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Many *de novo* donor-specific antibodies recognize β_2-microglobulin-free, but not intact HLA heterodimers

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Abstract

Solid-phase single antigen bead (SAB) assays are standard of care for detection and identification of donor-specific antibody (DSA) in patients who receive solid organ transplantation (SOT). While several studies have documented the reproducibility and sensitivity of SAB testing for DSA, there are little data available concerning its specificity. This study describes the identification of antibodies to β_2-microglobulin-free human leukocyte antigen (β_2-m-fHLA) heavy chains on SAB arrays and provides a reassessment of the clinical relevance of DSA testing by this platform. Post-transplant sera from 55 patients who were positive for *de novo* donor-specific antibodies on a SAB solid-phase immunoassay were tested under denaturing conditions in order to identify antibodies reactive with β_2-m-fHLA or native HLA (nHLA). Antibodies to β_2-m-fHLA were present in nearly half of patients being monitored in the post-transplant period. The frequency of antibodies to β_2-m-fHLA was similar among DSA and HLA antigens that were irrelevant to the transplant (non-DSA). Among the seven patients with clinical or pathologic antibody-mediated rejection (AMR), none had antibodies to β_2-m-fHLA exclusively; thus, the clinical relevance of β_2-m-fHLA is unclear. Our data suggests that SAB testing produces false positive reactions due to the presence of β_2-m-fHLA and these can lead to inappropriate assignment of unacceptable antigens during transplant listing and possibly inaccurate identification of DSA in the post-transplant period.

Introduction

Laboratory testing for donor-specific antibody (DSA) is a common technique to monitor immune status after solid organ transplantation (SOT). Its use as a surveillance tool complementary to the histopathologic biopsy and clinical health of the allograft has been recommended by the International Society of Heart and Lung Transplantation (ISHLT) since a consensus conference in 2008 (1). It is also recommended as a screening tool for monitoring renal transplant recipients independent of risk for AMR (2).

A major advance in human leukocyte antigen (HLA) antibody identification was the introduction of single antigen bead (SAB) arrays (3). Not long after the introduction of these assays, there were reports of the presence of alloantibodies to both HLA class I and II in nonalloimmunized males (4–6). Many of these 'natural' antibodies were subsequently shown to be due to peptide and β_2-microglobulin-free class I heavy chain molecules present on the surface of beads (7, 8). These antibodies are reactive with epitopes accessible to binding only when the heavy chain is not associated with β_2-microglobulin. In other words, β_2-microglobulin-free HLA (β_2-m-fHLA) antigens on the surface of the bead produce positive reactions that would otherwise not be detected on beads that consisted entirely of native HLA (nHLA). We (9) and others (10, 11) have described the frequency of these antibodies and their potential impact on organ allocation in patients awaiting cardiac and renal transplants. In general, these antibodies do not produce positive crossmatches against viable donor cells, nor do they fix complement. Anecdotal reports of transplantation across the barrier of β_2-m-fHLA antibodies suggest that they may not be important to long-term graft survival (12–14). A recent study of the potential clinical relevance of pre-formed donor-specific class I antibodies to denatured HLA following renal transplantation found no increased incidence of AMR or reduced 5-year graft survival (15). Thus, the presence of the aforementioned antibodies may produce false positive virtual crossmatches and needlessly exclude recipients from organ allocation, and have questionable clinical significance after SOT.

As an extension of our work in identifying these antibodies, we questioned to what extent *de novo* DSA to donor antigens in the post-transplant period were due to recognition of nHLA

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or β_2-m-fHLA. The present research compares conventional SAB analysis with acid treated (denatured) SAB to quantify the frequency of nHLA or β_2-m-fHLA, respectively among patients with *de novo* DSA after SOT. We also provide a small data set for the specificity for either clinical or histopathologic AMR among patients with positive DSA with nHLA or β_2-m-fHLA within a subset of these patients

Materials and methods

This study was performed under the oversight of the Institutional Review Boards of Aurora Health Care and Avera McKennan Hospital and University System. Serum samples were collected from cardiac or renal transplant recipients who were at least 30 days post-transplant, and were obtained either under routine protocol surveillance or for-cause as indicated by decline in cardiac or renal function. In cases where multiple post-transplant samples were available, we chose the sample that showed most recent to biopsy, otherwise, the sample with the highest mean fluorescence intensity (MFI) on SAB was chosen. All patients were negative for DSA by SAB assay (<500 MFI as defined below) and had negative flow cytometry crossmatches at the time of transplantation. Renal transplant recipients received basiliximab as induction therapy and were maintained on tacrolimus, mycophenolic mofetil (MMF), and steroids. Higher risk renal recipients (previous graft loss, high panel-reactive antibody (PRA), and African-American) were given thymoglobulin induction. Cardiac transplant recipients received bolus solumedrol at transplant followed by prednisone taper over an 11-week period along with MMF and tacrolimus maintenance immunotherapy. Histopathologic criteria for AMR were from the ISHLT Consensus Conference (16) and 2007 Banff (17) for heart and kidney grafts, respectively.

