ELSEVIER

HLA Class I Epitopes: Recognition of Binding Sites by mAbs or Eluted Alloantibody Confirmed With Single Recombinant Antigens

Nadim El-Awar, Jar-How Lee, Christine Tarsitani, and Paul I. Terasaki

Development of beads coated with single recombinant HLA antigens has permitted the confirmation and further definition of HLA class I epitopes. In this study, monoclonal antibodies (mAbs) or alloantibodies eluted from recombinant cell lines were tested for reactivity with Luminex® beads individually coated with 79 recombinant HLA class I single antigen (rHLA SA). Published amino acid sequences were used to map epitopes common to sets of antigens reactive with each antibody. While several epitopes have already been demonstrated, this study confirmed them by adsorption of allosera with transfectants or SA beads having a single HLA antigen and specific binding of the eluted antibody on SA beads. The allosera and mAbs used in this study recognized a total of at least 58 HLA class I epitopes, as demonstrated by their different adsorption/reactivity patterns. Of these, 25 epitopes were characterized by a single unique common amino acid, 30 shared 2 signature amino acids in close proximity, and 3 epitopes involved 3 specific amino acids in a non-linear sequence. Since these epitopes may be targets for antibody-mediated allograft rejection, epitope analysis should complement HLA and CREG assignment for defining complex antibodies and identifying suitable donors for highly sensitized transplant patients. *Human Immunology 68, 170–180 (2007).*

KEYWORDS: HLA Epitopes; HLA antibodies; HLA public antibodies; HLA crossreactions

ABBREVIATIONS

CREG	cross-reactive groups
HLA	human leukocyte antigen
mAb	monoclonal antibody
SA	single antigen

INTRODUCTION

Shortly after the discovery of the human leukocyte antigen (HLA) specificities in 1965, Svejgaard and Kissmeyer-Nielsen reported the first indication of an association between different HLA specificities, HLA A2 and HLA A28 [1]. The cross-reactivity between other specificities related to HLA B5 was noted in 1970 by Colombani *et al.* [2]. The relation between A1, A11, and A3 was demonstrated in 1972 by adsorption of a series of HLA antibodies with platelets, resulting in a table of the interrelation between specificities [3]. Rodey *et al.* [4] and Fuller *et al.* [5] used serologic cross-reactivity between HLA specificities to define cross-reactive groups (CREGs). These groups were based on the frequent association of certain HLA antigens reactive with numerous allosera. These cluster reactions were defined as *public epitopes*. The broadest groups were the 4a-4b (Bw4–Bw6) specificities first found by van Rood in 1963 [6], with each including several HLA specificities. In a study of 50,000 sera from multiparous women, a listing of public specificities was postulated to actually be directed against a single epitope [7].

Early observations of serologic cross-reactivity were later explained by elucidation of the molecular structure of the HLA molecule. For instance, the A2-A28 cross-reaction was demonstrated to likely be the result of a common epitope on the molecule [8, 9]. Further, the Bw4 and Bw6 public specificity could be traced to a

From One Lambda Inc., Canoga Park, CA, USA (N.E.-A., J.-H.L., C.T.); and Terasaki Foundation Laboratory, Los Angeles, CA, USA.

Address reprint requests to: Mr. Nadim El-Awar, One Lambda Inc., Research II, 11570 W. Olympic Blvd., Los Angeles, CA 90064; Tel: (310) 914-5240; Fax: (310) 914-5250; E-mail: nelawar@onelambda.com.

Received September 16, 2006; accepted November 15, 2006.

0198-8859/07/$–see front matter
doi:10.1016/j.humimm.2006.11.006

single location on the HLA molecule [10–12]. Bjorkman *et al.* discovered that the HLA-A2 molecule displayed multiple epitopes or antibody binding sites [13]. Many of the early CREGs were explained by common epitopes on the molecule [4, 5, 14, 15]. Duquesnoy *et al.* postulated that groups of three consecutive amino acid residues may act as immunogenic epitopes [16, 17].

Monoclonal antibodies produced in mice are assumed to react to a single epitope. One of the first epitopes to be identified by monoclonal antibodies (mAbs) [18] and by allosera [19] was A2-B17, which was later confirmed by studies of the molecular structure [20]. Many of the target epitopes of anti-HLA mAbs were found to be public in nature (*i.e.,* shared by multiple HLA specificities) [12, 21, 22]. Thus, each epitope may occur on various HLA molecules, and each HLA antigen may carry several different epitopes. Hence, there is a need for a more specific definition of the immunogenic HLA epitopes in terms of molecular structure versus serologic cross-reactivity.

The recent development of microbeads coated with single HLA antigens isolated from recombinant lines [23] has made it possible to analyze and define the epitopes recognized by mAbs (One Lambda Inc., Canoga Park CA), and to critically examine complex allosera. Methods of antibody analysis based on testing a large panel of cells, in use now for over 40 years, have recently been demonstrated to be inadequate for accurately identifying specificities [24]. We demonstrate here that adsorption of allosera with recombinant cells expressing a single HLA antigen, and testing of subsequently eluted antibody against single antigen (SA) coated beads, can provide information on the target epitopes for those antibodies.

We have confirmed and expanded upon the list of known HLA class I epitopes by analysis of the eluate versus single antigen immunobinding reactions using molecular modeling and amino acid sequence information. The first 58 epitopes, which we have thus far confirmed by this method, are described here. The importance of identifying these epitopes is that, according to the humoral theory of transplantation, they may be the transplantation antigens responsible for antibody-mediated transplant rejection [25, 26].

