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Epitopes of human leukocyte antigen class I antibodies found in sera of normal healthy males and cord blood

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ABSTRACT

This study defines 96 epitopes targeted by human leukocyte antigen (HLA) antibodies reported in the sera of normal healthy males with no history of deliberate alloimmunizations and in cord blood. These epitopes are accessible for antibody binding on either the intact or the dissociated forms of recombinant HLA class I single antigens. Sixty percent of the epitopes are accessible on dissociated antigens, are defined mostly by hidden amino acids, and are designated as *cryptic* epitopes. All 96 epitopes are located exclusively on A-, B-, or C-locus antigens except for one interlocus epitope. All sera in this study were tested in parallel, using single antigen beads that bear either intact or dissociated HLA antigens and antibodies with nearly identical specificities were identified in all tested sera. Because the specificities of these naturally occurring antibodies are unavoidably detected when testing for specificities of alloantibodies, it may be necessary to clearly differentiate the two forms of antibody. To date, the relevance of these antibodies in transplantation is unknown, but even if they are determined to be irrelevant to graft rejection, awareness of the newly identified epitopes could prove useful in avoiding the unnecessary exclusion of potential transplant donors.

1. Introduction

Recently, we reported the existence of what we termed *natural* antibodies to human leukocyte antigens (HLA) in the sera of normal healthy Mexican males who had no history of alloimmunization. Many of the target HLA antigens were rare [1]. We designed the present study to confirm and expand the previous study's conclusions and—perhaps more important—to define epitopes targeted by antibodies reported in normal sera to differentiate them from alloantibodies for which we already defined unique epitopes [2].

In an unpublished study, we treated the HLA antigens coupled to single antigen beads with an acid buffer to dissociate the β2-microglobulin and the peptide from the HLA class I heavy chain and accidentally discovered that the reaction strength of certain weak antibodies in one serum increased many fold when tested with dissociated forms of HLA class I antigens. This discovery generated the idea that these antibodies might target hidden epitopes on the dissociated form of class I antigens.

To explore this possibility, we again tested sera from the Mexican males, plus sera from normal healthy Japanese males, to demonstrate that these antibodies exist in other populations. We also tested cord blood from an Italian population to demonstrate that similar antibodies can be detected as early as birth. The study makes it clear that antibodies reacting with *dissociated* antigens are prevalent in the sera of healthy males and in cord blood. In our accidental discovery, we defined an epitope that was shared only by the A31 and A33 antigens and was located underneath the antigens' peptide. When the peptide was removed, the epitope was exposed, resulting in stronger reactions. We characterize all such epitopes as cryptic because they are defined by hidden amino acids—in this case, isoleucine at position 73. We also reported that not all antibodies target cryptic epitopes. Some antibodies react with exposed epitopes and cease to react with these target epitopes when the antigen is dissociated.

More than half of the 96 epitopes identified here are private epitopes (*i.e.*, unique to one antigen). The antibodies targeting these epitopes exist in all three groups of the tested sera. Public epitopes (*i.e.*, those shared by two or more antigens) were also reported, several of them almost as frequently as the private epitopes.

Determining the specificities of the antibodies and defining their epitopes may prove important in explaining what appear to be unexpected HLA specificities in the sera of transplantation and

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transfusion patients. These unexpected specificities could be the specificities of the nonalloantibodies, which have been reported to exist in pretransplant kidney and multitransfused patients [3,4]. Because the specificities of such antibodies are unavoidably detected in the same assays used to detect alloantibodies, it may be necessary to differentiate between the two sets.

It is not yet known whether these antibodies are clinically relevant to graft rejection. If so, they may have to be considered as having the same effect as alloantibodies, with the obvious significance for donor selection. Even if the antibodies prove to be irrelevant, it would still be necessary to clearly identify the specificities of these antibodies to avoid unneces-

Table 1
Fifty-eight mostly cryptic epitopes located on dissociated class I HLA antigen (heavy chains)

Dissociated antigen(s) with distinct epitope[a]	Epitope no. assigned[b]	Possible epitope site[c]	Epitope description[d]	Ag. form[e]	MX[f]	JP[f]	CB[f]	Total
A2	5001	(74H)	C	D	4	1		5
A23,A24	5002	(9S)+(70H)	C	D	3			3
A25,A26,A33,A3303,A34, A66,A6602,A68,A6802, A69	5003	(63N)+(67V)	C	D	1			1
A2901 & A2902	5004	(9T)+(114R)	C	D	1	1		2
A29,A43	5005	(63Q)	C	D	5			5
A3002	5006	(152R)	C	D	3			3
A31,A33	5007	(73I)	C	D	19	2	1	22
A3401	5008	(63N)+(66K)	C	D	1	2		3
A3402	5009	(63N)+(66K)+(156L)	C	D		1		1
A80	5010	(31S)	C	D	14	4	1	19
B2705,B2708	5011	(97N)	C	D	1			1
B27,B44,B47	5012	(24T)+(116D)	C	D	1			1
B2705	5013	(45E)+(77D)	C	D	3			3
B2708	5014	(70K)+(77S)	C	D	4			4
B27,B37	5015	(9H)+(77D)	C	D	1			1
B37	5016	(99S)+(116F)	C	D	11	1	1	13
B37,B47	5017	(70N)+(77D)	C	D	1			1
B4402	5018	(77N)+(156D)	C	D	3	1		4
B5501	5019	(97T)+(116L)+(152E)	C	D	1	1		2
B57,B58,B63	5020	(70S)	C	D		1		1
B65	5021	(11A)+(97W)	C	D	3	1		4
B67	5022	(70Q)+(116F)	C	D			1	1
B7,B48,B60,B81	5023	(178K)	C	D	1			1
B7,B42,B54,B55,B56,B67, B81,B82	5024	(66I)+(70Q)	C	D	8	3	1	12
B72	5025	(45E)+(77S)+(116S)	C	D	2			2
B75	5026	(67S)+(77S)+(95I)	C	D	2			2
B8	5027	(9D)	C	D	6	3		9
B8,B37,B42,B82	5028	(24S)+(156D)	C	D	3			3
B8,B42,B82	5029	(45E)+(156D)	C	D	2	1		3
B8,B42,B37,B41,B4102, B44, B45,B82	5030	(156D)	C	D	1			1
B82	5031	(24S)+(99F)	C	D	13	4		17
Cw1	5032	(6K)	C	D	1			1
Cw2	5033	(211T)	C	D	6			6
Cw1502	5034	(1C)+(116L)	C	D	4			4
Cw16	5035	(116S)+(156Q)	C	D	4			4
Cw17	5036	(116F)+(143S)	C	D	9	9		18
Cw4,Cw6,Cw17,Cw18	5037	(73A)+(77N)	C	D	3		1	4
Cw6	5038	(9D)+(97W)	C	D	5	1		6
Cw7	5039	(66K)+(99S)	C	D	2			2
Cw8	5040	(152T)	C	D	3			3
A1,A36	5041	(67M)+(70H)	C	D	3		1	4
A0203	5042	(114H)+149T	PC	D	4	2		6
A2501	5043	(81A)+90D	PC	D	1			1
A1,A26,A36,A29,A43	5044	(74D)+76A	PC	D	2			2
A1,A3,A11,A30,A31,A32, A36,A74,A80	5045	(63E)+71S+(95I)	PC	D	3			3
A25,A26,A34,A43,A66	4	(9Y)+149T	PC	D	8	1		9
A203,A25,A26,A34,A43, A66	5047	(152E)+184A	PC	D	4	2		6
A23,A24,A34	5048	43Q+66K+(74D)	PC	D	1			1
A6602	5049	(114Q)+163E	PC	D	2			2
A7401	5050	66N+(77D)+109L	PC	D	1			1
B4403	5051	(156L)+167S	PC	D	1			1
B76	5052	(70N)+166D	PC	D	1	1		2
A1,A3,A11,A30,A31,A32, A36,A74	5053	62Q	PC	D	2			2
A2,B57,B58	17	62G	PC	D		1		1
B44,B45,B82	5055	167S	E	D	4			4
A25,A26,A29,A31,A32, A33, A34,A43,A66,A74	5056	246S	E	D	1			1
A2,A203,A206,A25,A26, A29, A32,A34,A43,A66,A6602, A68,A6802,A69,A74	5057	184A	E	D		1		1
A2,A203,A206,A25,A26, A29, A31,A32,A33,A3303,A34, A43, A66,A6602,A68,A6802, A69,A74	5058	193A	E	D	1			1