All samples were obtained from transplanted patients with positive DSA based on HLA class I SAB. Class II DSA were not considered for analysis because in our hands, acid and/or heat treatment denatures the antigens to the extent that are no longer reactive with human serum (data not shown). Our centers consider samples positive for DSA when MFI is ≥500 for any single bead or the sum of beads within more broad serological specificities. Although the cutoff was not validated clinically, it is consistent with the range of MFIs related to kidney allograft failure reported by the Collaborative Transplant Study Report of Opelz and colleagues (18). Samples with negative control beads >300 MFI were treated with Serum Cleaner [LifeCodes, Stamford, CT (#628222)] according to the manufacturer's instructions and were spiked with fetal bovine serum (4% v/v) during incubation with beads. The HLA class I SAB arrays were purchased from Thermo-Fisher One Lambda (Canoga Park, CA) and used according to the manufacturer's instructions. Data were obtained on a Luminex© 200 instrument and analyzed with FUSION (v 3.1) software. Reactivity to β_2-m-fHLA was determined by testing on beads that were denatured by low pH. Briefly, 2.5 μl of class I SAB beads were treated with 50 μl Pierce IgG Elution Buffer pH 2.4 (#21004, Rockford, IL) for 10 min on a rotator, and the reactions were neutralized by the addition of 5 μl 1 M Tris, pH 9. Denatured beads were washed with PBS containing 2% bovine serum albumin and then blocked in the same solution for 30 min at room temperature. The beads were pelleted by centrifugation then used according to the manufacturer's instructions.

Antibodies were defined as reactive to β_2-m-fHLA when the MFI of acid-treated beads was ≥75% of the untreated bead MFI. Antibodies were defined as reactive to nHLA when the MFI of acid-treated beads was ≤40% of the untreated bead MFI. Antibodies were reported as mixed when the MFI of the acid-treated beads was between 40% and 75% of the untreated bead MFI, indicating reactivity with both β_2-m-fHLA and nHLA for the same antigen. Recipients with multiple DSA falling into more than one antibody category (β_2-m-fHLA, nHLA, or mixed HLA), were classified as multiple DSA. These were arbitrary assignments designed to provide a conservative estimate of the incidence of β_2-m-fHLA antibodies.

Selected samples from candidates for antibodies that were predominantly reactive with β_2-m-fHLA were tested by flow cytometry T- and B-cell crossmatch as previously described (9). Flow crossmatches were considered negative when channel shifts were ≤63 for T cells and ≤108 for B cells. These cutoffs represent three standard deviations from median channel fluorescence of a negative control serum validated for use in our clinical practice. Serum was considered positive for HLA class I only when supported by positive reactions for both T and B cells. In general, the flow crossmatch gives positive results on samples that provide 1000–2000 MFI on the SAB platform.

A one-way analysis of variance (ANOVA) test was used for comparison of differences in median values, and the chi-square or Fisher's exact statistic was used for comparison of proportions. Statistics were run on SIGMASTAT software (Systat Software Inc., San Jose, CA) for Windows Version 11.0 and on http://vassarstats.net/fisher2x3.html.

Results

The HLA antigens that coat HLA class I single antigen Luminex© beads are a mixture of both native and β_2-microglobulin-free molecules. Figure 1 illustrates the staining of SAB with the mouse monoclonal antibodies W6/32 and HC-10 that recognize intact (β_2-microglobulin containing) and β_2-microglobulin-free class I HLA molecules, respectively. All class I HLA specificities represented on SAB are recognized by the W6/32 antibody, whereas only those alleles previously identified to contain the HC-10 epitope (19, 20) were reactive with the HC-10 antibody. SAB staining of acid-treated beads are completely nonreactive with W6/32 antibody to nHLA with the exception of a small amount of residual activity to a single bead carrying HLA-Cw12. These data confirm that acid elution of SAB completely converts nHLA to β_2-m-free forms. In addition, acid-treated beads show a complete absence of

