). The antibodies therefore may show specificity to denatured antigen, which may have occurred during the manufacturing process of the SAB, or to epitopes not affected by the acid treatment. We favor the possibility of a denatured antigen being the targeted specificity of the antibody since an epitope not affected by the acid treatment, such as a linear one, would show crossreactivity with the FXM (if that linear epitope was exposed). The FXM test however was negative. Alternatively, the HLA B*44:02 epitope expressed on the beads may be due to some other manufacturer specific phenomenon during the production. The repeat testing, however, shows that the specificity is common in SAB from two manufacturers. The lack of FXM reactivity may be due to lack of expression of HLA B*44:02 on the donor cells. Unfortunately this theory could not be tested since no cells remained for testing from the deceased donor. (In case two, this theory was tested; see below).

One would also expect the MFI to increase if additional antigen epitopes became available by the denaturation process. That this did not occur might indicate there may be limiting amounts of antibody to the epitope in question or the susceptible epitope was also limiting in number.

Table 1
HLA typing of recipients and donors.

	Class I			
Recipient 1	A1	A2	B7	B62
Donor 1	A3	A2	B7	B44
Recipient 2	A1	A2	B8	B65
Donor 2	A2	A2	B65	B44
	Class II			
Recipient 1	DR4	DR4	DQ8	DQ8
Donor 1	DR4	DR15	DQ7	DQ6
Recipient 2	DR17	DR13	DQ2	DQ6
Donor 2	DR7	DR13	DQ2	DQ6

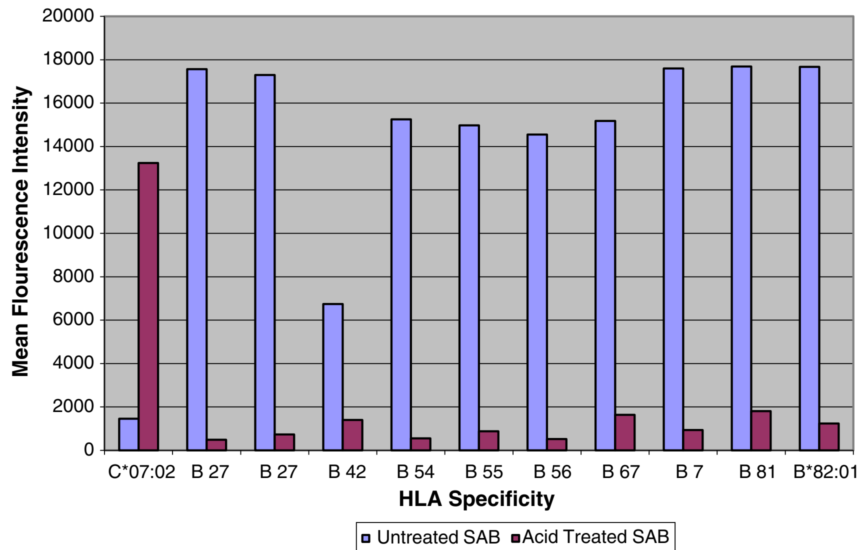

Fig. 1. Untreated and Treated SAB Assay with Control Serum. A marked decline in the fluorescence intensity for almost all of the specificities occurs when testing positive control serum using untreated and acid treated (denatured) SAB. Representative examples are displayed from one experiment.

In the 16 months since transplant, the patient has been hospitalized on multiple occasions- primarily for respiratory infections. This has required titering the patient's immunosuppression as appropriate for the infectious disease status. Cardiac biopsies were considered negative for rejection but did show endothelial swelling and C4d staining that was weak to moderate in intensity and numerous macrophages approximately 3 months after the transplant. This suggested antibody mediated rejection, although by this time the MFI for antibodies to HLA-B*44:02 antigens were only 3620, far lower than pretransplant levels. However, this might be explained by antibody binding to the graft and not being evident in the patient's plasma [7]. Steroid treatment and four therapeutic plasma exchanges were performed. Lung biopsies demonstrated no evidence of acute rejection and only focally positive C4d staining of the alveolar septa and endothelium 11 months after the transplant. At this time, suboptimal immunosuppression, due to another respiratory infection, was thought to be a contributing factor. The MFI with HLA-B*44:02 beads had climbed to 26,527, but since graft function was good clinically, no plasma exchanges were performed. Interestingly a cardiac biopsy performed two weeks later showed no evidence of rejection including negative C4d staining. The patient's course of treatment was complicated by multiple episodes of pneumonia and Pseudomonas bronchitis. Several lung biopsies did show loosely formed granulomas but were negative for organisms by various stains. Aspiration may have contributed to these findings. Approximately 17 months after transplant, lung biopsy did show “acute minimal rejection” (ISHLT grade A1, with no evidence of acute antibody mediated rejection evaluated by C4d staining. Admittedly, however, C4d staining may not be the most sensitive measure of antibody mediated rejection [8]). The patient's pulmonary status declined and she died approximately 22 months after transplant at an outside facility. No autopsy was performed. Table 2 shows the MFI of various antibodies during testing. The levels of the antibody did not correlate with the clinical scenario. In addition other specificities such as HLA B*45:01 are also seen to change over time.