MATERIALS AND METHODS

Monoclonal Antibodies

Eighteen mouse mAbs produced by conventional methods were tested as supernatants of cloned hybridoma cell lines or aliquots of ascites diluted 1:10 to 1:500,000. Because most of the mAbs were not purified, protein concentrations of the final dilutions were not determined. The mAbs used for this study were from repeatedly cloned hybridoma cell lines. Their specificity has been previously characterized by both serologic and immunobinding assays. Since mAbs by definition are monospecific, adsorption experiments were not generally conducted.

Allosera

Thirty-five anti-HLA alloantibody samples were obtained from multiparous women, placentas, or patients undergoing platelet transfusions or transplants. Sera were adsorbed by an appropriate rHLA SA cell line derived from the LCL712.2 B host cell line. The rHLA SA cell line used for adsorption of the antibody was selected based on the known serologic specificity of each sample. Forty μl of serum (diluted 1:3) was mixed with 3 to 5 $\times$ 10^6 cells and subsequently incubated for 30 minutes at room temperature (RT). The cells were then centrifuged to remove the adsorbed sera for testing. Some sera were adsorbed using a purified rHLA SA attached to microsphere beads; 0.5 $\times$ 10^6 beads were used per adsorption and the antibody was eluted as described below.

Antibody Eluates

After adsorbing the allosera with the cell lines or beads, the cells/beads were washed with phosphate buffered saline (PBS). The adsorbed antibody was then eluted by mixing 60 μl of ImmunoPure IgG Elution Buffer (Pierce, Rockford, IL USA. Catalog 21004) with the cells or beads and incubating for 10 minutes at RT. After incubation, the eluates were separated by centrifugation, removed, and neutralized by 3 μl of 1M TRIS-HCl pH 9.5. Most eluates were from an initial adsorption/elution step. However, in some cases the initial eluate was adsorbed by another recombinant cell line and the second eluate was tested with the SA beads.

Single Antigen Bead Assays

Monoclonal antibodies, or antibody eluates, were tested with 79 HLA class I (A and B-locus) rHLA SA individually coupled to different microsphere beads and with negative and positive control beads (LABScreen beads: LS1A01 and LS1A02, One Lambda Inc.) [23]. The HLA alleles represented in the SA bead panel are listed in Table 1. LABScreen assays were performed according to the manufacturer's protocol. Briefly, 20 μl of test serum was added to 5 μl each of LS1A01 and LS1A02 SA beads, incubated in the dark for 30 minutes at RT, and then washed with wash buffer. One hundred microliters of goat anti-human IgG or goat anti-mouse IgM or IgG secondary antibody conjugated with R-phycoerythrin was added to the beads, incubated for 30 minutes in the dark at RT, then washed and read on the LABScan 100 flow cytometer (One Lambda Inc.). With every assay, we ran a negative control serum for the allosera and PBS, or a mouse IgM control (Sigma-Aldrich, St. Louis, MO

TABLE 1 Single rHLA class I antigens coated on beads used for immuno-binding assay

Antigen	Allele	Antigen	Allele	Antigen	Allele	Antigen	Allele	Antigen	Allele
A1	A0101	A33	A3303	B13	B1301	B48	B4801	B64	B1401
A11	A1101	A34	A3401	B18	B1801	B49	B4901	B65	B1402
A11	A1102	A36	A3601	B27	B2705	B50	B5001	B67	B6701
A2	A0201	A43	A4301	B27	B2708	B51	B5101	B7	B0702
A2	A0203	A66	A6601	B35	B3501	B51	B5102	B71	B1510
A2	A0206	A66	A6602	B37	B3701	B52	B5201	B72	B1503
A23	A2301	A68	A6801	B38	B3801	B53	B5301	B73	B7301
A24	A2402	A68	A6802	B39	B3901	B54	B5401	B75	B1502
A24	A2403	A69	A6901	B39	B3905	B55	B5502	B76	B1512
A25	A2501	A74	A7401	B4005	B4005	B56	B5601	B77	B1513
A26	A2601	A80	A8001	B41	B4101	B57	B5701	B78	B7801
A29	A2901			B41	B4102	B57	B5703	B8	B0801
A29	A2902			B42	B4201	B58	B5801	B81	B8101
A3	A0301			B44	B4402	B59	B5901	B82	B8201
A30	A3001			B44	B4403	B60	B4001		
A31	A3101			B45	B4501	B61	B4002		
A32	A3201			B46	B4601	B62	B1501		
A33	A3301			B47	B4701	B63	B1516		

USA M5909 from tumor line MOPC-104E) for the mAbs to determine the background due to nonspecific binding.

Data Analysis

Data generated from the LABScan 100 were analyzed with computer software. Trimmed mean fluorescence values for the SA bead reactions were obtained from the output (.csv) file generated by the flow analyzer and were adjusted for background signal using the formula [(sample #N bead − sample negative control bead) − (negative control #N bead − negative control negative control bead)]. The adjusted reaction values were then normalized by multiplying each value by a corresponding normalization factor derived from the results of the mAb W6/32 with the same beads. Normalization factors were calculated by dividing the average value of all mAb W6/32 reactions by the adjusted fluorescence value for each bead. The data were then graphed with either Excel spreadsheets (Microsoft Corp., Redmond, WA) or HLA Visual software (One Lambda Inc.). All normalized reactions that were above zero were considered as potential positive reactions for this study, but values for the epitope-specific reactions were rarely below 500.