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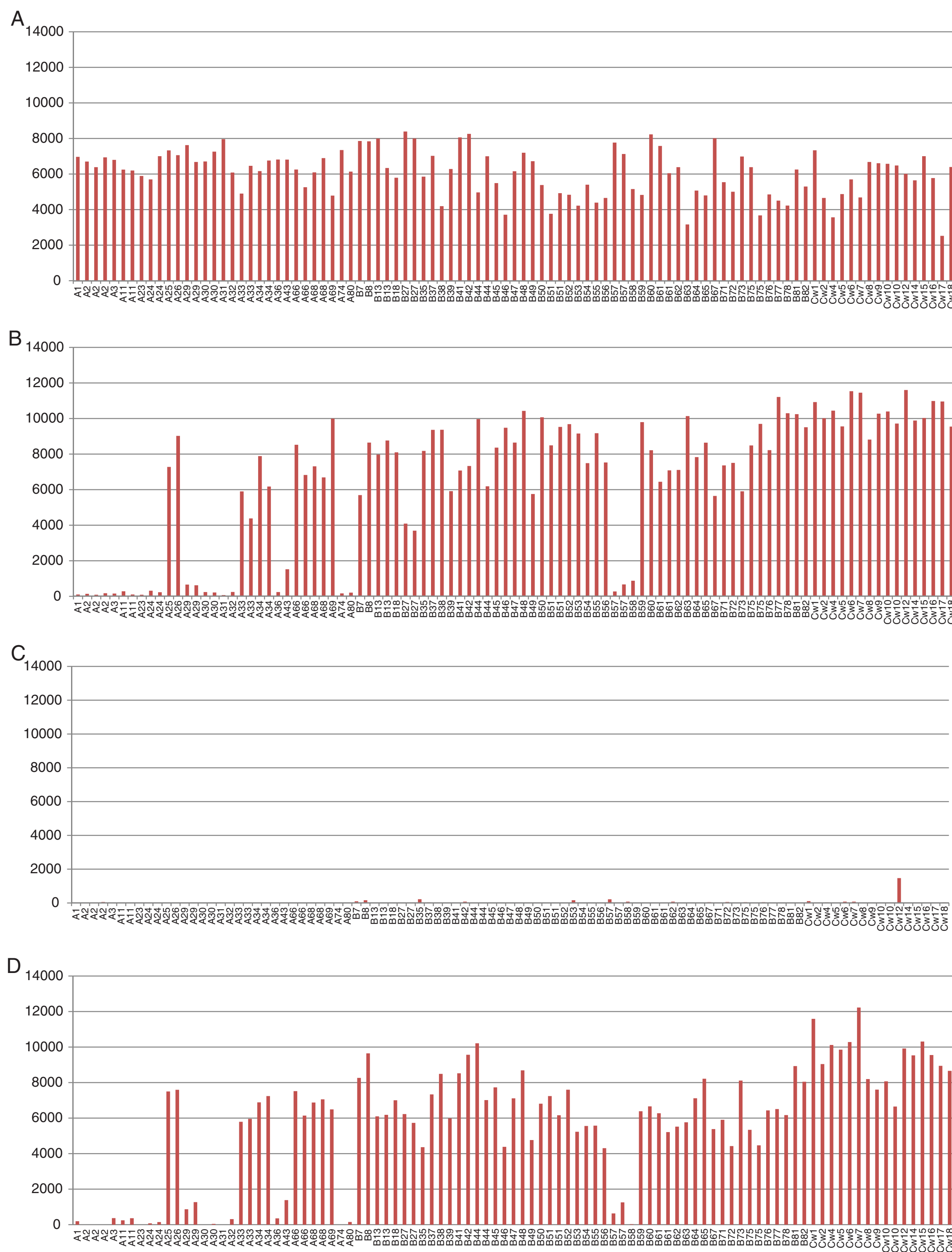

Figure 1 Binding of HC-10 and W6/32 antibodies to untreated and acid-eluted human leukocyte antigen (HLA) class I single antigen bead (SAB). (A) W6/32 antibody [reactive with native HLA (nHLA)]. (B) HC-10 antibody [reactive with β2-microglobulin-free HLA (β2-m-fHLA)]. Untreated beads were first reacted with 1 μg/ml monoclonal antibody to W6/32 (a gift of Dr Rico Buchli) or HC-10 [Nordic MUBio (Susteren, NL), MUB2037P], washed extensively, reacted with PE-labeled anti-mouse IgG [Jackson ImmunoResearch (West Grove, PA), 115-116-146] and then analyzed as described in *Materials and methods*. (C) Acid treated beads stained with W6/32 monoclonal antibody; (D) Acid-treated beads stained with HC-10 monoclonal antibody. The histograms shown are from single concentrations (1 μg/ml) obtained from a dilution series of each antibody, and were selected to assure that none of the beads reached saturated MFI. Notice the near complete absence of the native HLA (W6/32 reactivity) on acid treated beads.

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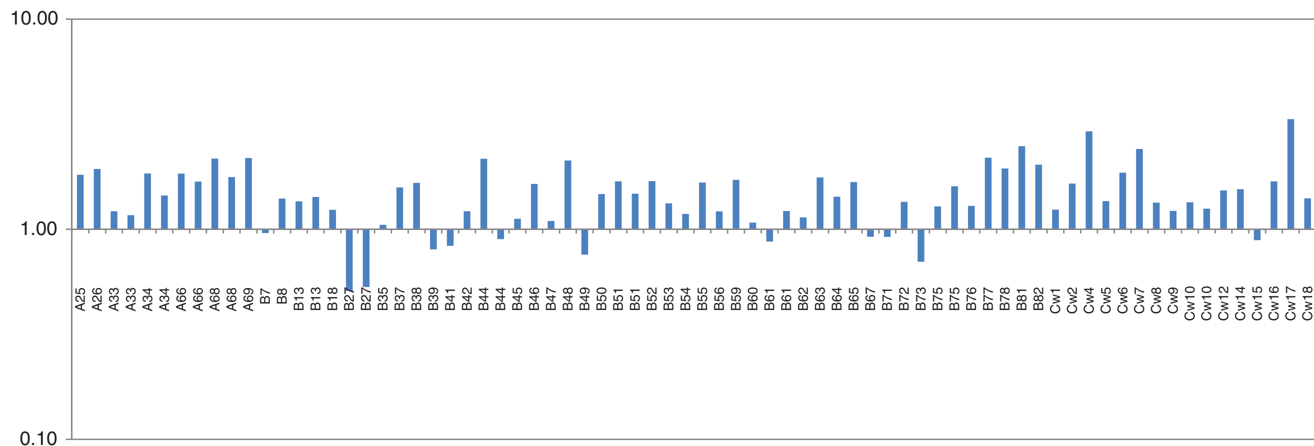

Figure 2 Ratio of HC-10:W6/32 MFI on different human leukocyte antigen (HLA) specificities on single antigen bead (SAB). The histograms shown are from single concentrations (1 μg/ml) obtained from a dilution series of each antibody, and were selected to assure that none of the beads reached saturated MFI. MFI obtained are at near 50% maximal signal and represent the average of three independent experiments. Only specificities that are known to be reactive with HC-10 are shown.