[a]One or more dissociated HLA class I antigens that share a unique epitope.
[b]Epitopes 4 and 17 have been assigned to alloantibodies in previous studies [2,11].
[c]Amino acids and their positions on the HLA dissociated antigens define each epitope. Hidden aa positions are given in parentheses. Epitopes that are defined by more than a single position/aa are separated by +. Although alternative definitions exist, for space considerations we list here only the most probable definitions based on distances among the aa and the orientations of their side chains.
[d]C = cryptic; PC = partially cryptic; E = exposed epitopes. These designations are in reference to the intact HLA antigen.
[e]D = dissociated HLA antigens (heavy chain of the HLA class I antigens).
[f]MX = Mexican; JP = Japanese; CB = cord blood.

sary donor exclusions or unnecessary alarms by falsely reporting specificities.

2. Subjects and methods

This study used sera from healthy males who had no history of deliberate alloimmunization. All donors indicated on the day their blood was collected that they had no immunization or blood transfusions within the past 6 months. Sera from cord blood samples were also used. We tested for HLA antibody specificities in 306 sera samples from healthy Mexican male blood donors used in a previous study [1], plus 95 sera samples from healthy Japanese male blood donors and 18 sera from Italian cord blood samples.

All sera were tested with 95 HLA class I (A-, B-, and C-locus) recombinant rHLA single antigens (SA) individually coupled to different microspheres and with negative and positive control beads (LABScreen beads: LS1A04 lot2; One Lambda Inc., Canoga Park, CA) and selected sera, positive with the SA beads, were tested with the LABScreen Panel Reactive Antibody (PRA) beads (LABScreen PRA beads: LS1PRA lot12; One Lambda, Inc., Canoga Park, CA) [5]. Each serum was tested in parallel using intact antigens (heavy chain, β2-microglobulin, and peptide) and the corresponding dissociated antigens (heavy chains only). To prepare dissociated antigens, SA beads or PRA beads were treated with ImmunoPure IgG elution buffer (EB; Catalog No. 21004; Pierce, Rockford, IL) and then blocked with 2% bovine serum albumin. All LABScreen assays were performed according to the manufacturer's protocol. To ensure that the antigens on the beads were dissociated before they were used to test the sera, dissociated beads were tested with the monoclonal antibodies W6/32 and anti β2m (both monoclonal antibodies [mAbs]: intact antigens, +; dissociated antigens, −).

Data generated by a LABScan 100 (One Lambda Inc., Canoga Park, CA) were analyzed using computer software. Trimmed mean fluorescence values for the bead reactions were obtained from the output (.csv) file generated by the flow analyzer and adjusted for background signal using the formula [(sample #N bead − negative control bead value of the sample) − (negative control #N bead − negative control bead value of the negative control serum)]. The data were graphed using an Excel spreadsheet. All adjusted values for the SA beads above 1000 and all adjusted values for the PRA beads above 500 were considered positive reactions.

Other selected sera positive with the SA beads, negative control serum, and positive control serum were tested with frozen T lymphocytes using a standard flow crossmatch assay. CD3-positive

Table 2
Thirty-eight mostly exposed epitopes located on intact HLA class I antigen

Dissociated antigen(s) with distinct epitope[a]	Epitope no. assigned[b]	Possible epitope site[c]	Epitope description[d]	Ag. form[e]	MX[f]	JP[f]	CB[f]	Total
A0101	5059	158V+163R	E	I		1		1
A1,A36	1	44K	E	I			1	1
A1102	30	19K	E	I	6	13	4	23
A2	201	43Q+62G	E	I			1	1
A2,A23,A24,A68,A69	19	127K	E	I			1	1
A23,A24	3	65G	E	I	2			2
A2403	5060	65G+166E	E	I	1	1		2
A2501	5061	76E+149T	E	I	2	1		3
A2601	5062	62R+76A	E	I	1	1		2
A25,A26,A33,A3303,A34, A66, A6602, A68,A6802,A69	214	43Q+62R	E	I	1			1
A2901 & A2902	5063	62L+163T	E	I	1	1		2
A30,31	31	56R	E	I	2			2
A3002	5064	17S+76E	E	I	23			23
A36	5065	150V+163T	E	I		1		1
A6602	5066	149T	E	I	5	2		7
A6901	5067	66N+107W	E	I		2		2
A80	5068	56E+	E	I	4	2		6
A32,B57,B58,A25,B63	5069	65R+76E	E	I		1		1
B2705	406	65Q+69A+80T	E	I	1			1
B57,B58	236	43P+62G	E	I	3			3
B57,B63	5070	46A+65R	E	I	1			1
B60	5071	41T+147L	E	I	1			1
B63	5072	43P+62R+65R	E	I	10	1		11
B76	5073	163L+166D	E	I	6	4		10
B82	5074	162D	E	I	5	7		12
Cw*0102,0302,0303,0304,1402,1802	5075	219W	E	I		1		1
Cw16	5076	193L	E	I	1			1
Cw17	5077	170G	E	I	6	6		12
Cw7	5078	273S	E	I	1	1		2
Cw8	5079	16S+90D	E	I		1		1
Cw9	5080	91R	E	I	3			3
Cw9,Cw10	5081	163L+173K	E	I	2	2		4
A2402	203	(156Q)+166D	PC	I	1	1		2
A2901	5082	62L+(102H)+163T	PC	I		1		1
A3401	5083	43Q+(66K)+90D	PC	I	3	2		5
B4501	5084	(9H)+167S	PC	I	7	5		12
B8	5085	(67F)+131R	PC	I	3			3
Cw6	5086	80K+90D+(114D)	PC	I	1			1

