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Research article

Determination of unacceptable antigens by summation of anti-HLA eplet antibody strength (MFI) based on single antigen bead assays: Excellent correlation with negative cell based cross matches

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ABSTRACT

The reliability of single antigen bead (SAB) assays and their use in predicting a negative cell based cross match (CBXM) is essential in the era of expanded organ sharing. A wide range of accuracy (80–95%) in predicting negative CBXM has been reported. We hypothesized that in SAB assays an antibody against an HLA eplet that was common among a number of different HLA alleles would be distributed among all of the shared eplet positive SABs. This would reduce binding to the donor specific SAB resulting in an under-estimate of antibody strength. We tested this proposal in adsorption studies using, instead of lymphocytes, a novel reagent, single-SAB (sSAB). Properties of SAB assays were examined that provided a basis for conducting adsorption – elution experiments with the sSABs. We found that incubation of sera with sA*02:01 or sB*42:01 not only depleted reactivity to these alleles but also depleted reactivity to beads that shared the reactive eplet. Anti-eplet strength from SAB data (sum of the MFI of eplet positive SABs (MFI-s) was compared with CBXM out comes in two case studies and with 99 proficiency testing sera. In these validation studies, an MFI-s above 11,000 was associated with a positive FCXM. This approach was placed into clinical practice for listing unacceptable antigens that shared a common eplet. CDCXMs (n = 3261) and FCXMs (n = 1012) were performed on patients listed in UNOS for deceased donor kidneys. All CDCXMs were negative and all FCXMs except one were negative. We conclude that summation of eplet strength provides a highly reliable method of predicting prospective negative CBXMs resulting in substantial savings of time and effort. Based on shared eplet summation data, CMS/NYSDOH has accepted our bead based XM (BBXM) method (aka, virtual XM) performed prior to transplant as fulfilling the regulation that XM results be available before kidney transplantation.

1. Introduction

The use of optical bead microarrays and analysis by Luminex technology has revolutionized diagnostic and prognostic laboratory tests for anti-HLA antibodies [1–4]. This technology has been used primarily in three circumstances; pre transplant determination of unacceptable donor HLA antigens for listing with UNOS/UNet^{SM}, evaluation of potential recipients for anti-HLA antibodies when a deceased donor kidney becomes available (the virtual cross match) and monitoring recipients for the development of donor specific anti-HLA antibodies (DSA) after transplantation [5–10].

Abbreviations: BBXM, Bead Based XM; BCM, background corrected MFI; CDCXM, complement dependent cytotoxicity XM; DD, deceased donor; CMS, Centers for Medicare & Medicaid Services; DSA, donor specific antibody; ELISA, Enzyme Linked-Immuno-Sorbent Assay; FCXM, flow cytometry XM; HLA, human leukocyte antigen; MFI, Mean Fluorescent Intensity; MFI-s, MFI-score; MCS, Mean Channel Shift; NYSDOH, New York State Department of Health; SAB, single antigen bead; SABs, single antigen beads; sSAB, preparation with only one type of HLA allele bound to beads; PBS, phosphate buffered saline; UA, unacceptable antigens; XM, cross match.

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Unacceptable antigens (UA) are those which the transplant program believes will cause a positive cell based cross match (CBXM) due to preexisting anti-HLA antibodies when a donor with such HLA becomes available for kidney transplantation and will be detrimental to the survival of the allograft [2,8,11]. The identification of UA has not been standardized and has been a center specific process [1,8].

Prior to the advent of single HLA antigen bead (SAB) assays using a Luminex platform, identification of anti-HLA antibodies was typically performed by complement dependent cytotoxicity cross matches (CDCXM), flow cytometry cross matches (FCXM) and/or ELISA The former two tests utilize lymphocytes and the latter assay used cellular extracts bound to a solid support as targets [2,12–14]. Currently two companies provide reagents for SAB assays. Each vendor has indicated that these assays are intended to determine the specificity of anti-HLA antibody present in the serum of patients and is not for quantitative analysis of the level of anti-HLA antibody, i.e., strength determinations. Never-theless SAB assay/Luminex reports not only indicate the presence or absence of an antibody to a specific HLA allele but also provide a semi-quantitative strength indicator, the Mean Fluorescent Intensity (MFI) value for each bead. Most laboratories worldwide use the MFI values in some manner as an indicator of the strength of the antibody and provide an assessment to transplant clinicians. Such reports range from an indication that the antibody when present against a particular HLA allele is, for example, weak, moderate or strong, to providing raw MFI data or a modified MFI "score" (MFI-s). In most cases it is clear that however the level of antibody is categorized, nil to weak donor specific antibodies (DSA) typically do not result in positive cell based cross matches (CBXMs), whereas "strong DSA" typically gives positive CBXMs [9]. However, moderate levels of anti-HLA antibodies and infrequently DSA characterized as weak may also give unexpected positive CBXMs. Inconsistent results in the latter circumstances decrease the reliability of assessing UA and DSA when a deceased donor's HLA type becomes available. The shipping of organs across the country based on unreliable SAB results is problematic and of great concern [1]. A wide range of accuracy (80–95%) in predicting a negative CBXM has been reported. Continued improvements in predicting CBXM outcomes based on SAB analyses are therefore needed for more reliable assessment of HLA antibody strength to avoid unexpected positive XMs [1,2,8].

In consideration of this objective, we developed an analytic approach for assessment of anti-HLA antibody strength derived from SAB data for use in our kidney transplant program. The rational employed in this endeavor ultimately took advantage of a fundamental property of antibodies to HLA in that they are induced by and bind to short stretches of 15–22 amino acids within the HLA protein termed an epitope [15]. HLA epitopes initially were characterized by serologic methods and were found on more than one HLA, and therefore were described as public epitopes [16]. Subsequently, Fuller and Rodey categorized the serologic cross-reactivity of these public HLA class I specificities by placing them into 8 cross-reactive groups (CREGs) [17,18]. With the advent of SAB/Luminex technology and nucleotide sequencing of HLA the exact amino acid sequence of HLA could be determined collectively revealing > 100 shared class I epitopes and additionally HLA class II epitopes [19–22].

Within the epitope is an immuno-dominant region consisting of at least one to three polymorphic amino acids. [15,23,24]. The term "eplet" was coined by Rene Duquesnoy to refer to these amino acids, which are located within 3.0–3.5 Angstroms of each other on the molecular surface of HLA [15]. In this report we use the term eplet when referring to this polymorphic region and the term epitope when referring to the larger polypeptide that contains the eplet. We and others have hypothesized that in SAB assays an antibody against an HLA eplet that was common among a number of SABs would bind to all of these beads thereby reducing the amount of antibody bound to a donor specific SAB [2,25–28]. This situation would likely result in an under estimate of antibody strength if one considered just the donor specific SAB. This shared-eplet phenomenon has been observed to result in under representing antibody strength or completely missing the specificity due to binding of the antibody to competing targets on multiple antigen beads, effectively "diluting" the resulting MFI readout on each SAB [25,28]. This situation, we believed, could be overcome by assessment of anti-HLA antibody strength by summing the MFI of all potential donor eplet positive SABs.

We investigated the above hypothesis by using a novel adsorption tool; SAB manufactured with only one attached HLA allele (single-SAB, sSAB). These beads are used to construct the multiplex standard SAB kits. If the specific antibody was in fact distributed among all eplet positive beads then incubation of sera with such a bead would not only deplete reactivity to the sSAB used for adsorption but would also remove reactivity to all non DSA beads that shared the reactive eplet. Adsorption – elution strategies using cell lines expressing single recombinant HLA have been a standard method to characterize HLA epitopes but this approach has not been used to characterize antibody strength [19,20,29]. This report provides novel data on the properties of SAB assays that were useful in adaptation of standard adsorption – elution assays with cells to sSABs. These findindings provided the basis for development of an analytic method for assessing unacceptable antigens by anti-HLA eplet strenght and subsequently to place this method into clinical laboratory practice. Six years of data on > 3,000 cell based cross matches are presented.

2. Materials and methods

2.1. Laboratory Tests.

This study was approved by the SUNY Downstate Institutional Review and Safety Board (Protocol 341403-1). All histocompatibility tests were performed as previously described [27] in accordance with ASHI/CAP/UNOS/CMS standards. HLA typing was performed by standard serologic and DNA, Sequence Specific Oligonucleotide Probes and Sequence Specific Primers methods using commercial reagents supplied by the following vendors: One Lambda/Thermofisher, West Hills, CA Transtype, Frederick, MD, Biotest Diagnostics Corporation, Rockaway, NJ and Lifecodes/Immucor, Peachtree Corners, GA. Anti-HLA antibody tests were performed quarterly with screening beads and/or SABs on a Luminex platform (Lifecodes/Immuncor). Cross matches were performed by CDCXM on T cells (AMOS one wash) and by flow cytometry (FCXM) on T and B cells using a FACSORT Flow Cytometer, Becton Dickinson, Franklin Lakes, NJ) as previously described [27,30]. A FCXM is considered positive if the MCS above the negative control serum is > than two SD for T cells and 3 SD for B cells (15 cells tested with the negative AB sera).