Figure 3 Single antigen bead (SAB) histograms of a serum sample with reactivity to both native and β-2-microglobulin-free human leukocyte antigen (β2-m-fHLA) epitopes. (A) Serum tested on standard SAB. (B) Same serum sample run on denatured SAB. Circles (○) represent DSA; squares (□) represent non-DSA.

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staining with a monoclonal antibody to β_2-microglobulin (not shown) further validating the effectiveness of the denaturation procedure. Figure 2 shows estimates of each species obtained by a titration of each monoclonal antibody. The data clearly illustrate that for each antigen tested, β_2-m-fHLA is present at significant amounts, and in many cases is the predominant species of HLA molecule on SAB. When expressed as a ratio of MFI obtained at sub-saturating levels of antibodies, the majority of HLA antigens show greater reactivity with the antibody that recognizes the β_2-m-fHLA form. We obtained qualitatively similar data with Luminex© class I microarrays purchased from a different vendor (LifeCodes/Immucor; data not shown). Thus, our observations are more likely due to inherent properties of HLA class I antigens, and not related to a specific vendors manufacturing process.

Having established that SAB contain both forms of HLA, serum samples from patients were tested on SAB under both standard and denaturing conditions. An example of data output obtained from a patient serum sample run under both standard and denatured conditions is shown in Figure 3. When run on SAB under standard conditions (Figure 3A), this serum shows antibody reactivity to DSA for HLA-A24 and HLA-B37, and non-DSA to HLA-A23, HLA-B44 and HLA-B45. Treatment of SAB with low pH (Figure 3B) resulted in a marked reduction in reactivity to HLA-A24, whereas reactivity to HLA-B37 was augmented. This reaction pattern confirms the presence of DSA to nHLA-A24 and β_2-m-fHLA-B37. The non-DSA in this example (HLA-A23, HLA-B44, and HLA-B45) all recognize nHLA as their reactivity is markedly diminished upon denaturation of SAB.

The distribution of patients identified with anti-nHLA and/or anti-β_2-m-fHLA is shown in Figure 4. Of the 55 patients tested, 29 had only anti-nHLA, 8 had only anti-β_2-m-fHLA, and the remaining 18 patients had mixed (both populations present) or multiple DSA. The frequency of antibodies exclusively to β_2-m-fHLA was similar among heart (17%) and renal (13%) allograft recipients. Table 1 shows the specificities of the DSA observed in the complete data set. The more common specificities of antibodies to β_2-m-fHLA included HLA-A1, HLA-B7, and HLA-B8, but there were no clear-cut associations between HLA antigens and whether or not the antibody reactivity was primarily reactive to β_2-m-fHLA or nHLA.

In order to further substantiate the reactivity of antibodies to β_2-m-fHLA and not nHLA, we performed flow cytometry crossmatches against viable lymphocytes from healthy volunteers ($n = 11$), which presumably express nHLA molecules as a majority. None of the antibodies interrogated yielded positive class I crossmatches (Table 2). The data substantiate that the antibodies identified as reactive to β_2-m-fHLA on denatured SAB are nonreactive with nHLA molecules. Selected β_2-m-fHLA antibodies ($n = 8$) were also tested for their ability to be blocked by pre-incubation of denatured beads with the HC-10 monoclonal antibody, which reacts with β_2-microglobulin-free heavy chains of many of the HLA-B and