HLA Amino Acid Sequences, Epitopes, and Distances Between Residues

Amino acid sequences of the HLA antigens or alleles were downloaded from the Anthony Nolan Web site [27]. Based on the data analysis mentioned above, we determined the positive antigens for each mAb or eluted alloantibody. An epitope search program was then utilized to identify distinguishing amino acids (aa) that are exclusively shared by the positive antigens at particular sequence positions. The program searched for one, two, or three common unique aa positions. Among the many possibilities generated, we selected for consideration positions that are exposed to the surface of the molecule and that are within the antibody binding span estimated at 494 $Å^2$ (19 × 26 Å) [28] or 750 $Å^2$ [29]. Approximate distances in angstroms between two amino acids were calculated using the Cn3D Viewer software [30] and the three-dimensional structure of an HLA-A0201 molecule 1QEW [31]. Unfortunately, three-dimension structural information for most HLA antigens is not available. Therefore, it was not possible to determine whether the side chains of the amino acids of a certain epitope are oriented in the correct direction, which would facilitate the binding of the antibody. Amino acids that were exclusively unique to a group of antigens that were reactive with a mAb or an eluted alloantibody, preferably exposed to the surface of the molecule and within the binding span of the antibody, were considered a distinguishing characteristic of the epitope. Epitope ID numbers were assigned depending upon the number of unique aa sites involved (1–200 for one aa, 201–400 for two aa, or 401–500 for three or more aa positions).

RESULTS

A total of 58 HLA class I epitopes were recognized by the series of monoclonal antibodies and eluted alloantibodies used in this study. Overall, we found 20 epitopes shared by A-locus antigens, 27 epitopes shared by B-locus antigens, and 11 interlocus epitopes shared by A and B locus antigens. Some of these epitopes have previously been described. However, this study is the first to con-

firm epitope sites using antibody eluted from single HLA antigen and then reacted with rHLA SA beads, and it is the first to define several of the epitopes.

The mAbs selected for this study recognized epitopes with either one or two unique aa at certain positions (Table 2). The combination aa positions were not contiguous but were within a conformational distance, allowing antibody binding. The interlocus epitope (204) is an example of an epitope defined by two uniquely shared aa positions. The reactive HLA antigens A32, A74, B8, B18, B37, B38, B39, B41, B42, B54, B55, B59, B64, B65, & B67 all were found to have leucine (L) at position 109 and threonine (T) at position 163. Although, several other HLA class I antigens have the 109(L) and 163(T), none of these antigens share both amino acids at these two positions, making the aa combination unique for this mAb-reactive group of HLA antigens.

For the allosera, eluates recovered from the recombinant rHLA SA cell lines were found to recognize epitopes consisting of unique aa residues at 1, 2, or 3 sequence positions (Table 3). Epitope 13 is an example of a single aa epitope that was defined by serum W7252.AO adsorbed with a recombinant cell line expressing only the HLA-A0101 antigen. The eluted antibody reacted with A1, A2, A3, A11, A24, A36, A68, A69, and A80 antigens. All nine antigens share the amino acid lysine (K) at position 144 in the alpha 2 domain of the HLA molecule. No other antigen among the 79 HLA-A and -B locus SA beads tested was positive with the eluted antibody, nor do they contain144 (K). Therefore, lysine at position 144 may define this epitope.

A few epitopes were defined by three unique aa positions in combination, such as epitope 401 (Table 3), shared by the antigens B7, B42, B67, B81, B63, B2705, B2708, B54, B55, B56, B5701, B5703, B58, and B82 antigens. This epitope is characterized by proline (P), alanine (A), and glutamic acid (E) at positions 43, 69, and 76, respectively, occurring together. All three residues are within the reach of the antibody-binding span Materials and Methods section. The furthest residues, 43(P) and 76(E), of this epitope are 20.5 Å apart.

Two different eluates for serum X9288.00 (Table 3) yielded two different specificities (epitopes 14 and 15). The eluate from the A2402 rHLA cell line reacted with the A1, A23, A2402, A80, and B76 SA beads, whereas a separate eluate from the A2902 rHLA cell line reacted with the A1, A26, A29, A36, A43, and A80 SA beads. These results indicate that this alloserum contains at least two antibodies that recognize different epitopes shared by two different groups of antigens. The fact that A1 and A80 each express both of these epitopes suggests that either antigen may have been the immunogen for

TABLE 2 HLA class I epitopes recognized by a series of mAbs

mAb tested	Single antigen[a] beads with positive reactions	Epitope no. assigned[b]	Position and unique aa for possible epitope[c] sites
Z3945.OL	A1,36	1	44K/150V/158V/
X3653.AP	A2,69	2	107W
Z1238.TO	A23,24	3	65G
Z8855.TO	A25,26,34,43,66	4	(9Y)+149T/(74D)+149T
X5518.TO	A29,43	5	62L
X8341.EO	A3	6	161D
F1398-2EH1	All B-locus antigens except B57,58,63	7	65Q
Z0693.TO	B13	8	145L/41T+46A[d]
Z7567.RO	B38,39,67	9	158T
Z5550.DM	B46	10	69R
X7768.TO	B8	11	(9D)
S8043	A2	201	43Q+62G/62G+66K/62G+76V/62G+79G
Z1060.TO	A23	202	65G+151R/127K+144Q/127K+151R
Z1022.HO	A2402	203	156Q+166D/156Q+167G
F760-5B5D8	A32,74,B8,18,37,38,39,41,42,54,55,59,64,65,67	204	109L+163T
F1119-9F4E7	A32,74,B7,8,4005,41,42,48,60,61,73,81	205	109L+131R
X7138.HO	A36	206	158V+163T/158V+166E/158V+167W
Z1203.TO	B57,58,63	207	43P+65R/65R+163L/66N+131S/66N+163L

Abbreviations: mAb = monoclonal antibody; aa = amino acid.