[a]One or more dissociated HLA class I antigen(s) that share a unique epitope.
[b]Epitopes 1, 30, 201, 19, 3, 214, 31, 406, 236, and 203 have been assigned to alloantibodies in previous studies [2,11].
[c]Amino acids and their positions on the HLA dissociated antigens define each epitope. Hidden aa positions are given in parentheses. Epitopes that are defined by more than a single position/aa are separated by +. Although alternative definitions exist, for space considerations we list here only the most probable definitions based on distances among the aa and the orientations of their side chains.
[d]E = exposed; PC = partially cryptic epitopes. These designations are in reference to the intact HLA antigen.
[e]I = intact HLA antigens (heavy chain + β2m + peptide).
[f]MX = Mexican; JP = Japanese; CB = cord blood.

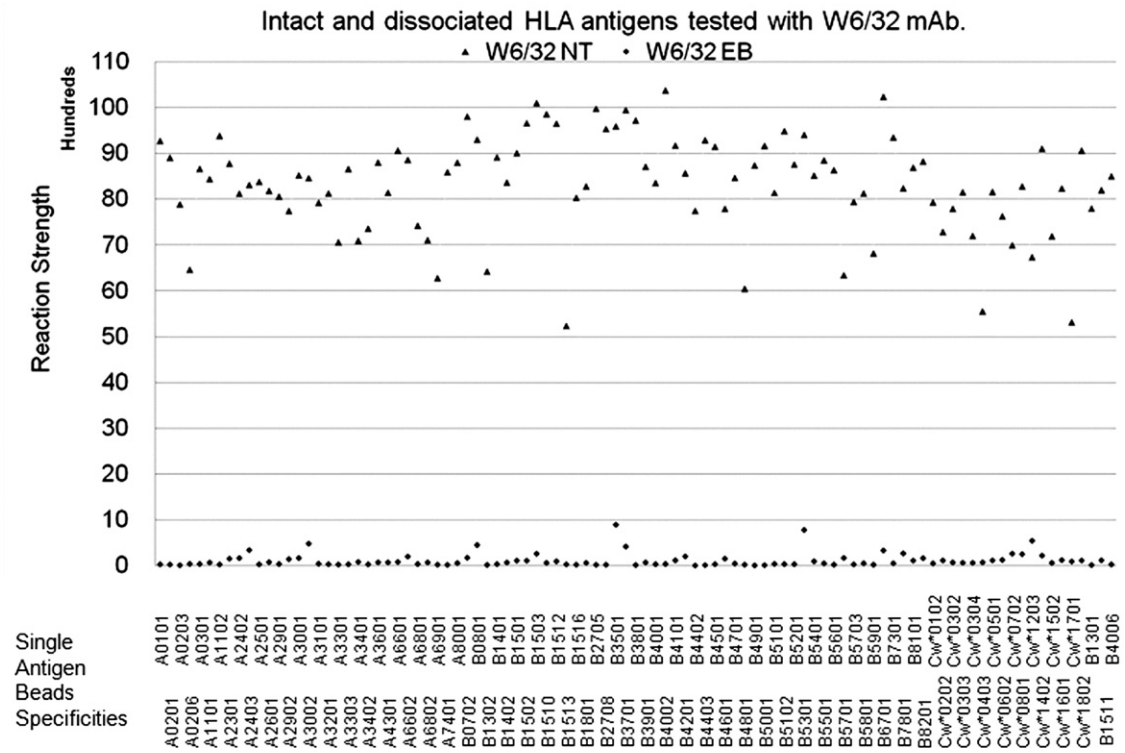

Fig. 1. W6/32 mAb reacts positively with intact HLA antigen (NT = nontreated) on the SA beads and negatively with SA beads treated with the acid elution buffer (EB), indicating antigen dissociation.

lymphocytes were gated and a mean channel shift of 50 or more channels from the negative control was considered positive.

Amino acid (aa) sequences of the HLA antigens or alleles were downloaded from the Anthony Nolan Web site [6]. Based on the data analysis above, we determined the positive antigens for each serum tested. An epitope search program was then utilized to identify distinguishing aa that are shared only by the positive antigens at particular sequence positions. The program searched for one or more unique aa positions. Among the many possibilities generated, we considered only the positions within the antibody-binding span, estimated at 494 $Å^2$ (19 × 26 Å) [7] or 750 $Å^2$ [8], approximate distances in Ångstroms between two aa calculated