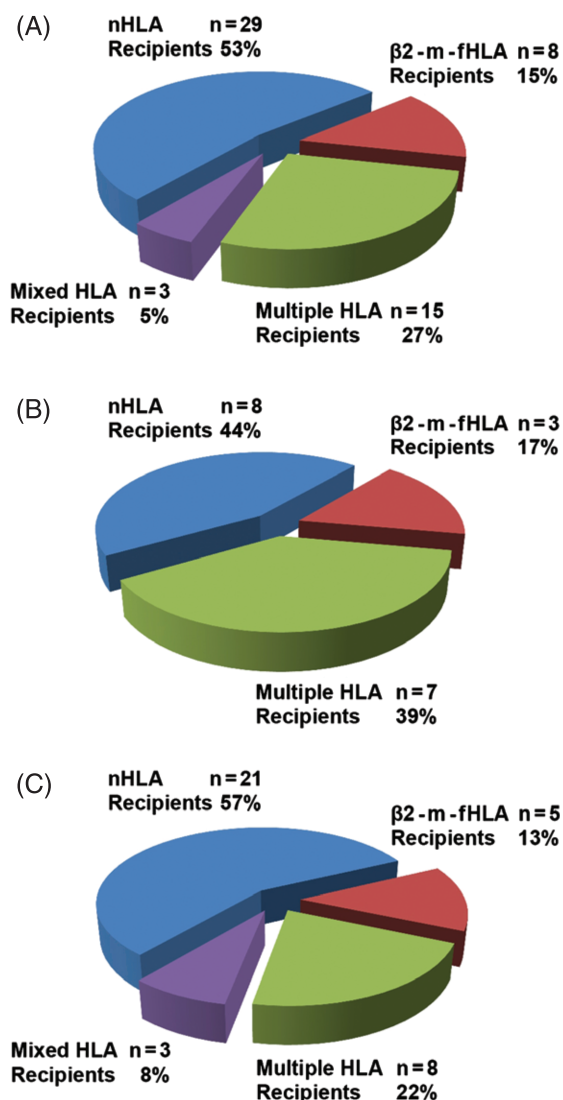

Figure 4 Distribution of recipients expressing *de novo* human leukocyte antigen (HLA) class I DSA. Recipients were classified by exclusive expression of donor-specific antibody (DSA) to β2-microglobulin-free HLA (β2-m-fHLA, red wedges), exclusive expression of DSA to native HLA (nHLA, blue wedges), expression DSA to a mixture of epitopes (mixed HLA recipients, purple wedges), or expression of multiple DSA to β2-m-fHLA, nHLA and/or mixed HLA (multiple HLA recipients, green wedges). (A) Distribution of DSA identified in all study recipients. (B) Distribution of DSA identified in heart recipients. (C) Distribution of DSA identified in kidney recipients.

HLA-C gene products. In most cases (seven of eight), HC-10 completely blocked binding of the β_2-microglobulin-free antigens in question (data not shown), further supporting the specificity of these antibodies for β_2-m-fHLA.

Table 3 shows the breakdown of demographic and clinical data of patients with and without antibodies to β_2-m-fHLA. There were no statistically significant associations with age, sex, type of organ transplant, or objective function (ejection

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Table 1 Types of DSA in cardiac and renal transplant recipients[a]

Patient ID	Graft	β_2-m-fHLA specificity[b]	nHLA specificity[c]	Mixed HLA specificity[d]
1	Heart	None	A68 (592)	None
2	Heart	None	B44 (1951)	None
3	Heart	B8 (514)	None	None
4	Heart	B37 (2345)	A24 (4900)	B8 (620)
5	Heart	None	A3 (3844), A11 (7170)	B27 (11,832)
6	Kidney	None	A2 (15,580)	A36 (8496)
7	Heart	None	B57 (1601)	B7 (11,005)
8	Heart	None	A3 (1941), B62 (1246)	A1 (3630)
9	Kidney	None	A2 (961)	None
10	Kidney	None	None	A11 (4328), B8 (3237)
11	Heart	None	A11 (3475), A24 (4748)	None
13	Heart	None	A2 (7682), A30 (1945), B13 (1570), B50 (1888)	None
16	Heart	None	B45 (650)	A34 (13,479)
17	Heart	None	A24 (2581)	None
19	Heart	None	A24 (3666)	None
20	Heart	B55 (910)	None	None
21	Heart	A11 (1468)	None	None
23	Heart	None	A1 (593), A31 (712), B8 (1960), B27 (1115)	None
25	Kidney	None	A1 (7370), A32 (3240), B57 (3949)	None
26	Kidney	B72 (1230)	None	None
27	Kidney	None	A11 (1062)	None
28	Heart	None	B53 (529), B57 (2747)	None
29	Heart	B8 (1138)	A1 (5199), A25 (1424), B18 (832)	None
31	Kidney	None	B51 (7872), B60 (6240)	None
32	Kidney	None	A2 (19,623), A32 (735), B8 (4352)	None
33	Kidney	None	A1 (1266)	None
34	Kidney	None	B49 (12,165)	None
35	Kidney	A31 (950)	B60 (2554)	None
36	Kidney	None	A2 (9673)	A24 (2618)
38	Kidney	None	Cw17 (3316)	None
39	Kidney	None	A11 (865), B51 (2725), Cw15 (8386)	None
40	Kidney	None	A29 (565), B44 (2460)	None
41	Kidney	A1 (940)	None	None
42	Kidney	None	A1 (2577), A2 (1111)	None
43	Kidney	None	A26 (5753)	None
44	Kidney	None	B27 (6154), Cw1 (1077)	None
45	Kidney	None	Cw10 (3702)	None
46	Kidney	None	None	A24 (2056)
47	Kidney	None	A2 (1595)	None
48	Kidney	None	A68 (2586), A69 (1048), B60 (1152)	None
49	Kidney	A24 (510)	Cw4 (1051)	None
50	Kidney	B44 (863)	None	None
51	Kidney	None	A2 (3294), B51 (3330)	Cw15 (1001)
52	Kidney	A1 (652)	None	A24 (963), B61 (739)
53	Kidney	None	A2 (6007), A23 (8922), B49 (1012), B51 (1987)	None
54	Kidney	A2 (1953), B7 (639)	None	None
56	Kidney	None	A2 (1270), B44 (7684)	None
57	Kidney	None	A2 (3333), B50 (1436)	None
58	Kidney	None	A31 (1546), B58 (811)	None
59	Kidney	None	None	A33 (1418)
60	Kidney	None	B51 (904)	B44 (3165)
62	Kidney	B7 (3983)	None	None
63	Kidney	None	A2 (29,694)	None
64	Kidney	None	Cw6 (1982), Cw7 (1477)	A1 (601)
65	Heart	None	A2 (7894)	B60 (992)

DSA, donor-specific antibody; β_2-m-fHLA, β2-microglobulin-free human leukocyte antigen; nHLA, native human leukocyte antigen; ND, not determined.
[a]MFI of specific bead, or the sum of beads within more broad serological specificities, observed during standard SAB assay is shown in parentheses for each antibody identified.
[b]β_2-m-fHLA specificity, antibody target identified as β_2-m-free HLA.
[c]nHLA specificity, antibody target identified as native HLA.
[d]Mixed HLA specificity, antibody target identified as both native HLA and β_2-m-free HLA.