[a] Alleles are designated only when other alleles of the same antigen did not react with the mAb.

[b] Epitopes 1–11 can be defined by a single amino acid at a unique position on the HLA molecule. Epitopes 201–207 involve two amino acids/positions considered in combination.

[c] Possible alternative epitope definitions are separated by "/". Epitopes that are defined by more than a single position/aa are separated by "+". Amino acids that are not exposed at the surface of the HLA molecule are between parentheses.

[d] Epitope 8: in addition to the unique aa leucine (L) at position 145, this epitope can also be defined by the two amino acids threonine (T) at position 41 and alanine (A) at position 46.

TABLE 3 HLA class I epitopes recognized by a series of anti-HLA allosera

Alloserum tested	rHLA[a] cells used for adsorption	Single antigen[a] beads reactive with eluted antibody	Epitope no. assigned[b]	Position and unique aa for possible epitope[c] sites
AS264	A2501	A1,11,25,26,43,6601	12	163R
W7252.AO	A0101	A1,2,3,11,24,36,68,69,80	13	144k
X9288.OO	A2402	A1,23,2402,80,B76	14	166D/167G
X9288.OO	A2902	A1,26,29,36,43,80	15	76A
W6409	A8001	A1,36,11,25,26,34,43,6601,80,B73	16	90D
X4632	B5801	A2, B57,58	17	62G
X4632	A6901	A2,68,69	18	142T/145H
Z6303	A6901	A2,23,24,68,69	19	127K
ARCJA	B0702	B7,8,41,42,48,60,81	20	177D/180E
W7248.BO	B4402	B13,4005,41,44,45,47,49,50,60,61	21 see Fig. 2	41T
Z7227.00	B1301	B8,13,18,35,37,38,39,3905,4005,41,44,45,47, 48,49,50,51,52,53,59,60,61,62,64,65,71,72, 75,76,77,78	22	69T
RGF19	B4901	A23,24,25,32,B38,49,51,52,53,57,58,59,63,77	23	80I
RGF19	B4403	All Bw4 associated antigens in SA bead panel	24 see Fig. 1	82L/83R
X1779.BO	B5501	All Bw6 associated antigens in SA bead panel	25	80N
X9174	A8001	A1,3,11,24,36,80	208	142I+144K/144K+145R
Z2076.@O	A2501	A11,25,26,43,6601	209 see Fig. 3	163R+166E/158A+163R/163R+167W
SE896	A1101	A2,3,11,68,69	210	76V+144K
RGF40	B76 (B1512)	A203,25,26,34,43,66,B46,62,76	211	(152E+156W)
X8059.C0	B5701	A23,24,32,B38,49,51,52,53,57,58,59,63,77	212	80I+149A/80I+90A
Z6895.00	A3101	A23,25,26,29,30,31,32,33,34,43,66,74	213	138M+144Q
Z3044	A6901	A25,26,33,34,66,68,69	214	43Q+62R/62R+109F
Z7227.00	B5401/A6801	A33,34,68,69,B8,18,37,38,39,41,42,54,55,59, 64,65,67	215	62R+163T
Z3038	B4102	All Bw6 associated except B46 & B73	216	76E+80N/76E+82R/76E+83G
RGF41	B4403	B13,2705,37,44,47	217	76E+80T/79R+80T/80T+82L/80T+83R
X9733	B76 (B1512)	B13,57,62,63,75,76,77	218	46A+76E
X6101	B1801	B18,35,37,51,52,53,78	219 see Fig. 4	45T+62R/45T+65Q/45T+66I/45T+69T/ 45T+71T
SE897	A3301	B18,51,52,64,65,78,A3301	220	90A+(171H)
RGF19	B5601	B53,35,63,49,50,75,77,4005,56,71,62,78,52, 51,72,58,46,57	221	163L+167W
X3245.AO	B7301	B7,13,27,47,48,60,61,73,81,A6602	222	163E+166E/163E+167W
RGF37	B0703	B7,13,27,47,48,60,61,81	223	76E+163E
X0786.00	B5801/B5502	B7,27,42,54,55,56,57,58,63,67,73,81,82	224	69A+43P
RGF38	B0801	B8,59	225	(67F)+163T
Z6895.00	B1801	B8,64,65,38,39,42,67,18,37,41,54,55,59	226	43P+163T
X4715.D0	B4501	B8,64,65,39,48,62,75,72,71,76,18,35,60,61, 4005,41,45,50,78	227	69T+80N/69T+82R/69T+83G
Z6331.00	B3905	B64,65,38,39,67,18,37,54,55,59	228	131S+163T
Z6331.00	B8101	B7,42,67,73,81,27,54,55,56,82	229	65Q+69A
Z2980.BO	B5201	B38,77,49,51,52,53,59	230	65Q+80I/69T+80I
Z6328.00	B0702	B7,42,67,81,63,27,54,55,56,57,58,82	401 see Fig. 6	43P+69A+76E
Z9009.A0	B8201	B7,42,67,81,54,55,56,82	402	65Q+69A+70Q
Z6845.B0	B76 (B1512)	B62,75,76,77,46	403	41A+46A+65Q

Abbreviations: aa = amino acid.