2.2. Comparison of anti-HLA antibody strength based on the sum of potential donor specific SABs or the sum of potential donor specific eplet positive SABs.

Calculation of antibody strength was performed by either addition of the MFI of the donor specific HLA SABs or the donor specific eplet positive SABs. For example in the former case, A*02:01, 3000 MFI + B*57:01 4000 MFI = 7000 MFI-s). For example, in the latter case the donor specific eplet positive 62GE SABs are summed (A*02:01 3000 MFI + A*02:02 MFI 2800 + A*02:05 MFI 2700 + A*02:06 MFI 2500 + B*57:01 MFI 2500 + B*58:01 MFI

2400 = MFI-s 15,900). We use the HLA Epitope Registry to determine which eplet(s) may be responsible for the observed antibody reactivity, http://epregistry.ufpi.br/index/index [31]. We use the background adjusted MFI values from all antibody reactive eplet positive HLA - A, B, C, DR, DQ and DP loci presented on SABs for summation. In a nuanced approach that was empirically developed from 2008 to 2011, we reduce MFI values of eplet positive DRB3, 4, 5 by 8 fold and C locus antigens by 4 fold, respectively, to take into account low expression in tissues when listing patients' unacceptable antigens. This procedure gives more weight to high expression HLA loci e.g., as we would not wish eliminate B locus antigens with a total summed eplet of 5000 MFI-s for example, by addition of 6500 non adjusted MFI-s from the C locus (ie MFI-s > 11,000). In another example of a nuanced approach to listing unacceptable antigens; if antibodies were present to DR15 but not to DR16, DR 51 would not be entered as unacceptable even though the DR51 SAB was reactive since a non-reactive DR16 donor would not be considered for transplant if DR51 were listed as an AU. Our transplant team will not perform a kidney transplant across a B cell positive XM involving DQ or DP DSA as well as DR DSA based on outcome data (see reference [26]) so no adjustments in MFI are made to eplet reactive DQ and DP loci. Additionally, if antibody is present to C locus eplets only the MFI is not modified and the eplet positive alleles are listed as unacceptable since we have observed positive T and B cell FCXMs > 11,000 MFI-s for the C-locus, which are a contra indication to transplantation at our center. Empirically these and other nuanced approaches to eplet summation have been quite successful in predicting negative CBXMs (see Results and Discussion).

2.3. Adsorption of sera with sSAB and subsequent SAB analysis.

In preliminary studies the optimal amount and incubation time with SABs was determined as this information had apparently not been published. Serum was incubated for 2 h at room temperature with an optical bead preparation that had only one type of HLA SAB. The sSAB s used for adsorption in this study were sA*02:01 or sB*42:01 (supplied at no cost by Lifecodes). These beads are routinely mixed with the other sSAB to yield the SAB kits used in clinical laboratories to assess anti-HLA antibody reactivity. We note that the incubation time of the standard SAB assay is 30 min. The beads were centrifuged and the supernatant and bead pellet collected. The beads were then wash two times with phosphate buffered saline (PBS) by centrifugation and then incubated in a glycine buffer, pH 4.0 for 30 min. The pH 4.0 extract was dialyzed against PBS, pH 7.4. The adsorbed serum and the bead extract were each tested for the presence of anti-HLA antibodies in the standard SAB assay.

3. Results

3.1. Kinetics of antibody binding to SAB

In order to design experiments to assess anti-HLA eplet strength as it relates to CBXM outcomes it was necessary to determine the kinetics of antibody binding to SABs as published data on this matter was not found. The optimal incubation time was necessary for adsorption – elution experiments using s SABs. Sera from sensitized patients were reacted with SABs for increasing incubation times before the MFI was determined. Fig. 1 shows representative binding assays for highly reactive sera with different antibody specificities (>45 reactive SABs). The reaction typically goes to completion in about 90 min. We note that the level of antibody at 30 min, the vendor recommended assay time, is only about 55% of the maximum binding of antibody at 90 min for antibodies

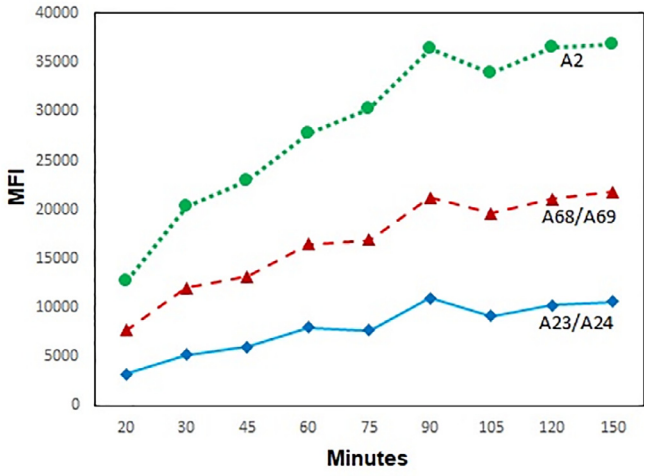

Fig. 1. Fig. 1.Kinetics of anti-HLA binding to SABs. SABs were incubated for the times indicated on the abscissa with serum from two different patients on the active list for a deceased donor (DD) kidney transplant (A and B). All other conditions for SAB analysis were performed in accordance with the manufacturer's instructions. Upper panel: Maximum MFI occurred in 90–120 min against the indicated SABs and are representative of 45 positive beads (>1500 MFI) in that serum. Maximum MFI occurred in 90–120 min against the A66 SAB (lower panel). In contrast, maximum binding of antibody to the B45 SAB occurred in 30 min. The serum in the lower panel had 55 positive SAB >2000 MFI.

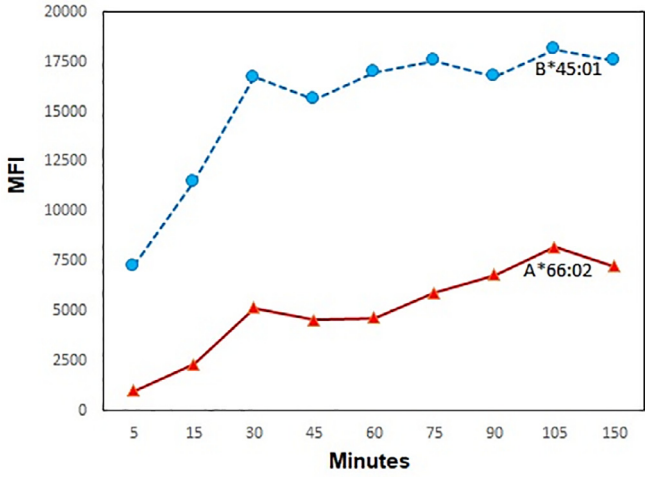

Fig. 1 (*continued*)

in this particular serum (Fig. 1 upper panel). In some sera the maximum MFI to a particular antigen was reached in only 30 min, whereas in the same serum the maximum MFI of an antibody to another antigen is not achieved until 90 min of incubation (Fig. 1 lower panel). The latter SAB MFI is 63% at the usual incubation time of 30 min similar to the binding experiment in Fig. 1 upper panel. These results are consistent with the understanding that binding of anti-HLA antibodies to SABs is an equilibrium reaction that goes to completion depending, in part, on the concentration and affinity of the antibody as well as other factors [32].