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Table 2 Surrogate crossmatching of sera reactive to β_2-m-fHLA

Patient ID	DSA identified to β_2-m-fHLA	MFI on Standard SAB[a]	T cell MDCS[b]	B cell MDCS[b]	Crossmatch result[c]
3	B8	514	14	56	Negative
20	B55	910	<1	<1	Negative
21	A11	1468	20	17	Negative
35	A31	950	20	79	Negative
41	A1	940	52	196	Negative
49	A24	510	42	100	Negative
50	B44	863	<1	<1	Negative
52	A1	65	1	<1	Negative
54	A2	1953	8	132	Negative
54	B7	639	2	50	Negative
62	B7	3983	4	<1	Negative

β_2-m-fHLA, β2-microglobulin-free human leukocyte antigen; MDCS, median channel shift.

[a]MFI of specific bead, or the sum of beads within more broad serological specificities.

[b]Data expressed as MDCS relative to negative control serum, and the value reflects the mean of duplicate assays.

[c]Negative result indicates MDCS ≤63 for T cells and ≤108 for B cells.

fraction or creatinine level) most proximal to DSA sample date. There was also no difference in the distribution of the various patterns of antibodies or the numbers of mismatched HLA-A, -B, and -DR antigens.

Although only a few patients in this study had evidence of AMR ($n = 7$), all of them had at least one antibody to nHLA, and none had solely antibody to β_2-m-fHLA (Table 4). Among the AMR negative group, 4 of 22 (18%) patients had only β_2-m-fHLA which is similar to the overall frequency of β_2-m-fHLA in this study (13%). The difference in the distribution of the types of anti-HLA observed in patients with or without AMR was also not statistically significant.

The distribution of the types of antibodies (β_2-m-fHLA, nHLA, or mixed) among all patients was not significantly different with respect to whether they were DSA or non-DSA (Table 5). This also appeared to be true when stratified by organ (heart or kidney). Thus, it appears that the prevalence of antibodies to β_2-m-fHLA is a common feature of allosensitization in general and may not be related to SOT *per se*.

In order to determine whether the MFI value observed with a given patient sample would reveal whether it was due to antibodies to nHLA or β_2-m-fHLA, we evaluated the fluorescence characteristics of each group. Table 6 compares the fluorescence intensity values obtained for antibodies to nHLA and the β_2-m-fHLA on both denatured *vs* conventional SAB. As a group, antibodies to β_2-m-fHLA are of lower mean and median fluorescence intensity in standard SAB assays than those that recognize nHLA specificities; however, there was considerable overlap between the two groups. In addition, antibodies to β_2-m-fHLA tend to produce higher MFI on denatured SAB than those observed on standard SAB, presumably due to conversion of all HLA antigens to the β_2-m-fHLA species upon denaturation.

Table 3 Demographic and clinical data of patients with DSA to β_2-m-fHLA *vs* native HLA.

	nHLA recipients[a]	β_2-m-fHLA recipients[b]	Mixed/ multiple HLA recipients[c]	*P*-value
Number of recipients	29	8	18	
Gender				0.316[f]
Male	16	5	12	
Female	13	3	6	
Age at time of sample				0.566[g]
n of known age =	28	8	17	
Median	53	45	53	
A/B/DR mismatches				NS
n =	29	8	18	
Median	5	5	5	
Ejection fraction[d]				0.313[g]
n with results =	8	3	7	
Median	63%	62%	55%	
Creatinine level[e]				0.443[g]
n with results =	16	3	8	
Median	2.4 mg/dl	2.0 mg/dl	2.2 mg/dl	

DSA, donor-specific antibody; β_2-m-fHLA, β2-microglobulin-free human leukocyte antigen; NS, not significant.

[a]nHLA, recipients express only antibodies to native HLA.

[b]β_2-m-fHLA, recipients express only antibodies to β_2-m-free HLA.

[c]Mixed/multiple HLA, recipients express mixed antibodies to both native HLA and β_2-m-free HLA or express multiple antibodies to native HLA, β_2-m-free HLA, and/or mixed HLA.

[d]Ejection fraction in cardiac transplant recipients.

[e]Creatinine level in renal transplant recipients.

[f]*P*-value determined using Fisher's exact test.

[g]*P*-value determined using one-way ANOVA.

Table 4 Incidence of histopathologic diagnosis of AMR among recipients expressing DSA to β_2-m-fHLA, native HLA, or multiple HLA

	nHLA recipients[a]	β_2-m-fHLA recipients[b]	Multiple HLA recipients[c]
AMR−	13	4	5
AMR+	3	0	4

DSA, donor-specific antibody; β_2-m-fHLA, β2-microglobulin-free human leukocyte antigen.

[a]nHLA: recipients with only antibodies to native HLA.