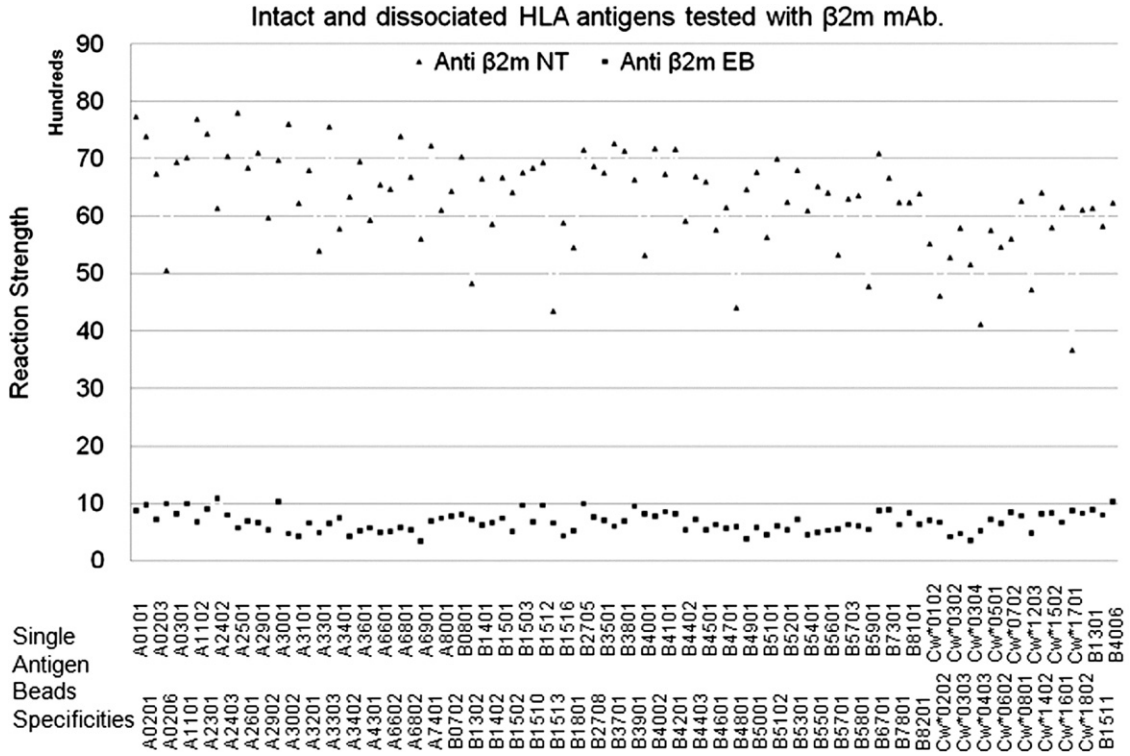

Fig. 2. Anti β2m mAb reacts positively with intact HLA antigen (NT = nontreated) on the SA beads and negatively with SA beads treated with the acid elution buffer (EB), indicating antigen dissociation.

with Cn3D Viewer software [9] and the 3D structure of an HLA-A0201 molecule 1QEW (Orth *et al.*, MMDB: Entrez's 3D-structure database on the National Center for Biotechnology Information Web site) [10]. Any aa unique to the antigen that was positive with a monospecific serum were considered distinguishing characteristics of the epitope, as were aa unique to all antigens positive with a serum and within the binding span of the antibody.

All sera tested had HLA specificities. However, for the definition of epitopes, we considered only the sera that were either monospecific or locus monospecific (only one specificity for the A-, B-, or C-locus)—plus certain multispecific sera in which all positive antigens for any one serum shared the same epitope (and only that epitope). We excluded other multispecific sera whose specificities could not be determined to share only one epitope.

3. Results

This study confirmed the existence and universality of HLA antibodies in otherwise normal healthy males. We identified and characterized a total of 96 HLA class I epitopes of such antibodies.

About 60% of all epitopes identified in this study were private epitopes, reported only on SA. Antibodies targeting any of these epitopes are most likely monospecific. The rest were public epitopes targeted by multispecific antibodies. Of the 96 epitopes, 58 (60%) epitopes were accessible for antibody binding only on the dissociated HLA antigens (heavy chains). Of these, 41 were defined by hidden aa and therefore designated cryptic epitopes. Thirteen were defined by at least one hidden aa in addition to exposed aa and designated partially cryptic. For four epitopes, no hidden aa were reported to define them; therefore, they were designated exposed epitopes. All hidden aa are listed in parentheses and are believed to be inaccessible in the intact form of the antigens (Table 1).

Table 1 lists the 58 mostly cryptic epitopes accessible only on the dissociated forms of a single HLA antigen—for example, HLA antigen A2 (epitope 5001) or B37 (epitope 5016). Epitopes shared by the dissociated forms of two or more antigens are also listed—for example, A23,A24 (epitope 5002), B7,B42,B54,B55,B56,B67,B81,B82 (epitope 5024), or Cw4,Cw6,Cw17,Cw18 (epitope 5037). One to four aa define the epitopes. In most cases, more than one alternative aa can define the epitope. However, for space considerations we list only the epitope defined by the smallest number of aa that are separated by distances not exceeding the antibody's binding span. Antibodies that target the most frequently occurring epitopes were reported in all three sera groups. For instance, A-locus epitope 5007 was defined by 22 sera (19 MX, 2 JP, 1 CB). B-locus epitope 5024 was defined by 12 sera (8 MX, 3 JP, 1 CB) and epitope 5031 was defined by 17 sera (13 MX, 4 JP). C-locus epitope 5036 was defined by 18 sera (9 MX, 9 JP).

Table 2 lists 38 epitopes accessible on intact HLA class I antigens. Thirty-two epitopes were defined by exposed aa, whereas 6 epitopes are defined by two or more aa, of which only one was hidden. All 38 epitopes were either unique to a single HLA antigen or shared by a group of two or more intact antigens. The most frequently occurring epitopes—30, 5064, 5072, 5073, 5074, 5077, and 5084—were located on antigens A1102 (23 sera), A3002 (23 sera), B63 (11 sera), B76 (10 sera), B82 (12 sera), Cw17 (12 sera) and B4501 (12 sera), respectively. Several of these epitopes were identified earlier as target epitopes of alloantibodies. For example, the epitope shared by A1,A36 HLA antigens and defined by aa 44K was designated earlier as epitope No. 1 [2,11].

Evidence for the dissociation of HLA antigens on the SA beads is illustrated in Figs. 1 and 2. mAbs W6/32 and anti β2m, both positive with the intact HLA antigens, have negative reactions with the dissociated antigens. With few exceptions of marginal values, all