[a] Alleles are designated only when other alleles of the same antigen did not react with the eluate.

[b] Epitopes 12–25 can be defined by a single amino acid at a unique position on the HLA molecule. Epitopes 208–230 and 401–403 involve either two or three amino acids/positions, respectively, considered in combination.

[c] Possible alternative epitopes are separated by "/". Epitopes that are defined by more than a single position/aa are separated by "+". Amino acids that are not exposed at the surface of the HLA molecule are between parentheses.

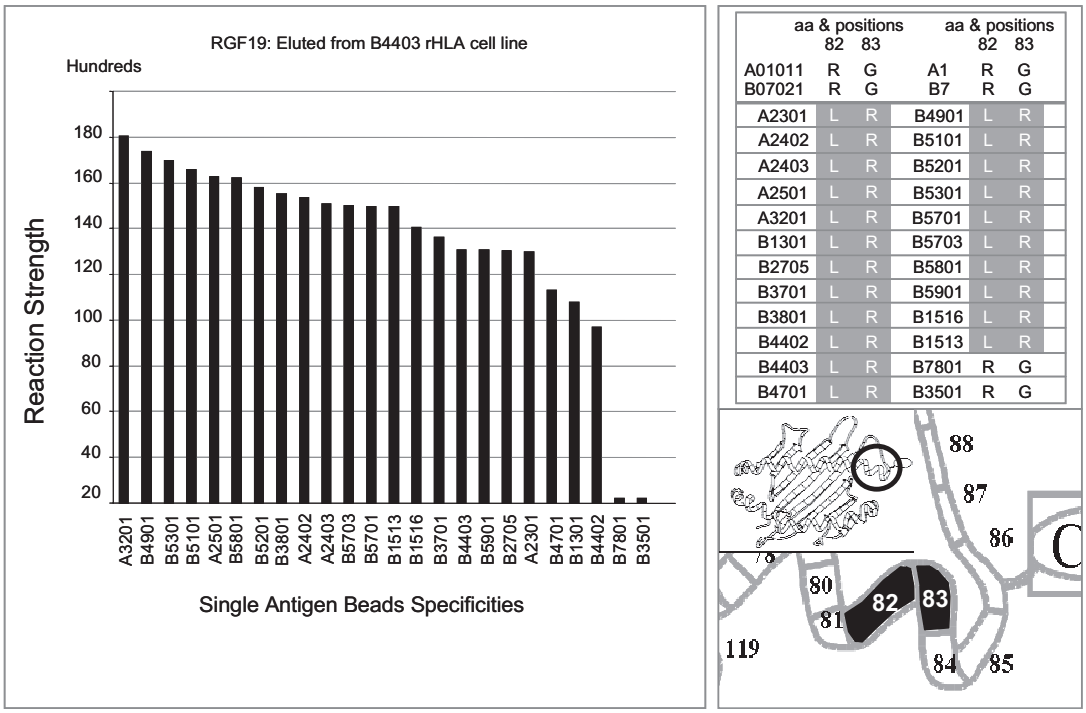


FIGURE 1 All single antigen (SA) beads coated with Bw4 associated antigens were positive with alloantibody from RGF19 serum adsorbed and eluted from a B4403 rHLA SA cell line. Only the reactive antigens have amino acids (aa) leucine (L) at position 82 and arginine (R) at position 83 in the alpha 1 domain of the molecule (epitope 24, Table 3).

this alloserum, although any other two antigens (one from each group) could also have resulted in this dual epitope specificity.

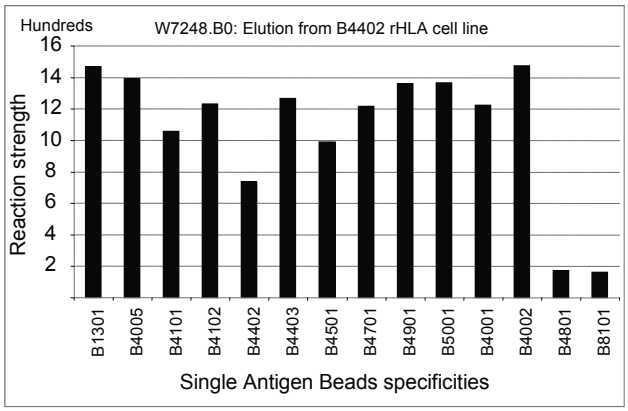
The actual fluorescence data for several of the immunobinding reactions are presented graphically (Figures 1–6) for the positive (epitope-specific) reactions for several allosera and one mAb (Figure 5). Only the highest negative reactions are included to demonstrate the marked drop in signal from the epitope-positive antigens. The other SA beads experienced little or no fluorescent signal. The figures also depict the relevant aa residues at the pivotal antibody-binding positions, as defined by their unique sequence composition for the reactive HLA alleles on the SA beads.