[b]β_2-m-fHLA: recipients with only antibodies to β_2-m-fHLA.

[c]Multiple HLA: recipients with multiple antibodies to native HLA, β_2-m-f HLA, and/or mixed HLA.

Discussion

This study provides several observations regarding the development of antibodies to HLA following SOT. First, the antibodies that are reactive to β_2-m-fHLA class I antigens are a common finding, as they were observed in 43% and 56% of patients with

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Table 5 Frequency of DSA and non-DSA, directed to native, β_2-m-fHLA, or mixed HLA[a]

	nHLA[b]	β_2-m-fHLA[c]	Mixed HLA[d]
DSA	72% (76)	13% (14)	15% (16)
Non-DSA	71% (542)	18% (137)	11% (83)

DSA, donor-specific antibody; β_2-m-fHLA, β2-microglobulin-free human leukocyte antigen.
[a] *N* value is given in parentheses and represents number of antigens in each category.
[b] nHLA, antibody target identified as native HLA.
[c] β_2-m-fHLA, antibody target identified as β_2-m-free HLA.
[d] Mixed HLA, antibody target identified as both native HLA and β_2-m-free HLA.

kidney and cardiac transplants, respectively. Second, approximately 30% of the HLA antigens among these DSA are due to antibodies to β_2-m-fHLA, whereas 70% are reactive with nHLA. Finally, although the number of patients in our study with AMR was low, we did not observe AMR in any patient whose DSA was exclusively reactive with β_2-m-fHLA. These observations suggest that many of the DSA antibodies identified on SAB arrays recognize only β_2-microglobulin-free forms of HLA and these antibodies may be unrelated to the pathogenesis of AMR.

AMR is an important cause of allograft dysfunction contributing to poor patient outcomes, and is often refractory to currently available treatments (21). In addition, the diagnosis of AMR is challenging, especially in patients without allograft dysfunction. While there are subtleties in the histopathologic findings in renal (22) and cardiac (16) allografts, there are several common features. These include intravascular accumulation of $CD68^+$ cells of the monocyte/macrophage lineage, endothelial swelling, and sub-endothelial infiltration with mononuclear cells. These lesions are sometimes associated with deposits of C4d and the presence of circulating DSA (16, 23). Damage to vascular endothelium is mediated, in part, by complement activation and damage to the microvasculature, antibody-dependent cellular cytotoxicity involving Fc receptors on natural killer (NK) cells and cells of the monocyte/macrophage lineage, and type I activation of endothelial cells leading to enhanced inflammatory cell adhesion and aggregation, and damage to the microvasculature (24–26).

DSA testing has been widely adopted as an adjunct to other clinical and laboratory findings in the post-transplant monitoring of organ recipients. Up to 25% of transplant patients are reported to develop *de novo* DSA (27–29), and approximately one-third of patients with *de novo* DSA will experience AMR within the first year of transplant (28, 29). In addition, DSA and subsequent rejection episodes are associated with a higher risk of chronic rejection and late graft loss (30–32). These observations highlight the clinical importance of DSA testing and the critical need to accurately identify *de novo* DSA and establish their potential clinical relevance once identified.

Solid phase SAB arrays for HLA antibody identification have proved to be a major technical advance in the interpretation of complex serological data (33–35). Their use has provided improved algorithms for predicting positive crossmatches, and is primarily responsible for the now almost universal practice of virtual crossmatching in the setting of allocation of SOT (15). SAB arrays are a highly sensitive testing platform with excellent negative predictive values, especially when compared with the historical use of complement-dependent cytotoxicity previously performed by most HLA labs (34, 35).

The specificity of SAB arrays in DSA testing, however, has not been extensively evaluated, nor has the assay been formally validated against any clear clinical endpoint. A report of the Antibody Consensus Group of the Transplantation Society (2) suggests that each laboratory establish its own threshold for antibody specificity assignment and clinical interpretation but did not provide guidance on how to achieve this end. Our survey of the literature provides a range of cutoff values between 300 and 1000 MFI (18, 29, 36, 37). The cutoff of 500 MFI used in our study is within the range of reported cutoffs.

Several investigators have reported that denatured HLA on SAB may contribute to a lack of specificity (9, 10, 15). Initial reports of SAB testing of nonimmunized healthy blood

Table 6 Fluorescence characteristics of DSA observed on standard and acid-treated SAB

	MFI measured on standard SAB			MFI measured on acid-treated SAB		
	nHLA[a]	β_2-m-fHLA[b]	Mixed HLA[c]	nHLA[a]	β_2-m-fHLA[b]	Mixed HLA[c]
N[d]	76	14	16	76	14	16
Average	4018	1292	3544	395	2690	1954
Median	2507	945	2337	96	1807	1264
Range	529–29,694	510–3982	601–11,832	0–5852	591–11,952	352–6202

DSA, donor-specific antibody; β_2-m-fHLA, β2-microglobulin-free human leukocyte antigen; nHLA, native HLA; SAB, single antigen bead.
[a] nHLA, antibody target identified as native HLA.
[b] β_2-m-fHLA, antibody target identified as β_2-m-free HLA.
[c] Mixed HLA, antibody target identified as both native HLA and β_2-m-free HLA.
[d] *N* represents number of different HLA specificities identified as DSA from 55 patients in the study. Data represents average, median, and range of MFI observed for all DSA characterized as due to nHLA, β_2-m-fHLA, or a mixture of both.