We examined this issue further by incubation of sera for 30 min or 120 min with SAB and then assayed the supernatant for reactivity with fresh beads in 30 and 120 min assays. Representative data are shown in Fig. 2. These experiments demonstrate that there is substantial HLA specific antibody remaining in the supernatant after 30 min incubation and at 120 min incubation though more antibody is bound to the beads when incubated for the longer per-

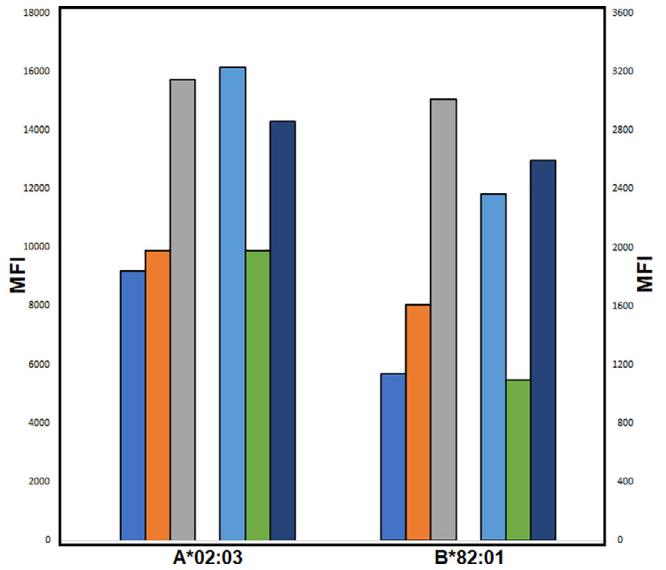

Fig. 2. Anti HLA antibodies partition between the reactive SAB and the serum. Two sera with specificity to A*02:03 and B*82:01, respectively, were used in this study. The sera were first incubated in the standard assay for 30 min with SABs (medium blue bars) or 120 min, light blue bars and the MFI determined. After centrifugation, the sera from the 30 min incubations were then analyzed with a new aliquot of SABs in the standard 30 min assay (orange bars) and for 120 min incubation (grey bars). The reactive sera from the 120 min SAB assay (light blue bars) also underwent centrifugation and then was analyzed with fresh SABs in the standard 30 min assay (green bars) and for 120 min (dark blue bars). Similar results were obtained with serum reactive to A*02:03 and B*82:01. These experiments demonstrate that 10–30 percent more of the reactivity as measured by the MFI value in the 30 min assay is present in the serum after the initial 30 min assay. (Compare the medium blue bars to the orange bars). However, when SABs and serum are first incubated for 120 min there is 46–62 percent less reactivity remaining in the incubated serum as measured by the MFI value in the 30 min assay (compare the light blue bars to the green bars). This finding likely occurs as the maximum binding of antibody to SABs is usually not achieved until 90–120 min of incubation (Fig. 1). These findings indicate that a substantial quantity of anti-HLA antibody remains in the serum after the standard SAB assay is performed. The amount of antibody that partitions between the serum and on the beads at equilibrium is likely a function of the antibody concentration, time of incubation and its affinity.

iod. Apparently, an equilibrium is reached between the bead - bound antibody and the antibody remaining in the sera as the reaction goes to completion. Importantly, the amount of SABs recommended for standard SAB assays was not sufficient to remove the majority of anti-HLA antibodies with low to moderate MFI values (1000–7500) from the sera. Therefore, the SABs in the standard kits are not useful for adsorption – elution experiments. Consequently we used sSAB where we could obtain higher concentrations of eplet specific beads.

3.2. Establishment of additional conditions for adsorption – elution of eplet specific anti-HLA antibody with sSAB

To assess whether antibody against specific eplets would be distributed over all SAB that had an antigen which expressed the eplet, we designed adsorption experiments using beads that had only a single attached HLA antigen (sSAB). These individual beads are routinely manufactured by the vendors to express single HLA alleles. They are then combined to yield the mixture of SABs for the assay kits. If antibody were distributed among all SABs that expressed a specific eplet then we reasoned that adsorption of serum with a sSAB containing the reactive eplet would, under the proper conditions, deplete the reactivity to all SABs that expressed that eplet. The amount of sSAB needed to deplete eplet specific antibody to sSAB A*02:01 and B*42:01is shown in Fig. 3 upper panel and 3 lower panel, respectively. Based on these experiments and proprietary information from the vendor we concluded that about a 30 fold excess of sSAB that are represented in the assay kit was needed to deplete substantial antibody reactivity from the serum.

3.3. Sera adsorbed with sSAB will remove reactivity to itself as well as other SABs that express the identical eplet

To investigate whether anti-HLA antibody directed against a particular eplet will be distributed among all eplet positive SABs in the bead set, we designed adsorption – elution experiments where sera was incubated with beads that had only one bound HLA allele expressing the reactive eplet (sSAB). If anti-eplet antibody is distributed among all eplet positive beads then incubation of serum with a sSAB that contained the eplet would not only deplete reactivity to the adsorption bead but would also deplete reactivity to all other SABs that contained the same eplet as the sSAB. Below we provide evidence that the DSA in SAB assays is in fact adsorbed by all beads that express the reactive eplet even though a bead(s) is not specific to the donor's HLA type. The serum studied in Fig. 4 contains antibodies directed to eplet 62GE (A*02, B*57, B*58) and to eplet 144TKH (A*02, A*68, A*69, respectively. We chose to use sSAB A*02:01since it expresses multiple eplets for adsorption. Two hours incubation with sSAB A*02:01 removed most of the reactivity (85%) in the serum to A*02:01, the other A2 SABs but also depleted reactivity to SAB B57, B58, A68 and A69 with which they share the above indicated common reactive eplets (Fig. 4 upper panel). Incubation of sera with reactivity to sSAB B*42:01, (likely eplet 65QIA) removed reactivity to 65QIA positive SABs (Fig. 5 upper panel). The anti-eplet reactivity was recovered from the sSABs, A*02:01 and B*42:01 after adsorption by elution in acidic buffer (Fig. 4 and Fig. 5, lower panels, respectively). Importantly, adsorption with non-eplet positive beads failed to remove most of the eplet specific reactivity.

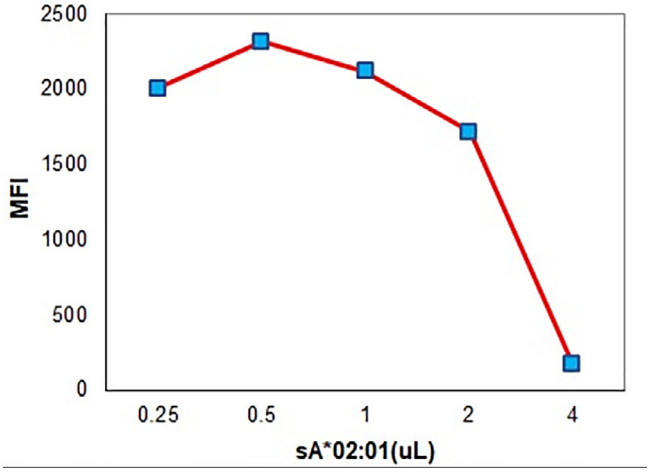

Fig. 3. Concentration dependent adsorption of anti-HLA antibody by sSAB. sSABs were obtained from Lifecodes/Immuncor without charge. The exact quantity of beads per µl was considered proprioritary by the vendor and was therefore not provided. However, we understood that 1ul of a particular sSAB allele was added to make up the SAB mixture for the standard multiplex assay. Increasing amounts of sSAB A*02:01 or B*42:01 were incubated for two hours at room temperature with 10 µl of serum (in 50 µl) containing the reactive antibody. The supernatants were then analyzed in the standard SAB assay. Antibody to A*02:01(upper panel) and antibody to B*42:01 (lower panel) was depleted from the patients serum by incubation with increasing amounts of sSAB A*02:01 or sSAB B*42:01, respectively as indicated on the abscissa.

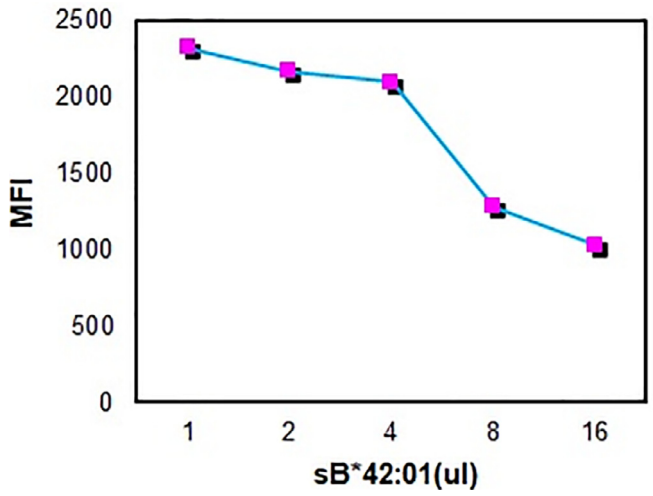

Fig. 3 (*continued*)

3.4. Anti-HLA antibody strength determined by the sum of antigen positive SABs: Correlation with FCXM results

We initially evaluated patients DSA with SAB MFI data in comparison to FCXM results (Fig. 6). If the DSA was against more than one antigen the MFI values of the highest bead in each antigen serologic group were added to give a net MFI score (MFI-s). For example, if the DSA reacted against A*11:01 at MFI 2300 and A*11:02 at 2450, the A*11:02 MFI value was used in the calculation along with the highest B7 SAB of 3680 MFI to give a combined MFI-s of 6130. Based on representative data shown in Fig. 6, an initial predicted positive FCXM cutoff of 11,000 MFI was selected with the understanding that 10,000–11, 000 MFI-s was a "grey area".