Figure 1 illustrates the reactions of SA beads with an antibody eluted from the B4403 antigen. All positive antigens are the Bw4-associated antigens among the 79 antigens tested. They uniquely have adjacent amino acids leucine (L) at position 82 and arginine (R) at position 83, which defines epitope 24 (Table 3). The fact that all Bw4-associated antigens (and no Bw6 SA beads) reacted to antibody eluted from a single rHLA cell line is a clear demonstration of the power of SA beads in confirming epitope-specific antibody reactions.

Figure 2 illustrates an epitope defined by one single amino acid. All positive antigens B13, B4005, B41, B44, B45, B47, B49, B50, B60, and B61 reacted with an eluate from the rHLA cell line expressing B4402. They all exclusively share the amino acid threonine (T) at position 41, which defines epitope 21 (Table 3).

Figure 3 illustrates the results of the SA beads with an alloantibody that was eluted from the A2501 recombinant cell line. Antigens A11, A25, A26, A43, and A6601 show strong positive reactions with the eluate and exclusively have the amino acids arginine (R) at position 163 and glutamic acid (E) at position 166, defining epitope 209. Although other antigens have arginine at position 163 or glutamic acid at position 166, none have both aa at the two positions, indicating that the unique combination may create this epitope. In this case, however, additional possibilities include 158A + 163R/ or 163R + 167W as alternative configurations that could be the binding site for the antibody (Table 3).

Figure 4 illustrates a conformational epitope defined by two amino acids 17 residues apart. The single antigens B18, B35, B37, B51, B52, B53, and B78 were positive with an eluate from a B1801 single antigen recombinant cell line, and all share exclusively the amino acids threonine and arginine at positions 45 and 62, respectively, defining epitope 219 (Table 3). Although linearly the two positions may seem too far apart for the antibody to bind both, their conformational positions are close and within the antibody binding span. The two residues are only 9 Å apart.



aa & position	41
B07021	A
B1301	T
B4005	T
B4101	T
B4102	T
B4402	T
B4403	T
B4501	T
B4701	T
B4901	T
B5001	T
B4001	T
B4002	T
B4801	A
B8101	A

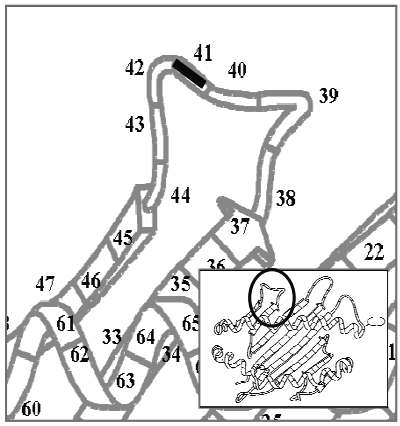


FIGURE 2 SA beads B13, B4005, B41, B44, B45, B47, B49, B50, B60, and B61 were positive with alloantibody eluted from a B4402 rHLA SA cell line. The positive antigens all share the amino acid threonine (T) at position 41 on a side loop of the human leukocyte antigen (HLA) molecule (epitope 21, Table 3). aa = amino acid.

Figure 5 illustrates the reactions of the SA beads with a mouse mAb. Antigens A32, A74, B4005, B4101, B4102, B42, B48, B60, B61, B7, B73, B8, and B81 show strong positive reactions with mAb F1119-9F4E7 and exclusively share the amino acids leucine and arginine at the combined positions 109 and 131, respectively, defining epitope 205 (Table 2). The two amino acids are located in two side loops of the HLA alpha chain and are about 15 Å apart.

Figure 6 illustrates an epitope defined by three amino acids. The eluate from the B0702 antigen reacted positive with B54, B5701, B55, B5703, B56, B63, B58, B82, B7, B67, B81, B2708, B42, and B2705 antigens. All antigens share exclusively the amino acids proline (P), alanine (A), and glutamic acid (E) at positions 43, 69, and 76 respectively, defining epitope 401 (Table 3). The antigens in this group also share 2 unique aa at positions that are not exposed at the surface of the HLA molecule, but these were presumed to be inaccessible as either immunogens or targets for antibody. Thus, the conformational epitope recognized by this antibody is more likely defined by the three aa residues and positions indicated. The farthest two residues 43(P) and 76(E) are 18.9 Å apart and are within the binding span of the antibody.

Since high panel reactive antibodies (PRA) sera may contain antibodies with multiple distinct epitope specificities (*e.g.,* from multiple transfusions), we investigated the feasibility of identifying such antibodies with the SA beads. For four of the allosera, we did one or two additional adsorption-elutions with different rHLA SA cell lines and obtained an eluate that was positive to another epitope (see Table 3). For X9288 (discussed above), X4632, and Z6331, the second group of reactive antigens always included at least one of the antigens from the first group of positive antigens. The common antigens thus contained both epitopes, and could have generated antibody to either one.

For alloserum RFG19, adsorption with B4901 separated out an antibody against a subgroup of Bw4-associated antigens with epitope 23 (80I). A separate adsorption with B4403 produced an eluate reactive with all Bw4 positive single rHLA in the bead panel (epitope 24, 82L/83R, Figure 1). The third eluate, from a B5601 rHLA SA cell line, recognized epitope 221 (163L + 167W). The reactive antigens included B49, 53, 63, and 77 from the first positive antigen set (epitope 23), plus some other Bw4 antigens from the second set (epitope 24), and the Bw6-related antigens B35, B50, B75, B4005, B56, B71, B62, B78, B72, B46, unique to the third set of antigens (epitope 221). This exemplified the power of adsorption with rHLA single antigens to analyze such complex allosera.