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donors described unexplained and undefined reactivities to relatively rare HLA alleles (4). The data presented here and in several other publications (9, 10, 38) document that these reactions are not limited to rare HLA specificities, but in fact are frequent among common HLA alleles. While our finding of antibodies to β_2-m-fHLA in the post-transplant period are perhaps not surprising, prior publications on the topic of antibodies to denatured HLA have evaluated specimens obtained in the pre-transplant period (9, 10, 12–15, 38). Thus, our work is consistent with prior reports and extends them into the post-transplant clinical setting. An obvious extension of our work would be to perform serial studies on the development of antibodies to β_2-m-fHLA in the post-transplant period and examine their relationship to the development of AMR.

A plausible mechanism for the production of antibodies to β_2-m-fHLA stems from early observations of the structure of class I HLA; these proteins can be arranged on cells in at least two forms. First is the classical heterodimer of the heavy chain in association with β_2-microglobulin that is recognized by conformation-dependent antibodies such as W6/32 (39), and second are β_2-microglobulin-free class I alpha chains recognized by antibodies such as HC-10 (19) and LA45 (40). The latter were first described by Schnabl et al. (41) in activated human T cells, and their expression was shown to require internalization via the endosome compartment (42, 43). Thus, β_2-microglobulin-free class I heavy chains appear to be generated during the process of class I heterodimer recycling. Because manufacturing of SAB involves covalent conjugation of class I antigens *en masse*, it likely leads to loading of a mixture of classical heterodimer and β_2-microglobulin-free forms of heavy chain in proportion to the content of each species that were expressed on the cells from which the antigens were extracted.

As β_2-microglobulin-free class I molecules represent natural alternative forms of alloantigen, we speculate that the 'cryptic' epitopes that arise from conformational changes in the heavy chains provide immunogenic stimuli to distinct populations of B cells in ways similar to conventional intact class I molecules. Our finding that many DSA represent a mixture of antibodies to both nHLA and β_2-m-fHLA is supportive of the aforementioned concept. Given that some samples contained only one population of HLA molecules (nHLA or β_2-m-fHLA) might also suggest different mechanisms of immune activation. For example, the predominance of the β_2-m-fHLA form might be expected to arise under natural conditions of cell degradation from processes of apoptosis and/or necrosis, as both processes involve acidic and proteolytic microenvironments of the endocytic and phagocytic pathways (44). It is also possible that soluble forms of HLA found in blood plasma (45) contain a greater proportion of β_2-m-fHLA, thus supplying novel epitopes to antigen presenting cells. It is also feasible that antibodies to HLA proteins could recognize linear epitopes within the HLA molecule. As such, these antibodies could recognize both nHLA and/or β_2-m-fHLA. These antibodies would, of course account for the observation of specificities identified as 'mixed'. Although not the focus of this study, we have performed a preliminary series of experiments with sera that recognize β_2-m-fHLA of HLA-A2 tested against overlapping 30-mer linear peptides of the A2 molecule and where unable to detect any reactivity in an enzyme-linked immunosorbent assay (ELISA) format (unpublished data). If further studies of other HLA specificities hold true, this would suggest that the epitopes recognized by antibodies to β_2-m-fHLA are discontinuous. Thus, alloimmunization to these forms would likely be due to antigen presentation of intact heavy chains, not simply degradation products thereof.

The identification of antibodies to β_2-m-fHLA might be of practical importance as patient samples with DSA exclusive to β_2-m-fHLA type had no clinical or histologic evidence of AMR. Additionally, sole reliance of current SAB testing platforms to assist in the diagnosis of AMR without consideration of whether the DSA recognize nHLA or β_2-m-fHLA could confound the diagnosis of AMR. Both of these implications will need to be validated and reproduced in larger sets of SOT patients with and without AMR. Nonetheless, our observation of a lack of AMR in patients with DSA exclusively to β_2-m-fHLA is consistent with observations of the lack of clinical importance of pre-formed antibodies to β_2-m-fHLA at the time of transplant (10, 12–15). If our findings are validated and extended by other labs and it holds true that antibodies to β_2-m-fHLA have no clinical relevance, we think it would prove appropriate to perform similar acid elution studies on all patients to rule out false positive SAB. Our findings also illustrate the need for manufacturers to develop platforms that consist only of native forms of HLA, or in the absence of that, provide users information regarding the relative amounts of intact *vs.* β_2-m-fHLA.

In summary, while the SAB antibody detection platforms are highly sensitive and represent a dramatic improvement in the detection of antibodies to HLA, an unintended consequence of their use is the identification of antibodies that lack specificity for nHLA antigens. Our data add to previous observations that pre-transplant antibodies to β_2-m-fHLA are not a barrier to organ transplantation and support the concept that DSA to β_2-m-fHLA does not appear to confer a pathologic role in the development of clinical or histologic AMR. The rapid adoption of these SAB platforms has already led to changes in clinical management of waiting lists for SOT, and their use in post-transplant monitoring is widespread. The data presented here raise concerns about the specificity of DSA testing and highlight the need for a reassessment of the clinical relevance of many HLA antibodies in the diagnosis and management of AMR.

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Conflict of interests

The authors have declared no conflicting interests.

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