3.5. Comparison of SAB/eplet summation to DSA summation of patient samples at the time of deceased donor cross matches

SAB results were used for real time evaluation of two recipients for different deceased donor kidney transplants (Fig. 7). We note that these cases occurred before UA were listed based on eplet summation of SAB data. In the first case (Fig. 7 upper panel), the only donor reactive SAB for which there was detectable antibody was B42. The SAB, B*42:01 (bead 261) had a background subtracted MFI of 1,100, a value well under the cutoff of 11,000 MFI that our initial studies had indicated would be associated with a positive CBXM (Fig. 6). The T cell CDCXM was negative but the T and B cell FCXM were positive consistent with the understanding that FCXMs are more sensitive than non-augmented CDCXMs [2,13,27]. The question of how to account for the positive FCXM when the SAB assay showed such a weak level of DSA against the B*42:01 can be resolved by summation of the donor HLA eplet reactive SABs that share a common eplet. Examination of the pattern of the bead reactivity for the above patient suggested that the antibody was directed to an eplet(s) that was common to the donor specific SAB but was also reactive to non-DSA beads that shared eplets with the donor HLA allele. This included, B7, B27, B42, B54, B55, B56, B81, B82, eplet 65QIA and eplet 66AA, B7, B42, B57, B58 (Fig. 7A). In this situation, it appeared that a relatively low level of DSA was spread over 10 eplet positive beads that was sufficient in cumulative strength above 11,000 MFI-s that resulted in a positive FCXM (MFI-s 11,973). This conclusion was supported by experiments shown in Figs. 4 and 5, where incubation of donor reactive beads adsorbed antibody to non-donor SABs that expressed the eplet. In the above example, one may speculate that if there was only one eplet positive bead instead of 10 it would have an MFI of >11,000 not 1100 [25,26] but this is difficult to test as the vendor software is set to analyze all of the different allele specific SABs or it will fail to provide results.

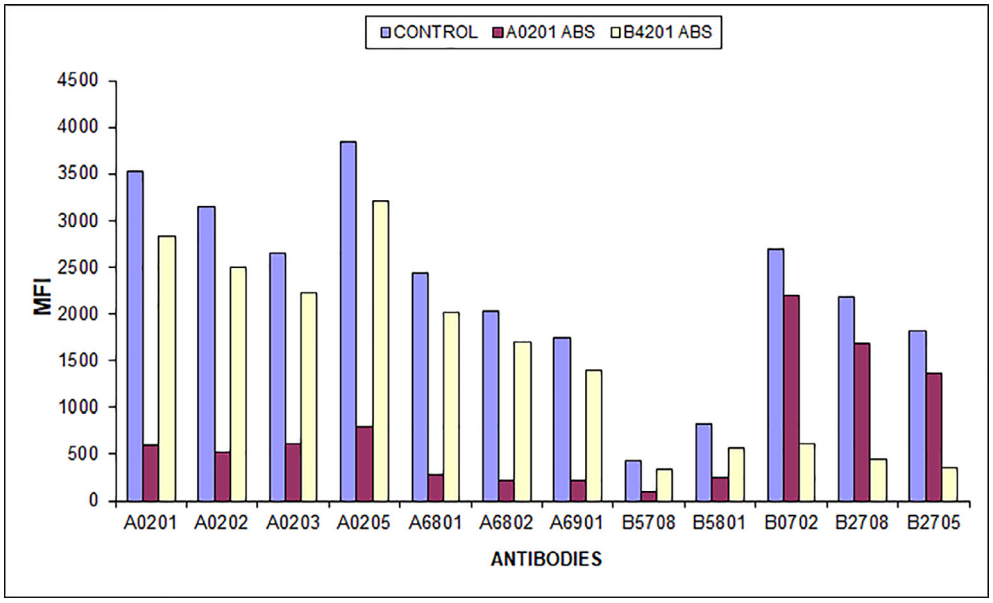

Fig. 4. Depletion of antibody to eplet 62GE and 144TKH by adsorption of serum with sSAB A*02:01. Serum was diluted 1:4 and 10 µl containing reactivity to 62GE, 144TKH and perhaps 62GK was incubated with 30 µl of sSAB A*02:01in a volume of 50 µl for two hours at room temperature. The adsorbed serum (upper panel) and the antibody eluted from the adsorbed sSAB (lower panel) were tested in the standard SAB assay. Results of pre adsorption serum with reactive SABs (eplets 62GE, 144TKH and 62GK) are shown by the blue bars in the upper panel. The red bars in the upper panel show reactivity against eplets 62GE, 144TKH after adsorption with sSAB A*02:01. The yellow bars in the upper panel show reactivity of serum incubated with sSAB B*42:01. Serum incubated with sSAB A*02:01 removed most of the reactivity (85%) in the serum reactive to A*02:01 and the other A2 SABs but also depleted reactivity to SABs, A68, A69, B57 and B58, with which they share the common reactive eplets, 144TKH and 62 GE. The anti-eplet reactivity is recovered from the sSAB A*02:01 after elution in acidic buffer (lower panel). Importantly, adsorption of this serum with the B*42:01 sSAB did not remove most of the reactivity to SABs specific for eplets 62GE and 144TKH (yellow bars) since B42 does not express either of these eplets. Adsorption of this serum with B*42:01 sSAB depleted reactivity to SABs that share an eplet found on B*07:02, B*27:05 and B*27:08. (For visual confirmation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

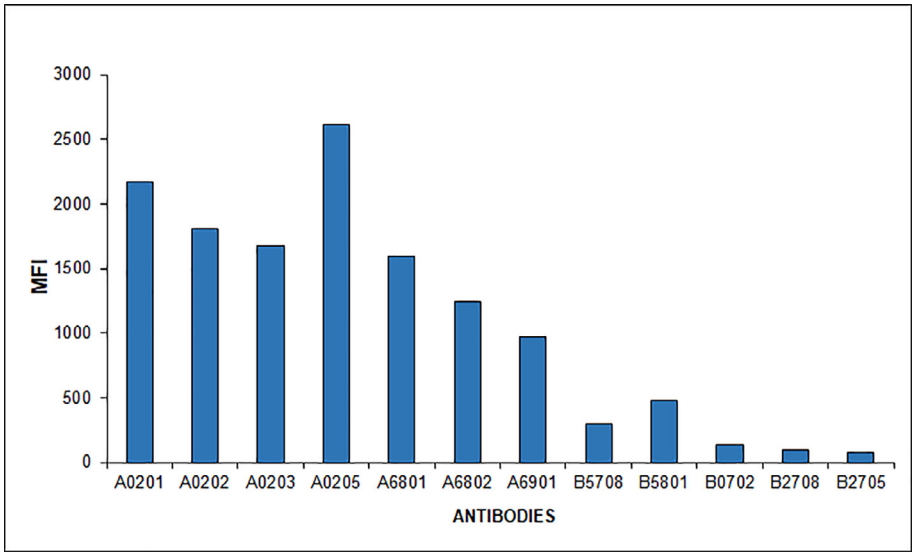

Fig. 4 (*continued*)