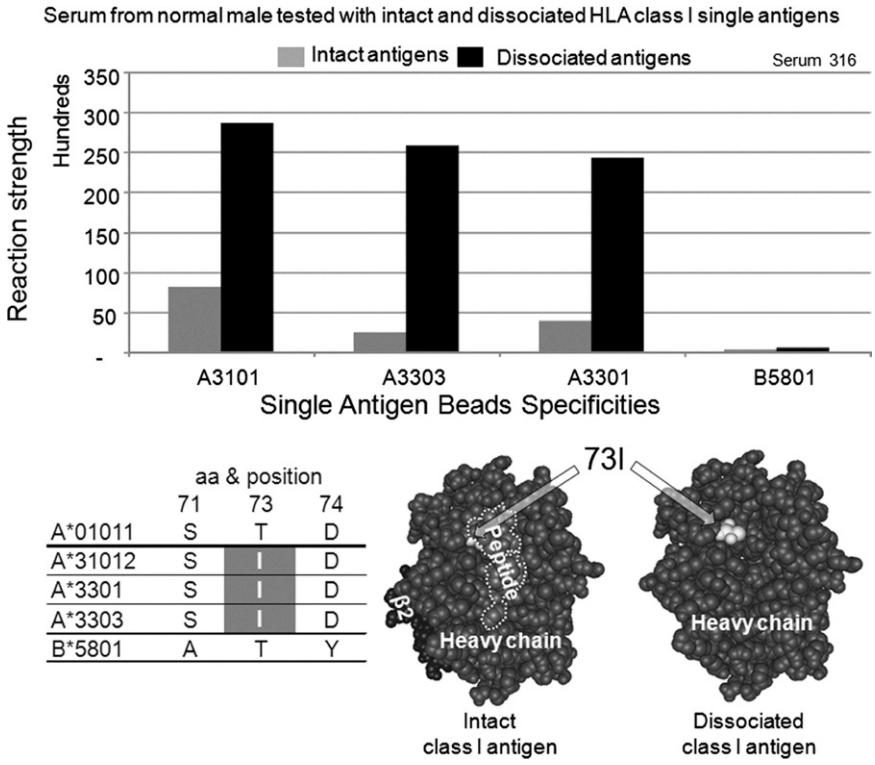

Fig. 3. Serum 316 demonstrates weak to moderate positive reactions with intact HLA antigens A3101, A3301, and A3303 and very strong positive reactions with the dissociated forms (heavy chains) of the three A-locus antigens. A3101, A3301, and A3303 are the only antigens that share the aa isoleucine (I) at position 73. 73I lies beneath the peptide in the intact antigens and becomes fully exposed after the antigens are dissociated, resulting in stronger binding with the antibody. Isoleucine at position 73 defines a cryptic epitope on the dissociated antigens (epitope 5007, Table 1).

reactions with the dissociated antigens were below the 1000 mean fluorescence intensity (MFI) cutoff point. The dissociated antigens on the LABScreen PRA beads also had negative reactions with the same mAbs (data not shown).

Figs. 3–6 graphically present the fluorescence data that demonstrate the positive reactions of dissociated and intact antigens with defined cryptic or exposed epitopes. Only the highest negatives are included to clearly demonstrate the marked drop in signal from the epitope-positive antigens.

Fig. 3 illustrates the reactions of serum 316 with the A3101, A3303, and A3301 intact and dissociated antigens of the SA beads. It is clear from these data that the strength of the reactions when using dissociated antigens is up to 10 times greater than with intact antigens. Only A3101, A3303, and A3301 share the aa isoleucine (I) at position 73. As demonstrated in the 3-D antigen illustrations, this position is located underneath the peptide. These findings strongly suggest that aa 73(I) defines a cryptic epitope that becomes accessible for antibody binding only when the peptide is removed. The weak reactions indicated with the intact antigen beads are most likely caused by the presence of dissociated antigens produced during the purification or coupling process along with the intact antigens on the same beads (epitope 5007, Table 1). Similar observations for eight B-locus antigens sharing the cryptic epitope 5024 and four C-locus antigens sharing the cryptic epitope 5037 are clear (Figs. 4 and 5).

A total of 48 monospecific sera were reported, of which 30 had antibodies recognizing exposed epitopes and 18 recognizing cryptic epitopes. Fig. 6 illustrates five examples of monospecific sera. Two sera (3 and 30) seem to react with a unique cryptic epitope (stronger reactions with the dissociated antigen than with the intact antigen), whereas the other three sera (488, S503, and 243) seem to react with a unique exposed epitope (a positive reaction with the intact antigen, which becomes negative when tested with the corresponding dissociated antigen; Tables 1 and 2).

Eighty-one percent of all sera exhibited increased reactions (Δ = 505 to 23,360 MFI) and 61% of the sera exhibited decreased reactions with the dissociated antigens (Δ = 21,602 to zero MFI). An increase or decrease in reactivity with the dissociated antigens was not absolute for every serum. Some sera exhibited increased reactivity for certain antigens and others exhibited decreased reactivity. The actual MFI values for 46 sera for a cryptic epitope on the A3301 antigen are presented in Table 3. As an example, the reaction strength for serum 316 increased from 3888 MFI with the intact beads to 24,291 with the dissociated beads.

To insure that the positive reactions of the dissociated antigens on the SA beads are not caused by altered molecular structures of antigens as a result of their recombinant cell line origin, selected sera were tested with the LABScreen PRA beads, which have antigens from a different source, namely transfected cell lines presumed to have antigens in their native forms.

Table 4 clearly indicates that three sera with the A31and A33 specificities, which exhibit stronger reactions with the dissociated antigens on the SA beads, also exhibit stronger positive reactions with the dissociated antigens on the PRA beads bearing either the A31 or the A33 antigens. These and the results shown in Table 5, illustrate that the anti HLA antibodies in normal males react in the same way with HLA antigens from different sources.

Fig. 7 illustrates the crossmatch results of four sera, positive with intact and dissociated antigens on the SA beads, and lymphocytes bearing similar antigens. For example, the flow crossmatch between lymphocytes expressing the A3401 HLA antigen and serum AlbT137, determined by intact SA beads to have A3401 specificity (MFI = 9392), resulted in 107 mean channel shift. On the other hand, sera exhibiting strong reactions with the dissociated antigens on the SA beads were negative with lymphocytes bearing the same antigens. All sera had a negative flow crossmatch with lymphocytes that have irrelevant antigens (data not shown).