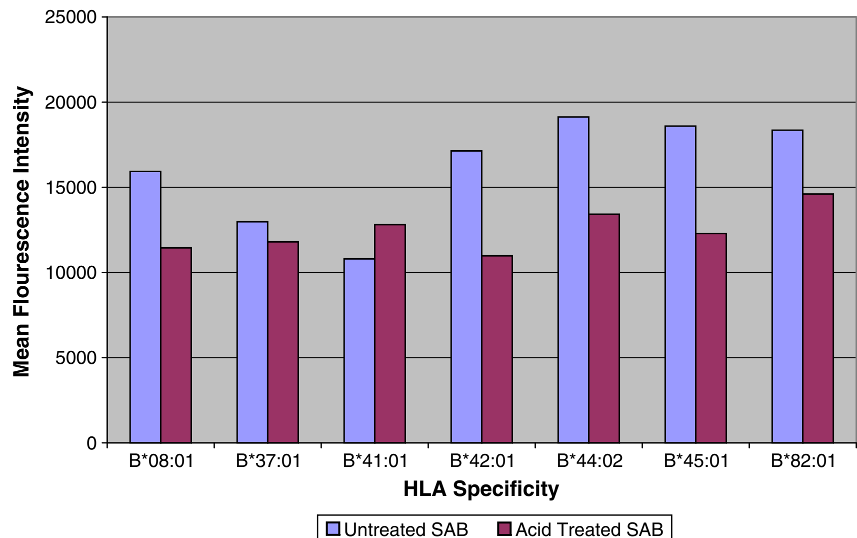

Fig. 2. Untreated and Treated SAB Assay with Recipient 1 Serum. The specificity and fluorescence remains basically unchanged before and after acid denaturation when testing with recipient serum in case one.

Table 2
SAB testing longitudinally of recipient 1 serum.

		MFI									
	Day (relative to transplant)	−832	−127	−1 *	1‡	3	51	99	340	361	399
Allele	B*44:02	5127	6503	13.486	4750	10.558	2.279	3620	26.527	5535	9776
	B*44:03								1944	810	504
	B*45:01	10.809	8739	9615	2693	6.915	8323	9707	19.429	1409	15.118

* Plasma exchange performed, ‡ day of transplant. On day 98, four additional plasma exchanges were performed.

2. Case two

As in the first case presented, the second case demonstrates discordant results between the FXM and SAB testing. The patient is a 64-year-old male with idiopathic membranous glomerulonephritis who at the time was being evaluated for possible living donor kidney transplantation. Testing of this recipient showed that he had formed an antibody to B*44:02 with a MFI of 7914. High resolution typing of his potential donors, his spouse and daughter, showed that they typed as B*44:02 (Table 1). As in our previous case, the FXM results using cells from both the spouse and daughter were negative. Denaturation of the HLA antigens on the SAB was again performed using the acid treatment protocol. Testing of the recipient's sample with the acid treated beads showed persistent reactivity to HLA B*44:02.

To show that the potential donor's cells expressed HLA B*44:02, two control samples with known HLA B*44:02 antibody specificity were used in FXM studies using the lymphocytes from the daughter. Both samples reacted positively. No other DSA were present. Unlike our initial case in which donor cells were not available for testing, this finding demonstrates the cells used in the FXM do express HLA B*44:02.

An adsorption was performed using platelets isolated from several donors that were positive for HLA-B*44:02. The adsorbate was then used in an SPA which showed the anti-B44:02 antibody was still present with a MFI of 8259. When an identical adsorption was performed using one of the control serums with known anti-B44:02 specificity, the MFI for B44:02 decreased from 8985 to 47 (Table 3).

Subsequently this individual was transplanted with a kidney from his daughter (2 antigen mismatch of 6). Although the follow-up period is too short for adequate evaluation, 4 months after transplantation he does not show signs of rejection.

The clinical significance of DSA in solid organ transplants has been well documented in some but not all solid organ transplant types. For heart transplants the importance of antibody mediated rejection is becoming more apparent [2,4,5]. The true clinical significance of the antibodies described in this report, detected only by SPA with a negative FXM, is not entirely clear. The antibody reactivity to specificity HLA-B 44 by SPA remained consistent despite acid treatment of the testing antigen in both cases presented. The antibodies may show specificity to denatured antigen or to epitopes not affected by the acid treatment. We favor the possibility of a denatured antigen being the targeted specificity of the antibody. In addition, antibody levels (MFI) do not correspond to clinical instances of antibody-mediated rejection as seen in the first case.

For these reasons, it seems unlikely that the anti-B 44 antibodies seen in our cases have clinical significance. What is of definite clinical relevance however is the impact such findings (negative FXM in the presence of a positive VXM) may have during the organ allocation procedure. Since the presence of a clinically relevant DSA might be concluded from a VXM using the SPA single antigen bead technology, a compatible organ may be passed over when in reality this may represent false positivity to a denatured antigen. Discordant results between the FXM and VXM using the SPA technology must be scrutinized to rule out the possibility of specificity to denatured rather than native antigen. The importance of such antibodies is unclear and warrants continued study as to their clinical relevance and origin.

Others have shown that the immune system can react to denatured antigens [9,10]. In addition it has been theorized that this may represent the specificity of some “natural” HLA antigen antibodies [11]. Virtual crossmatch is a clinically important tool in the armamentarium of the transplant team. Possible causes for false positive and false negative findings must be considered in all cases but particularly when discordant results are found [12]. We present the first reported cases of antibodies directed against the denatured form of an HLA antigen but not it's native form [13]. (Recently, another such case was presented) [14]. Importantly, this antibody did not correlate with rejection. It is curious that the same HLA antigen, B*44:02, is the specificity in both of our cases. One might theorize that the protein sequence of this antigen is particularly susceptible to denaturation or exposure of the epitope in question in vivo and that at least some individuals are capable of forming antibodies to this epitope. The presence of this antibody specificity in detectible amounts prior to the transplants would indicate that they were exposed to this epitope some time during their clinical course.

Table 3
SAB Testing Recipient 2 Serum Adsorbed with HLA B*44:02 Positive Platelets.

	MFI	
	Unmanipulated Serum	Adsorbed Serum with HLA B*44:02 Platelets
Recipient 2 Serum	7914	8259
Control anti-HLA B*44:02	8985	47

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