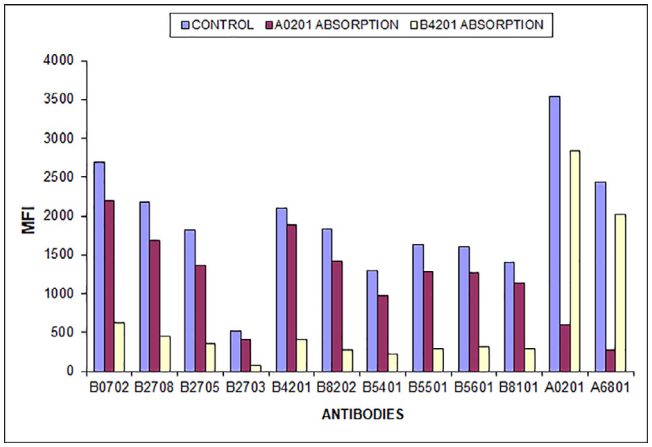

Fig. 5. Depletion of antibody to eplet 65QIA by incubation of serum with sSAB B*42:01. Serum was diluted 1:4 and 10 µl containing reactivity to eplet 65QIA was incubated with 30 µl of sSAB A*42:01in a volume of 50 µl for two hours at room temperature. The adsorbed serum (upper panel) and the antibody eluted from the adsorbed sSAB (lower panel) were tested in the standard SAB assay. Results of pre adsorption serum with reactive SABs (eplet 65QIA) are shown by the blue bars in the upper panel. The yellow bars in the upper panel show reactivity of serum adsorbed with sSAB A*42:01. The red bars in the upper show reactivity after incubation with sSAB A*02:01. The adsorbed serum (upper panel) and the antibody eluted from the adsorbed sSAB (lower panel) were subsequently tested in the standard SAB assay. Sera incubated with sSAB B*42:01 removed most of the reactivity (>80%) in the serum to B*42:01 but also depleted reactivity to other SABs with which they share the common reactive eplet 65QIA (yellow bars, upper panel). The reactivity was recovered (lower panel) from the sSAB B*42:01 as well as all other eplet 65QIA positive SABs after elution in acidic buffer (B, B*07:02 to B*81:01 shown on the abscissa). Importantly, adsorption of this serum with the A*02:01 sSAB did not remove most of the reactivity to SABs specific for eplet 65QIA (Fig. 4) since A*02:01 does not express this eplet. Incubation of this serum with sSAB B*42:01 did not remove reactivity to the 144TKH eplet positive beads A*02:01, A*68:01. (For visual confirmation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

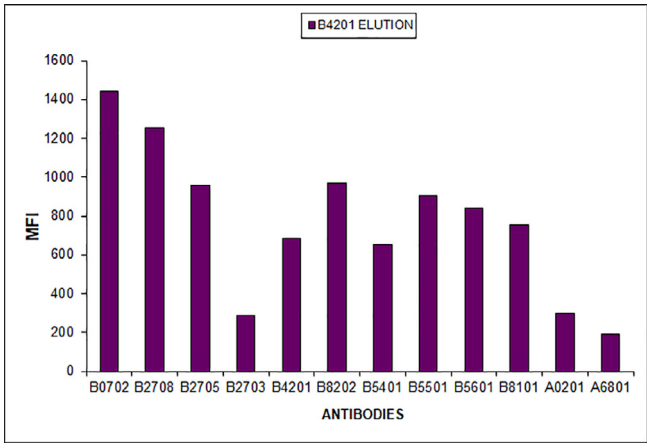

Fig. 5 (*continued*)

SAB data from a second case is shown in Fig. 7 lower panel. A Luminex SAB report is shown where the deceased donor cell HLA mismatch was HLA A*74:01 and B*45:01. The MFI-s for eplet summation included all beads with the donor eplet 66NV (n = 19 beads) and eplet 162GLS (n = 4 beads) was 33,176. In contrast, addition of the MFI values of the donor HLA SABs (HLA A*74:01 MFI 1,456, plus B*45:01, MFI 1,378) totaled 2,834 MFI-s well below our summed 11,000 cutoff. Laboratories using a cutoff of 3000 MFI for donor specific SABs would predict a negative cell based FCXM in the latter situation. The eplet/SAB calculation method correctly predicted a positive T and B cell FCXM.

3.6. Prediction of XM outcomes of proficiency testing sera based on the sum of the MFI of the antigen reactive SABs compared to the sum of the MFI of the eplet positive SABs

We subsequently analyzed a larger number of samples, tested in our laboratory, using HLA class I and Class II CAP proficiency testing sera for prediction of FCXM outcome based on SAB assays. We compared the two methods of predicting CBXMs, one by antigen summation of donor HLA reactive beads and the other by summation of donor eplet positive SABs (Fig. 8). A comparison of antigen calculated – SAB data to eplet calculated – SAB data associated with FCXM outcomes was performed in 65 HLA class I and 36 HLA class II proficiency testing sera from 2010 to 2017. We confirmed the HLA typing of the cells provided in the CAP surveys for XM tests. The sum of the MFI-s of eplet positive SABs (show by the red bars, Fig. 8 upper panel) correctly predicted positive T cell FCXMs in 58 of 59 sera (98.5%) of the HLA class 1 reactive sera (n = 58/59) and a negative FCXM in 6/6 sera when a cutoff of

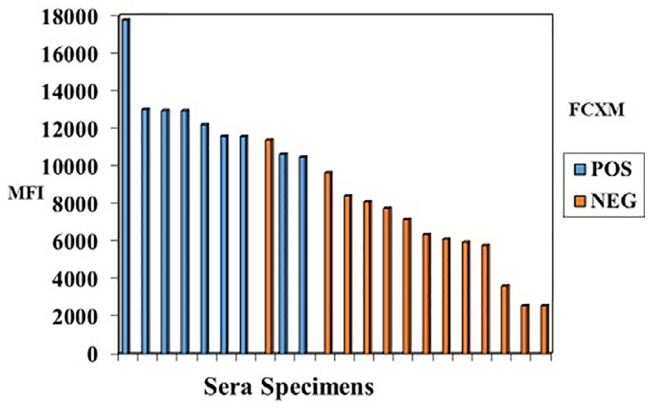

Fig. 6. Donor specific HLA SAB reactivity correlated with FCXM results. The total of the donor specific SAB MFI is shown on the y axis. If more than one reactive allele specific bead was present from a serologic group, the bead with the donor allele or if unknown the bead with the highest MFI value was used in the calculations. The MFI of donor reactive SABs from different serologic groups were added (e.g., A*02:01, MFI 2389, B*15:12, MFI, 3481 = 5870 MFI-s). The method of performing FCXMs is described in detail in a previous publication [27]. T cell FCXM negative results for each serum are shown in orange and positive XMs are shown in blue. In general the FCXMs were positive when the MFI was in the 10,000–11,000 range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

11,000 MFI-s was used. In contrast, calculation of the sum of the DSA positive SAB (shown in blue), only predicted a positive T cell FCXM in 47% (28 of 59) of the sera. Even if the cutoff for the sum of DSA calculations of positive SAB is decreased, the predictive value is not superior to the eplet based method. For example, prediction of a positive T cell FCXM is observed in 79.7% of the sera when the cutoff is adjusted to 5000 MFI, 84.7% when the cutoff is set to 2500 MFI and 93.2% if the cutoff is set at 1000 MFI. Comparison of the two methods of predicting positive FCXMs for sera with HLA class II reactivity is shown in Fig. 8 lower panel. Summation of the DSA SABs demonstrated 36.7, 70.0, 80.0 and 86.7 percent accurate prediction of a positive FCXM when the cutoffs are set at 11,000 MFI, 5000 MFI, 2500 MFI and 1000 MFI, respectively, whereas summation of the donor eplet positive SABs predicted 100% positive FCXM with an MFI cutoff of 11,000.

3.7. Use of SAB/eplet summation for determination of unacceptable antigens: prediction of negative cross matches

Based on the evaluation of proficiency testing specimens and patient samples, our center has successfully used donor eplet summation of SAB MFI data in clinical practice as demonstrated below. From 2012 to 2017 the epitope positive SAB MFI were summed to give an MFI-s of patients sera. Similar numbers of patients were cross matched on a yearly basis during this period (Table 1A).There were approximately 300–350 patients on our active list during this period of which about 35% were sensitized to HLA. All of the eplet positive antigens that had an MFI-s equal to and above 11,000 were listed as unacceptable in UNET so that most patients on a match run for a DD would have no DSA. Alleles that contained the positive eplet below 11,000 MFI-s were not listed as UA. However, at the time the laboratory was asked to evaluate recipients against a specific deceased donor, the eplet based MFI-s were provided to the transplant service for use in considering this deceased donor kidney for transplantation. A representative example of an initial eplet based prospective donor specific SAB report to the transplant team is shown in Fig. 9. This real time information allows the surgical team to accept a deceased donor kidney for transplantation even if the recipient has DSA, though at a level below that which would result in a positive XM. Note that the retrospective FCXM was negative as were the donor specific SABs of this patient.