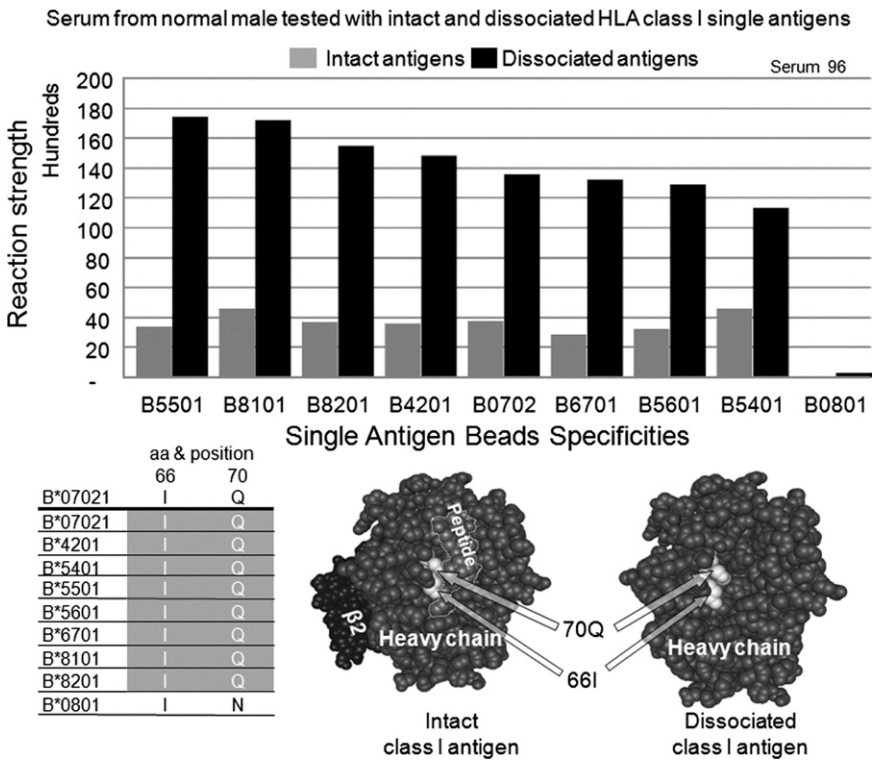

Fig. 4. Serum 96 exhibits weak to moderate positive reactions with intact HLA antigens B5501, B8101, B8201, B4201, B0702, B6701, B5601, and B5401 and very strong positive reactions with the dissociated forms (heavy chains) of the eight B-locus antigens. These are the only antigens that share the aa isoleucine (I) and glutamine (Q) at positions 66 and 70, respectively. The two positions appear to be partially exposed in the intact antigens. In the dissociated forms of the antigens, the two aa become fully exposed, binding strongly with the antibody. The two aa define a cryptic epitope (5024, Table 1).

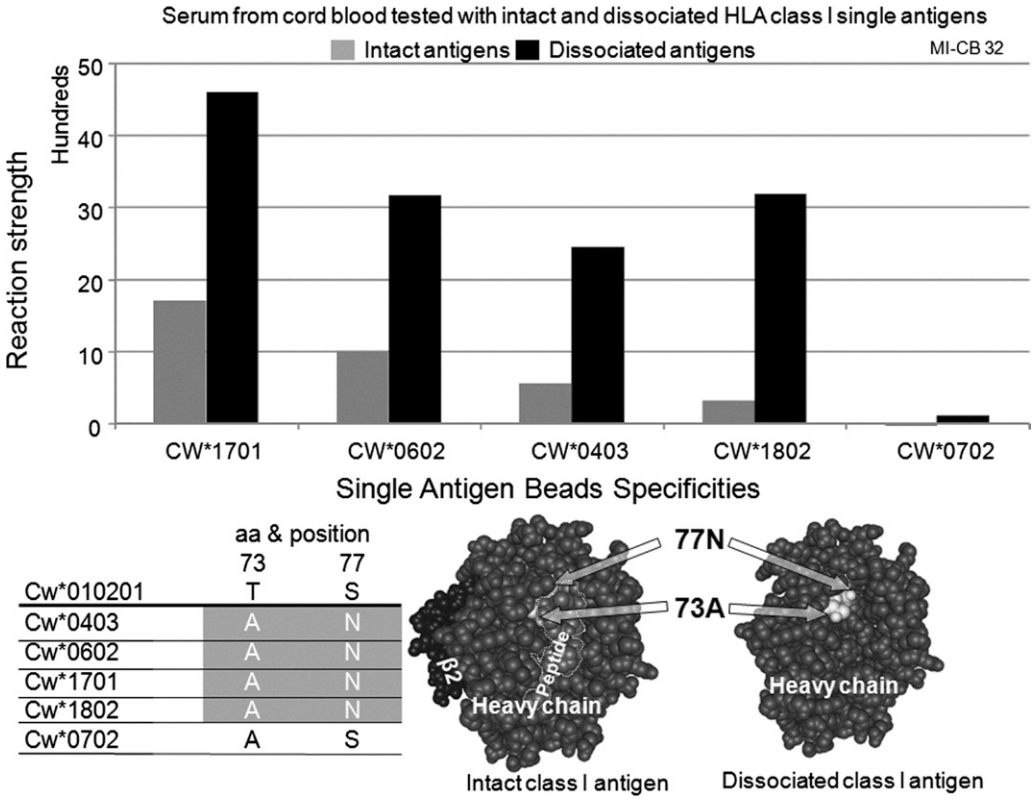

Fig. 5. Serum MI-CB32 exhibits weak positive reactions with intact HLA antigens Cw1701, Cw0602, Cw0403, and Cw1802 and moderate positive reactions with the dissociated forms (heavy chains) of the four C-locus antigens. These are the only antigens that share the aa alanine (A) and asparagine (N) at positions 73 and 77, respectively. The two positions are hidden beneath the peptide in the intact antigens. In dissociated antigens, the two aa become fully exposed, binding with moderate strength with the antibody. The two aa define a cryptic epitope on the dissociated antigens (epitope 5037, Table 1).

4. Discussion

Anti-HLA antibodies have been identified in sera from nonalloimmunized healthy males using purified HLA antigens attached to microspheres [1]. Many of these antibodies were directed against a limited number of rare HLA antigens, which indicates they are unlikely to have arisen as a result of allogeneic antigen exposure.

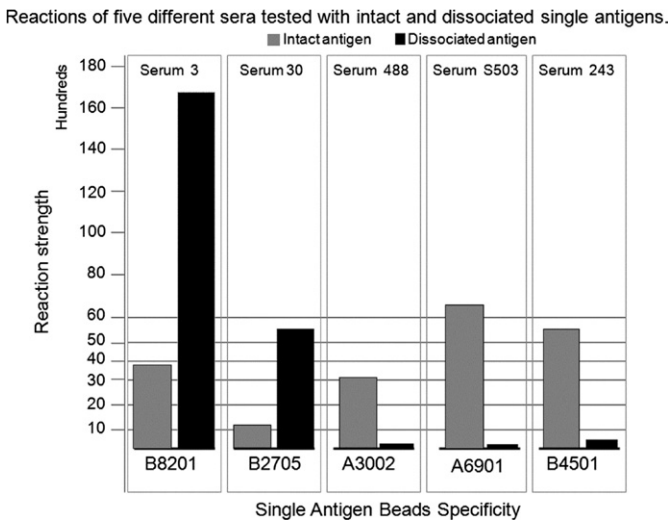

Fig. 6. The separate reactions of five monospecific sera with intact and dissociated antigens are illustrated. Three sera have antibodies that recognize only unique cryptic epitopes (3 and 30); the other three sera (488, S503, 243) have antibodies that recognize only unique exposed epitopes. Sera 3, 30, and 49 react more strongly with the dissociated antigens, whereas sera 488, S503, and 243 only react with the intact antigens, with all three becoming negative when tested with the dissociated antigens (Tables 1 and 2).