DISCUSSION

We describe here the unique ability of SA beads to identify the epitopes recognized by mAbs and alloantibodies. Even an anti-Bw4 alloantibody (recognizing epitope 24) reacted to 22 separate SA beads coated with single recombinant Bw4-associated HLA antigens, after adsorption and elution from a B4403 rHLA SA cell line (Table 3 and Figure 1). Interestingly, the epitopes defined by murine mAbs are often precisely reflected by allosera, such as the reaction to A2, B57, B58 epitope. We have demonstrated here the elution of antibodies from SA-bearing recombinant cell lines (or beads) and the testing of the eluates with SA beads as a powerful new way to define immunogenic epitopes. The eluted antibodies have been demonstrated to react to groups of HLA single antigens that share unique amino acids at specific sequence positions, reflecting the composition of the target epitopes. With this method, conformational epitopes composed of two or three noncontiguous aa

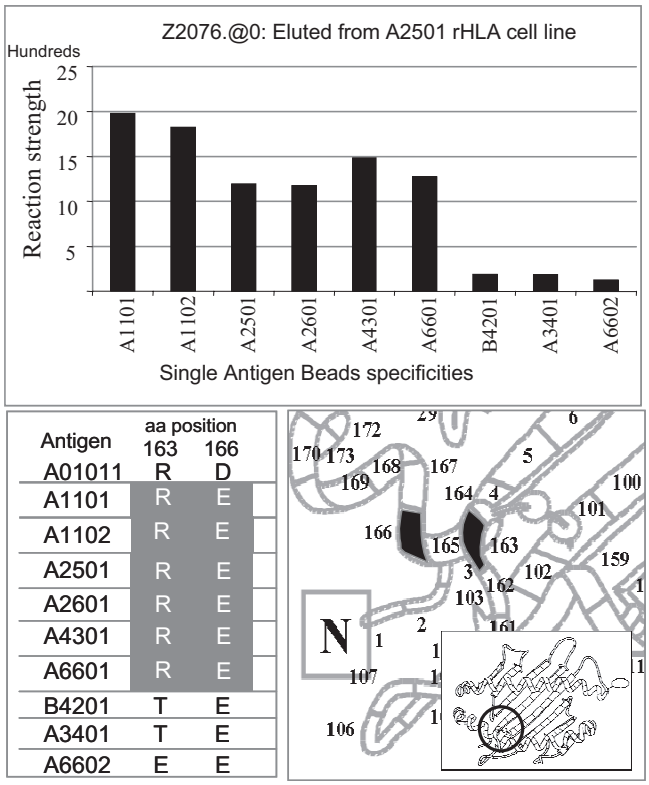


FIGURE 3 Antibody eluted from the A2501 rHLA SA cell line reacts strongly with only A11, A25, A26, A43, and A6601 SA beads. These antigens have the unique combination of arginine (R) at position 163 and glutamic acid (E) at position 166 on the HLA molecule (epitope 209, Table 3). aa = amino acid.

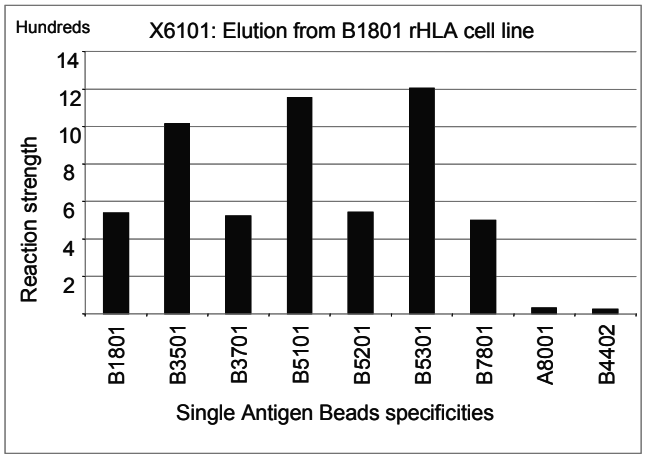
positions were demonstrated, and many previously recognized single aa epitopes were confirmed.

Earlier attempts to match all possible epitopes formed by unique combinations of amino acids were unsuccessful [32], emphasizing the need to identify the immunogenic-epitopes. Duquesnoy has proposed triplet epitopes taking three amino acids around variable positions on the HLA molecule [16,17]. However, this method fails to identify many epitopes, which are conformational in nature. Our current study using antibodies to identify the epitopes demonstrates clearly that many epitopes are conformational in nature. For example, two amino acids that are separated linearly may be sufficiently close in the three dimensional structure to act as an epitope for some antibodies. As an example, the epitope for A2402 is on positions 156 and 166 or 156 and 167 (Table 2).

All of the epitopes described here have been defined by antibodies directed against them. These epitopes are of potential importance in transplantation since they are the antigens against which the antibodies had been made. Thus, it is reasonable to suggest that the humoral responses to an allograft, which cause hyperacute, acute, or chronic rejection, are directed against these epitopes [26]. According to the humoral theory [25, 26], we hypothesize that these epitopes are the key factors that need to be matched for transplants, as noted earlier [33]. This study focused only on the HLA-A and -B locus class I epitopes. A similar study of class II epitopes has also been conducted in our laboratory [34]. Analysis of larger numbers of mAb or allosera reagents may lead to the identification of additional immunogenic epitopes. For convenience, we sequentially numbered the epitope in this series based on the number of unique amino acids shared by a set of reactive antigens. However, as more information becomes available, a formal consensus nomenclature may be needed to systematically encompass the complex array of HLA epitopes.