Comparisons of eplet based MFI-s with 3012 CDCXM and 1012 FCXM were performed in 2012–2017 (Table 1). All patients during this period were XM by CDC whereas only patients likely to be offered the kidney were tested with a FCXM as well. Table 1B shows the expected negative CDCXM and FCXM results based on the actual SAB/HLA eplet analyses that were provided to the transplant service in the recipient selection period (Fig. 9). All XMs that were predicted to be negative by the SAB/eplet summation method were negative in the CDC T cell XMs. Remarkably, only one FCXM that was predicted to be negative by the SAB/eplet method was positive in a B cell FCXMs. In this case the MFI-s was 7,483 against an HLA class II eplet (less than the11,000 MFI-s cutoff). We considered that a high level of complement binding was blocking the secondary PE labeled antibody from binding to the IgG anti-donor HLA antibody attached to the SABs. On further analysis after heat treatment of the serum to inactivate complement (63 °C, 2 min) the sum of the eplet positive beads was 78,811 MFI-s, a value that would have predicted a positive FCXM. Subsequently all of the antigens with the reactive eplet were listed as unacceptable. Patients are now tested in SAB assays at room temperature and after treatment at 63 °C for 2 min. If the heated specimen gives an MFI-s greater than 2,500 above the non - heated specimen then further SAB tests are performed with heat treated serum.

4. Discussion

Adsorption – elution studies with cell lines that express single recombinant HLA alleles and theoretical investigations have identified numerous HLA eplets [15,19–21,33], thereby greatly expanding an understanding of the molecular basis of cross reactive groups [17,18]. The previous cellular adsorption studies do not, however, address properties of SAB assays as they relate to the kinetics and distribution of anti-eplet antibody binding, nor have they been used routinely to assess the strength of anti-HLA antibodies or predict CBXM outcomes. Experiments in the current report demonstrate properties of SAB assays that should be considered when evaluating the strength of anti-HLA antibodies in a patient's serum. In standard 30 min assays maximum binding of the antibody to the specific bead is typically not achieved but rather maximum binding is observed at 90–120 min incubation although maximum biding may occur in 30 min for highly reactive antibodies. Anti-HLA antibody partitions in a relatively equal amount between the reactive SABs and the serum in standard 30 min assays. This is an important factor since small differences in incubation times may have a large effect on observed MFI from run to run.

It is well known that incubation of sera containing anti-HLA antibodies with cells expressing the reactive HLA eplet will remove the antibody to all cells expressing the eplet [19,20]. This phenomenon has not been demonstrated in peer reviewed reports for SABs. The above referenced adsorption – elution studies were a starting point for performing SAB adsorption studies to test whether eplet specific antibodies were distributed among all eplet positive SABs. It was clear from these experiments that the incubation of SABs with sera should be for at least 90 min to obtain maximum binding in adsorption studies. Several experiments indicated that the number of beads in the standard SAB assay would be insufficient to adsorb >95% of eplet specific anti-HLA antibodies. However, available sSABs preparations would provide adequate amounts of eplet specific beads for adsorption studies. Adsorption – elution experiments demonstrated that antibody directed against specific HLA eplets when adsorbed with sSABs expressing the reactive eplet would not only remove antibody to the sSAB used for

Bead	AdjVal1	AdjVal2	AdjVal3	Assigned	Raw Val.	Class I Antigens			
207	5545.5	28.15	33.12	Positive	5906	A0201			
209	4116	20.89	22.71	Positive	4510	A0203			
230	4087	20.75	22.07	Positive	4421	A6801			
232	3207	16.28	18.5	Positive	3567	A6901			
231	2902	14.73	17.54	Positive	3303	A6802			
210	2515	12.77	26.6	Positive	2861	A0205			
208	2009	10.2	23.18	Positive	2394	A0202			
279	1652	7.31	8.12	Positive	1971		B7801		Bw6
236	1573	6.96	7.18	Positive	1827		B0702		Bw6
254	1420	6.28	6.68	Positive	1710		B3508		Bw6
253	1411	6.24	6.5	Positive	1717		B3501		Bw6
238	1372	6.07	6.13	Positive	1727		B0801		Bw6
272	1192	5.27	6.21	Positive	1457		B5301	Bw4	
252	1107	4.9	5.16	Positive	1465		B2708		Bw6
261	1099.5	4.87	5.07	Positive	1356		B4201		Bw6
244	1065	4.71	4.76	Positive	1310		B1503/72		Bw6
277	1057.5	4.68	6.32	Positive	1382		B5801	Bw4	
274	1047	4.63	4.88	Positive	1335		B5501		Bw6
251	1036	4.58	5.04	Positive	1412		B2705	Bw4	
249	1025	4.54	4.93	Positive	1450		B1801		Bw6
275	1021.5	4.52	5.08	Positive	1410		B5601		Bw6
276	1000	4.42	5.53	Positive	1328		B5701	Bw4	
280	763	3.38	4.33	Positive	1049		B8101		Bw6
242	709.5	3.14	3.17	Positive	1008		B1501/62		Bw6
270	702	3.11	4.09	Positive	985		B5101	Bw4	
243	680.5	3.01	3.14	Positive	1056		B1502/75		Bw6
245	523	2.31	2.41	Positive	815		B1512/76		Bw6
281	423	1.87	2.18	Negative	729		B8202		Bw6
255	393	1.74	2.48	Negative	690		B3701	Bw4	

Fig. 7. Comparison of two methods for predicting a CBXM result from SAB assays on evaluation of recipients for deceased donor kidney transplantation. Two representative cases are shown in the upper and lower panels . We did not initially list these antigens as unacceptable as we evaluated these patients before the establishment of the MFI-s cutoff of 11,000 using eplet summation. In the first case, B*42:01 was the only donor specific antibody that was detected (blue arrow). The net MFI of this bead was 1,011, far below the 11,000 MFI cutoff that we believed would predict a positive FCXM (see Fig. 6). However, the FCXM was positive. One explanation for this result was that the DSA was spread over 10 eplet positive SABs shared with the donor HLA, B*42:01. Examination of the pattern of the bead reactivity in the above patient's serum suggested that the antibody was directed to an eplet(s) that was common to the donor specific SAB but was also reactive to non-DSA beads that shared eplets with the donor HLA allele. This included eplet 65QIA, B7, B27, B42, B54, B55, B56, B81, B82, and eplet 66AA, B7, B42, B57, B58 (upper panel). In this situation, it appeared that a relatively low level of DSA was spread over 10 eplet positive beads that was sufficient in cumulative strength above 11,000 MFI-s that resulted in a positive FCXM (MFI-s 11,973). SAB data from a second case is presented in the lower panel. A Luminex SAB report is shown where the deceased donor cell was mismatched for HLA A*74:01 and B*45:01. The MFI-s for eplet summation was 33,176, including all beads with the donor eplet 66NV (n = 19 beads) and eplet 162GLS (n = 4 beads). In contrast, addition of the MFI values of the donor HLA SABs (HLA A*74:01 MFI 1,456, plus B*45:01, MFI 1,378) totaled 2,834 MFI-s. Laboratories using a cutoff of 3000 or less may predict a negative CBXM. The eplet/SAB calculation method correctly predicted a positive T and B cell FCXM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adsorption but would deplete reactivity to other beads which expressed the shared eplet. The anti-eplet antibody was recovered from the eplet positive sSAB by elution in acid buffer. Subsequently, We demonstrated with proficiency testing sera and sensitized patients' sera that assessing the reactivity of an antibody against a donor antigen bead would occasionally under estimate the prediction of a positive FCXM if the antibody was relatively weak and spread over a number of eplet positive beads. In contrast, eplet summation of SAB data correlated 100% with negative FCXMs in 99 proficiency testing sera.

These results support our original proposal and that of subsequent investigators that anti-eplet antibodies would be distributed among all SABs that contained the reactive eplet [2,25,27,28]. However, one may occasionally observe significant differences in MFI between SABs carrying the identical eplet. This situation may occur by a number of different mechanisms. Differential presentation of an attached antigen on one of these beads, which may occur in the manufacturing process, could alter the binding, i.e., reducing the MFI, of the anti-eplet antibody. Also, reduced MFI of an anti-eplet antibody binding to a non-immunizer eplet positive SAB could occur relative to the inducer allele due to alternative amino acid residues near the eplet changing its configuration [29,34]. Furthermore, binding of an anti-eplet antibody, for example, shared by two beads but binding of a second anti-eplet antibody to just one of those SABs could cause a substantial difference in the observed MFI of the two beads. There are several methods that are discussed below which we have used in an attempt to resolve the above situations.