This study demonstrated that these antibodies occur in populations from Mexico to Japan to Italy and can be identified in cord blood, casting further doubt on their development as a result of alloimmunization. However, we cannot rule out a maternal origin in the cord blood samples because most mothers also have one or more of the same specificities. One expects to find anti-HLA antibodies in multiparous women and in patients who have had a transplant or transfusion, but finding anti-HLA antibodies in over 50% of a test group of healthy males, none of whom had a history of deliberate immunization, was surprising. Many of the same antibody specificities identified in the previous study of Mexican samples [1] were also reported in the Japanese and the cord blood samples at similar frequencies. These antibodies targeted similar epitopes in all three sample populations, confirming their universality and that they can be detected as early as birth (Tables 1 and 2).

The possibility that these antibodies react to HLA antigens with altered molecular structures formed in the process of their production in recombinant cell lines, purification, or adsorption to the SA beads is unlikely because the same sera reacted positively to the same dissociated antigens on the SA as well as the PRA beads. Similarly, other sera were positive to the same intact antigens on both types of beads. Furthermore, crossmatches of lymphocytes and sera that were positive with intact or dissociated antigens were only positive for sera that were positive with the intact antigens.

In transplantation, we expect that alloantibodies against HLA class I antigens are directed against intact HLA class I antigens on the cell surface—with the presumption that alloantibodies react with exposed epitopes. After defining the hidden epitope 73(I), shared by A31 and A33 antigens, we made the assumption that all epitopes targeted by the antibodies in nonalloimmunized individuals are cryptic. However, only 58 (60%) of the 96 epitopes described here were cryptic epitopes accessible for antibody binding

Table 3
Reaction strength of 46 normal sera tested with EB-treated (dissociated) and nontreated (intact) A3301 antigen on the SA bead

Serum	Intact antigen	Dissociated antigen	Serum	Intact antigen	Dissociated antigen
316	3,888	24,291	341	799	2,100
497	56	16,277	224	791	2,084
450	5,638	13,243	9	519	2,073
418	7,744	11,528	126	428	1,991
381	6,586	11,289	431	405	1,876
198	4,060	10,618	380	335	1,759
5	2,590	8,362	490	269	1,728
119	2,464	7,672	271	313	1,554
417	5,822	7,631	223	592	1,494
MICB-36	895	7,388	118	82	1,487
S468	1,081	7,113	222	439	1,398
122	1,326	6,648	448	345	1,384
310	457	6,520	86	—	1,309
436	1,728	5,515	478	455	1,307
125	2,455	4,808	320	295	1,270
S437	676	4,388	131	623	1,261
446	1,383	4,124	313	317	1,235
166	469	3,083	349	98	1,087
S258	388	3,033	387	126	1,082
206	252	3,012	242	194	1,081
114	1,572	2,876	370	246	1,079
347	571	2,627	291	—	1,067
55	—	2,107	404	131	1,026

on dissociated antigens only. Strong reactions of antibodies targeting these 58 epitopes became obvious only when the HLA antigens were dissociated—β2-micoglobulin and peptide were removed with acid treatment. The other 38 epitopes (40%) were recognized by antibodies reacting with the intact form of the antigens. Once the antigen was dissociated, the antibodies no longer bound.

Few studies of anti-HLA antibodies in normal males exist. Two studies describe the detection of anti-HLA-B8 [12,13] and another study [14] describes the detection of anti-HLA-A2 in normal sera of healthy males. Perhaps the weak sensitivity of the assays used in the past, plus the fact that 60% of the epitopes targeted by the antibodies described in this study are cryptic, is the main reason these antibodies have not received more attention. Only recently has a full study of HLA specificities in normal sera from healthy males been published [1].

Sixty percent of the epitopes defined in the current study are private epitopes. We were surprised to report several public epitopes shared by 2 to 18 HLA antigens—many at the same frequency as private epitopes. Two such epitopes are especially noteworthy. Twenty-two sera, representing all three sample populations, appear to have one antibody targeting an epitope shared only by the A31 and A33 HLA antigens. Similarly, 12 sera appear to have one antibody targeting an epitope shared only by the B7, B42, B54, B55, B56, B67, B81, and B82 antigens, indicating that these antibodies are not restricted to private epitopes on rare HLA antigens. It is also interesting to note that interlocus epitopes—not uncommon for alloantibodies—were almost entirely absent.

We utilized only sera that were monospecific for A-, B-, or C-locus antigens or were multispecific, but with all positive antigens clearly sharing only one epitope. A monospecific serum is presumed to have only one antibody recognizing a unique epitope on a SA, whereas a multispecific serum—with all positive HLA antigens sharing one or more unique aa—is also presumed to have only one antibody recognizing one epitope defined by these unique aa. We excluded other multispecific sera that were presumed to have multiple antibodies because such sera would require the use of absorption/elution assays, used in previous studies [2], to isolate one antibody for testing with the SA beads. To absorb and then elute, antibodies targeting the cryptic epitopes would require the use of recombinant SA cell lines bearing dissociated antigens. These assays, although currently under development, do not exist at this time.

The immunizing antigens and events that may lead to the production of these antibodies are unknown. Evidence that bacterial antigens cross-reactive with HLA are immunizing antigens has been discussed by Morales et al. [1]. Other plausible immunizing antigens include polypeptides from ingested foods and inhaled allergens.

Table 4
A31, A33 sera positive with the dissociated SA beads (EB treated) are tested with dissociated and intact (nontreated = NT) LABScreen PRA beads

A31,33 sera SA+	SA beads			PRA bead antigens including A31,33[a]						
	A3101	A3301	A3303	Neg. bead	Pos. bead	A2,31 B39,48 BW6 CW7,8	A11,3303 B75,58 BW6,4 CW10,8	A3303,36 B63,53 BW4 CW4,14	A3301,74 B72,78 BW6 CW2,16	A11,3303 B18,52 BW6,4 CW7,X
AlbT-9NT	1,175	519	403	23	19,888	101	145	199	76	142
AlbT-9EB	6,244	2,484	3,758	809	20,400	1,755	1,259	1,497	1,130	1,292
AlbT-114NT	3,531	1,572	883	30	21,658	102	125	220	85	146
AlbT-114EB	19,964	8,719	15,543	571	22,445	2,120	1,394	1,400	784	1,353
AlbT-198NT	8,195	4,060	2,309	63	21,150	199	454	294	162	224
AlbT-198EB	24,037	19,072	22,790	771	20,611	5,868	6,685	5,912	3,640	5,700

Both types of beads exhibit positive reactions with these sera. SA+ = positive with SA beads; NT = nontreated beads; EB = elution buffer-treated beads; ND = not done.
[a]All beads with irrelevant antigens were negative.