While we wished to put this analytic approach using eplet quantitation of SAB data into clinical practice, we recognized a number of issues that could confound the application of this method as mentioned above. Highly sensitized patients present a

Sample ID: 529791 **Lot ID:** 03203F 02283A-SA1 **Donor Center #:** **Accessio**

Patient HLA Type: **Report**

Method Type: Standard Calculation

Positive MFI:	CON 1 MFI:	CON 2 MFI:	CON 3 MFI:	Analysis Mode:	MFI Value Cutoff:
16,198	123	495	267	Manual	

Bead	Bead Count	BCM	BCR	AD-BCR	Assign	Raw	Serology	A/B/Cw	Bw	Epitopes
226	73	4049	47.08	56.79	Positive	4326	A34(10)	***A*34:02***		66NV
281	73	3193	29.16	30.47	Positive	3487	Bw6	***B*82:02***	Bw6	162GLS
225	67	2877	33.45	38.67	Positive	3239	A33(19)	***A*33:03***		66NV
231	98	2823	32.83	37.47	Positive	3112	A68(28)	***A*68:01***		66NV
230	107	2461	28.62	36.92	Positive	3071	A66(10)	***A*66:02***		66NV
245	80	2446	22.34	24.05	Positive	2824	B76(15)	***B*15:12***	Bw6	163LG
232	91	2275	26.45	31.8	Positive	2729	A68(28)	***A*68:02***		66NV
233	97	2464	28.65	30.45	Positive	2713	A69(28)	***A*69:01***		66NV
224	71	2083	24.22	31.54	Positive	2658	A33(19)	***A*33:01***		66NV
229	74	2144	24.92	30.47	Positive	2468	A66(10)	***A*66:01***		66NV
213	92	1792	20.83	29.18	Positive	2028	A11	***A*11:01***		66NV
298	75	1091	5.74	11.58	Positive	1899		***C*17:01***		184R3
264	84	1378	12.59	16.43	Positive	1768	B45(12)	***B*45:01***	Bw6	162GLS
219	70	1279	14.87	18.45	Positive	1738	A26(10)	***A*26:01***		66NV
218	98	1354	15.74	21.89	Positive	1708	A25(10)	***A*25:01***	Bw4	66NV
214	96	963	11.2	16.07	Positive	1685	A11	***A*11:02***		66NV
234	60	1456	16.92	20.34	Positive	1660	A74(19)	***A*74:01***		66NV
212	87	1402	16.3	20.51	Positive	1650	A3	***A*03:01***		66NV
221	91	1381	16.06	17.84	Positive	1634	A30(19)	***A*30:01***		66NV
222	93	1095	12.73	14.81	Positive	1607	A31(19)	***A*31:01***		66NV
220	81	865	10.06	13.23	Positive	1302	A29(19)	***A*29:02***		66NV
262	94	664	6.07	14.08	Positive	1240	B44(12)	***B*44:02***	Bw4	162GLS
263	83	607	5.54	11.95	Positive	1141	B44(12)	***B*44:03***	Bw4	162GLS
223	83	814	9.47	13.58	Positive	1108	A32(19)	***A*32:01***	Bw4	66NV
228	85	605	7.03	11.06	Positive	1042	A43	***A*43:01***		66NV
291	69	309	1.63	2.06	Negative	985	Cw7	C*07:02		
299	96	562	2.96	3.52	Weak	943		C*18:01		
288	76	376	1.98	2.65	Negative	912	Cw5	C*05:01		
240	78	66	0.6	0.96	Negative	872	B64(14)	B*14:01	Bw6	

Fig. 7 (*continued*)

major problem in that it may be difficult to determine the identity of the reactive eplets. While the Immucor software lists possible eplets associated with positive SABs (a useful starting point) it is not programed to filter out recipient eplets and may not be up to date. Consequently, we always review possible eplets listed in the HLA Epitope Registry where such a filter can be used for this purpose (https://www.epregistry.com.br/). Furthermore, many sera have complex mixtures of anti-HLA antibodies where multiple anti-HLA eplet antibodies bind to many reactive SABs. In this circumstance antibody to more than one eplet is bound to reactive SABs, which may cause substantial differences in their MFI as mentioned in the above paragraph.

We have developed several approaches to address the latter situation. For example, if one wishes to calculate the strength of a donor specific eplet and there were more than the donor eplet listed on each of 10 SABs but an 11th bead had only the donor specific eplet, then the MFI of that bead would be multiplied by 11 to give an estimated MFI-s. If the value was above 11,000 MFI-s we would record all antigens with that eplet as unacceptable. If the MFI-s was below 11,000 the antigens expressing the eplet would not be recorded as unacceptable but would be reported to the transplant service when the patient was listed for a deceased donor kidney transplant (Fig. 9). In this circumstance the surgeon has the option to accept the kidney for transplantation after, for example, pretransplant plasmapheresis.

In other circumstances where a patient had a reactive A locus eplet of 7000 MFI-s for example and a B locus antigen of 5000 MFI-s, we would not record either of these antigens as unacceptable but would report the MFI-s of 12,000 at the time a deceased donor was under evaluation for this patient (indicating a likely positive FCXM). A typical report form is shown in Fig. 9. Another method of discerning the reactive eplet among multiple SABs is to perform a XM with a sentinel cell. This is a useful technique if there is a SAB that expresses one eplet that is part of one set of beads that would give an MFI-s >11,000 and a positive XM but if it was part of another eplet group that would give an MFI-s below 11,000 and therefore a negative XM. If the difference in the MFI-s for the two possible eplets in the above example would not give different XM results than one can perform an adsorption study with a sentinel cell or with sSABs if the latter should become an available reagent from the vendors. In this situation, the adsorbed serum would be tested in a SAB assay to determine which group of eplet positive beads had reduced MFI. Another circumstance that may reduce the observed strength of anti-HLA antibody is blocking of the secondary labelled antibody by complement thereby reducing the estimate of MFI-s strength. This issue can be addressed by heat treatment or addition of EDTA to inactivate complement [35].

In this report we demonstrate a highly effective method that uses eplet analysis and quantitation of SAB MFI data to predict the outcome of the XM against donor specific HLA. The use of this analytic technique has been highly effective in the identification of UA for listing in UNet and to advise the transplant service, at the time of the deceased donor match run, regarding the presence of donor specific anti-HLA antibody reactivity and the likelihood of

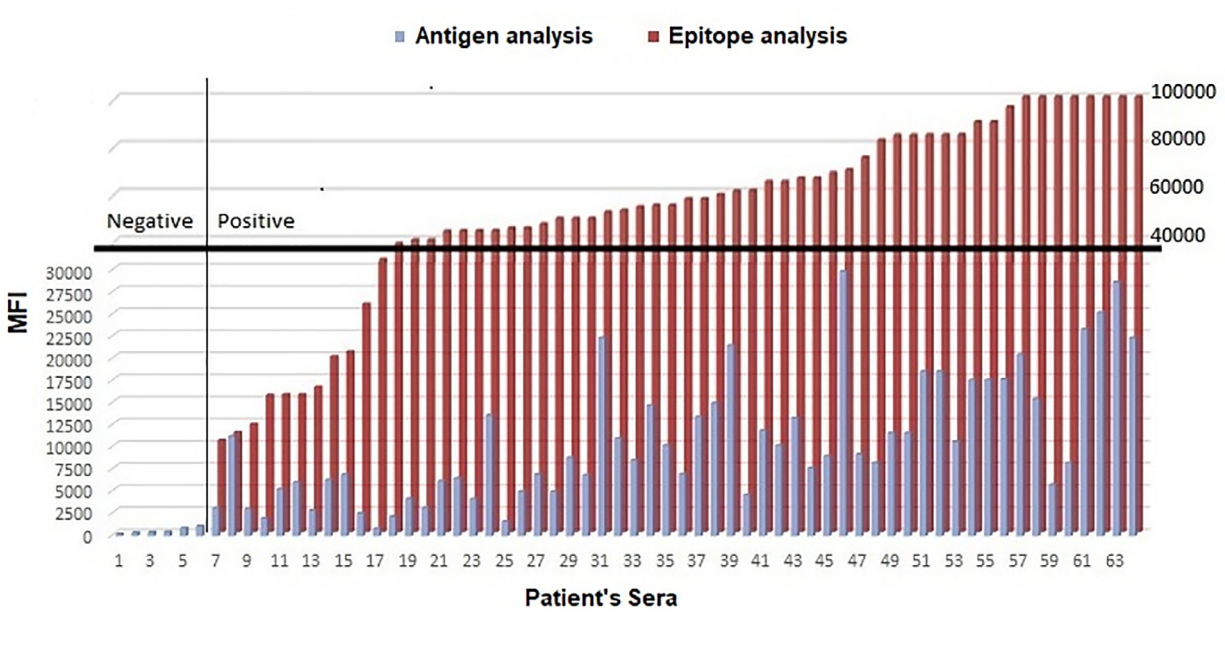

Fig. 8. Two methods of analyzing proficiency testing sera by SAB: comparison in predicting positive FCXM results. The results of SAB assays and the FCXM assays are those obtained by our laboratory and not the consensus results reported by the participating laboratories. Summation of donor HLA antigen positive SAB (blue bars) is compared to summation of the donor eplet positive SAB (MFI-s, red bars) for correlation with T and B cell FCXMs. FCXMs were performed as previously reported [27]. FCXMs were considered positive when the mean channel shift (MCS) of the patient's serum was 2 and 3 standard deviations above the control AB serum for T and B cells, respectively. This typically was a MCS of 100 for positive T cell FCXMs and a MCS of 120 for B cell FCXMs. The upper panel compares HLA class I SAB assays with FCXM results. The lower panel compares HLA class II SAB assays with FCXM results. CAP Proficiency Testing sera that had antibodies to HLA class II and not to HLA class I were provided by the survey. Sera to the left of the vertical line had negative FCXMs. Summation of the MFI of eplet positive SABs had a greater positive predicted value then summation of the MFI of the donor HLA reactive SABs. Our FCXM results were graded 100% correct according to the laboratory consensus. (For visual confirmation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

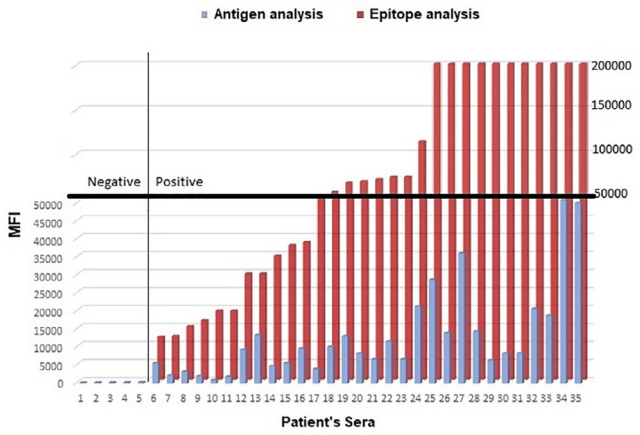

Fig. 8 (*continued*)

a negative XM. Nearly 100 percent correlation has been achieved, using an 11,000 MFI-s cutoff derived from SAB/eplet calculations, with negative CDCXMs and FCXMs. Studies presented in this report have used the SAB kits from Lifecode/Immucor. In principle, the method of assessing anti-HLA antibody strength using eplet quantitation should be applicable to the One Lambda/Thermofisher SAB kits as well, since the property of eplet sharing by HLA molecules is universal. We submit, therefore, that the results of the present investigation will also apply to One Lambda SAB eplet calculations, provided that the detected antibody reacts against a defined eplet on the native HLA trimer [2,36–40]. In this regard One Lambda

Table 1
Prospective CDCXM were performed against T cells. FCXM were performed against T and B cells of 506 recipients. All patients were XM by CDC whereas only patients likely to be offered the kidney were tested by a FCXM as well as a CDCXM. About 25% of our patients had >50% cPRA. There were 506 T cell FCXMs and 506 B cell XM performed concurrently on the same patients for a total number of 1012. (A) Cross matches performed on a yearly basis. An increase in the number of XMs occurred from 2012 to 2017 though UAs were listed by the eplet summation method. (B) Comparison of predicted negative XM outcomes by eplet calculation of SAB data. All T cell CDCXMs that were predicted to be negative by the SAB/eplet calculation method were negative. All but one FCXM that was predicted to be negative by the SAB/eplet calculation method was positive in a B cell FCXM. In this case the MFI-s was 7483 against an HLA class II epitope. We presumed that this discrepant result was due to a high level of complement binding to the donor eplet positive SAB. After heat treatment to inactive complement (63 °C, 2 min) the sum of the eplet positive beads was 78,811 MFI, a value that predicts a positive FCXM.

(A)

Year	T cell CDCXMs Performed	Recipient FCXMs Performed
2012	339	71
2013	380	47
2014	435	59
2015	598	102
2016	620	99
2017	889	128
Total	**3261**	**506**

(B)

Predicted Negative T Cell CDCXM	**Actual Negative T Cell CDCXM**
3261	**3261**
Predicted Negative T and B Cell FCXM	**Actual Negative T and B Cell FCXM**
1012	**1011**

SABs express denatured HLA class I and class II and may therefore give false positive or false negative reactions in some patient's serum [38–40]. The MFI-s cutoff established in our program has

Donor Specific Anti-HLA Antibody Report

DONOR MR#:	UNOS #:	TECH:	DATE:
XXX	AVV	PL	02/23/2021

				RESULTS*		T/B CELL FCXM	
RECIPIENT NAME	SEQ/RANK#	MRN#	Serum Date	Class I	Class II	Prospective	Retrospective
ABC	17/2	xx	xx	Neg	Neg		Neg
DEF	42/21	xx	xx	Neg	Neg		ND
HIJ	107/98	xx	xx	Neg	1728		ND
KLM	467/366	xx	xx	3678	Neg		ND
NPO**	778/744	xx	xx	Neg	22,555		ND
RST877	877/804	xx	xx	Neg	Neg		ND

* An MFI Score of more than 11,000 is likley to be FCXM Positive. The RESULTS of class I and Class II tests are based on data obtained from reacting the patient's serum with donor specific HLA bound to a solid support (Immucor-LSA, Luminex Platform).

COMMENTS:

**Patient just activated, UA not evaluated.

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Fig. 9. An example of a pretransplant report of a list of patients for evaluation against a deceased donor on request by the transplant service. Eplet positive antigens that had an MFI-s equal to or above 11,000 were listed as unacceptable in UNET and were therefore not on the match run list. SABs that contained a positive eplet that was below 11,000 MFI-s were not listed as unacceptable and therefore patients could be listed on the match run even if the donor HLA was positive for the eplet. However, at the time the laboratory was asked to evaluate recipients for a deceased donor, the MFI-s were provided to the transplant service for use in selecting recipients(s) to receive the kidney for transplantation. A representative example of a deceased donor specific SAB evaluation report is shown in this figure. FCXMs are now only performed retrospectively on patients that are designated to receive the kidney for transplantation as a quality assurance measure unless the surgical team or the laboratory director requests a prospective FCXM. The latter circumstance may occur for example, if the eplet is not clearly identified and the choice between eplets would result in one eplet above the 11,000 MFI-s cutoff and one eplet below the cutoff. Occasionally, a patient with an MFI-s above 11,000 is listed. This occurs when a patient is newly activated but has not been evaluated for UA.

proved efficacious and therefore has provided a basis for a quantitative approach to accessing UA. Clearly, differences in laboratory and transplant service practices warrant individualized validation studies for selection of center specific cut points based on HLA eplet quantitation. Though not formally performed in the current study, ROC analyses may be useful in establishing cutoffs for programs that wish to investigate the use eplet summation for selecting UA.

Fig. 9 of this report gives an example of a real time donor eplet specific bead based prospective XM with transplant service selected patients against a deceased donor. Also shown is a retrospective FCXM of the patient that received the deceased donor kidney. By federal regulation, 42 CFR 493.1278 f (2), kidney transplant programs are required to have available results of cross matches before the kidney is transplanted. This regulation has been interpreted that a donor – cell based XM must be performed in a timely manner and that a prospective virtual XM cannot be substituted for a CBXM. However, the preceding Centers for medicare & Medicaid Services (CMS) standard defines the types of XMs that satisfy the above regulation, 42 CFR 493.1278 e (2) (ii): "Crossmatching. The laboratory must do the following: (2) Have available and follow written criteria for the following: "(ii) The preparation of donor cells or cellular extracts (for example, solubilized antigens and nucleic acids), as applicable to the cross match technique(s) performed." As transmitted by email to A. Norin, from CMS/New York State Department of Health (NYSDOH), a "bead based cross match" (BBXM), is permissible under 42 CFR 493.1278 e (2) (ii) when an extract from cells contains HLA and the HLA is then covalently bound to individual beads by a manufacturer, i.e., SABs, and is mixed with a patient's serum.

In order to obtain this determination it was a requirement to provide these agencies with data showing a high degree of correlation of negative donor eplet reactive BBXM results compared to negative CBXM results. The data provided showed that our SAB-eplet based XM method had a very high degree of correlation (>99.9%) with CBXMs. Consequently, CMS/NYSDOH approved of retrospective BBXM as satisfying the above stated regulations (received by A. Norin on February 22, 2019 by email). Our BBXM has been reviewed subsequently during routine inspections by ASHI, CAP, and the NYSDOH and the laboratory recertified under this DD XM provision. Email documentation of this string of communications is available on request by appropriate individuals.

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Declaration of Competing Interest

The author declare that he have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data from this study is available on reasonable request to the corresponding author.

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