Table 5
Sera positive with intact antigens (NT) on the SA beads are tested with intact antigens on the LABScreen PRA beads

Serum no. (SA bead specificities)	SA beads				PRA beads bearing B8, 45, 76, or B82 specificities[a]								
	B8	B45	B76	B82	Neg. bead	Pos. bead	A2,11 B51,76 BW4,6 CW10,14	A2,24 B62,8 BW6 CW7,9	A34,66 B8,35 BW6 CW7,16	A23,80 B8,18 BW6 CW2,10	A1,29 B8,45 BW6 CW6,7	A68,74 B72,45 BW6 CW2,16	A1,2 B57,82 BW4,6 CW6,10
(B8, B76)													
AlbT-156 NT	2,579		1,990		48	21,890	654	462	824	1,946	1,273	82	93
AlbT-156 EB	363		0		229	19,240	5	19	557	126	120	136	73
(B4501)													
S322 NT		11,335			40	20,420	39	661	71	283	656	503	93
S322 EB		188			508	20,951	0	0	0	0	0	0	0
(B8201)													
S186 NT				21,858	24	23,629	43	42	107	97	72	61	4,424
S186 EB				257	401	25,416	0	0	0	0	0	0	0

Each serum reacted positively with both types of intact antigens and negatively with the corresponding antigens on the dissociated beads (EB). SA+ = positive with SA beads; NT = nontreated beads; EB = elution buffer-treated beads; ND = not done. Reactions equal to or less than zero after adjusting for the negative control are shown as zero.
[a]All beads with irrelevant antigens were negative.

Specificities of antibodies reported in normal sera are similar to those found in pretransplant kidney and multitransfused patients. This indicates that these antibodies may be reported in other than healthy males and that they are detected by the same assays used to detect alloantibodies; therefore, they could pose challenges in transplantation. If such antibodies are determined to be clinically relevant to graft rejection, the possible consequences of their existence in patient sera must be determined, just as with alloantibodies. However, even if they are irrelevant to graft rejection, specificities of these antibodies must still be identified to prevent unnecessary exclusion of potential transplant donors and unnecessary alarm occasioned by reports of false specificities. In a recent study, we have demonstrated that antibodies to denatured antigen are not relevant to long-term kidney graft survival, whereas antibodies to intact HLA antigens lower long-term graft survival [15]. This new awareness of epitopes of these antibodies, together with the knowledge of what epitopes are targeted by the alloantibodies, now makes it

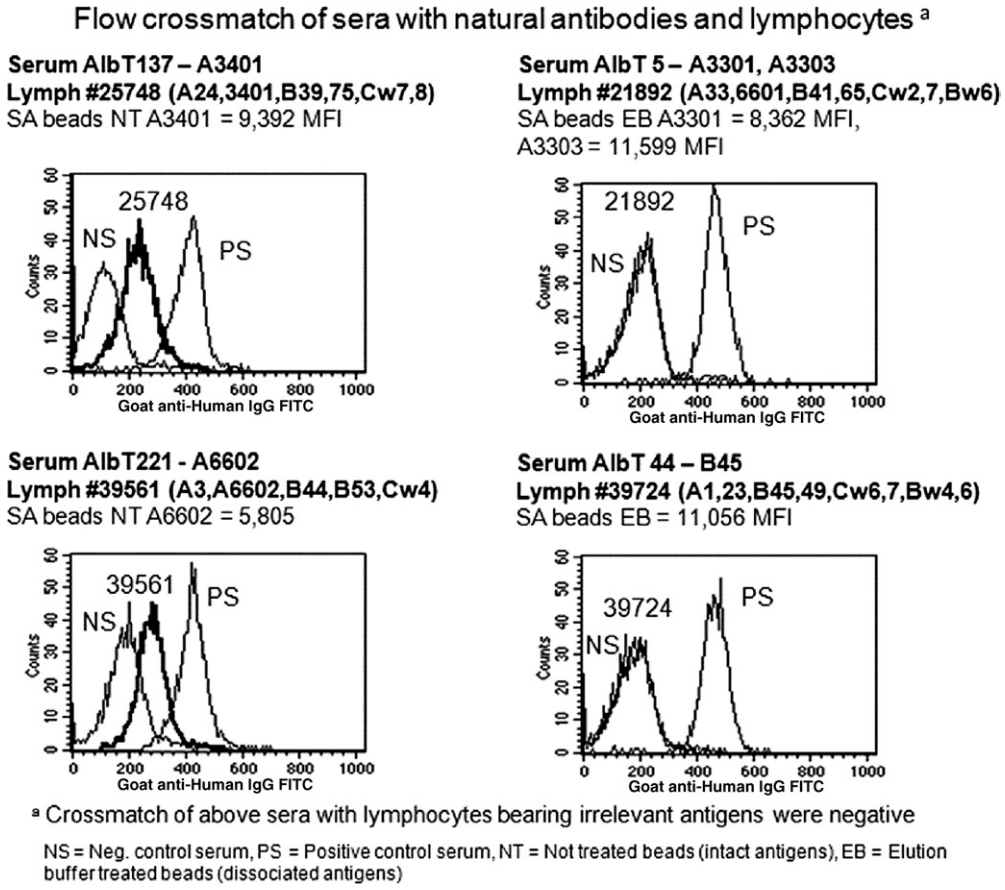

Fig. 7. Crossmatches of sera positive with intact antigens and sera positive with dissociated antigens on the single beads with lymphocytes bearing corresponding specificities are illustrated. Sera AlbT137 and AlbT221 positive with the intact SA A3401 and A6602, respectively, and negative with the dissociated forms of these antigens exhibit positive reactions with lymphocytes bearing these antigens. On the other hand, sera AlbT5 and AlbT44 positive with the dissociated SA (A3301, A3303) and B45, respectively, and weak (A3301, A3303) or negative (B45) with the intact forms of these antigens exhibit negative reactions with lymphocytes bearing these antigens.

possible to differentiate among the specificities of these antibodies and alloantibodies.

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