Prior attempts to determine which antigens might be stronger than others and which might be permissible mismatches can now be reinvestigated with consideration of epitopes [35]. This effort should be of importance both for solid organs as well as for unrelated bone marrow transplants, where one or two epitopes may be mismatched when a complete match cannot be found.

The allosera selected for this study generally exhibited multiple HLA specificities. We did not exhaustively define all of the specificities in each serum. However, it is noteworthy that in all of these cases the antibody eluted from a single recombinant HLA antigen reacted with antigens exclusively sharing the amino acids defining a single epitope. The experiments with more than one adsorption of a serum (using different rHLA) identified multiple epitope-specific antibodies. These data support the concept that the different epitopes presented by an HLA protein molecule are the actual immunogenic sites of importance and demonstrate the value of single



Antigen	aa position 45	62
B07021	E	R
B1801	T	R
B3501	T	R
B3701	T	R
B5101	T	R
B5201	T	R
B5301	T	R
B7801	T	R
A8001	E	E
B4402	K	R

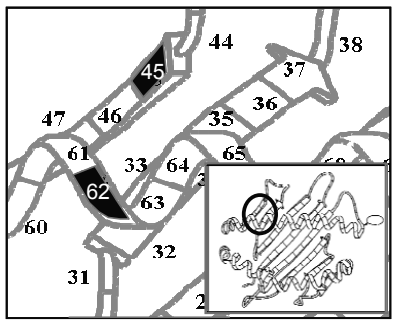


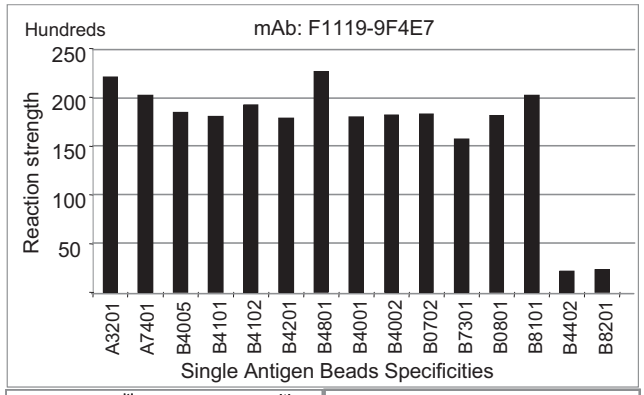
FIGURE 4 Example of a conformational epitope dependent on threonine (T) at position 45 and arginine (R) at position 62. Eluate from the B1801 recombinant SA cell line recognized only B18, B35, B37, B51, B52, B53, and B78 single antigens, which are the only HLA molecules with this amino acid (aa) signature. Although separated linearly by 17 aa residues, the two amino acids are 9 Å apart and within the antibody's binding span (epitope 219, Table 3).

recombinant HLA antigens in dissecting these epitope-specific reaction patterns.

These immunogenic epitopes will be useful in the classification of antibodies. We have previously called attention to the difficulty in finding all the antibodies in a serum by conventional methods and the need for testing with SA technology [24]. The complex series of antibodies identified by the SA beads can be best understood when they are defined by the epitopes actually recognized. Thus, rather than describing the series of antigens reacting with an antibody, we propose to assign the antibody specificity to the target epitopes. Further, by applying our knowledge of the reactive epitopes, the often-encountered technical problem of how to determine the cutoff between positive and negative reactions when testing SA beads is also addressed.

SA bead testing can also refine current understanding of antibodies to HLA CREG antigens. Specifically, the well-known CREGs established by the reactions of antibodies can now be more precisely defined as being antibodies against the epitopes that underlie these CREGs [4]. No two listings of CREGs by different authors are identical. The common CREGs often identified in different listings are confirmed in this study, and some other groups have been added. Epitopes identified here explain many of the complex antibodies previously evidenced in the sera of multiparous women, multitransfused patients, and patients who had rejected an organ transplant. For example, a mismatch to HLA-A1 could result in an antibody to A1, A2, A3, A11, A24, A36, A68, A69, and A80, because all of these share one epitope designated as epitope 13 (Table 3). In addition, antibodies could be generated against other epitopes on the HLA-A1 antigen. Screening for the presence of donor-specific antibodies in solid organ transplant patients often reveals that even a single antigen mismatch can result in the production of antibodies to many other antigens. This phenomenon can now be understood when considering the epitope that was mismatched and the corresponding immune response to the epitope.

To date, we cannot determine if some antibodies are reacting to the peptide alone on the HLA antigen. Minor histocompatibility antigens have been postulated by



	aa position 109	131		aa position 109	131
A01011	F	R	A01011	F	R
B07021	L	R	B07021	L	R
A3201	L	R	B4002	L	R
A7401	L	R	B0702	L	R
B4005	L	R	B7301	L	R
B4101	L	R	B0801	L	R
B4102	L	R	B8101	L	R
B4201	L	R	B4402	L	S
B4801	L	R	B8201	L	S
B4001	L	R			

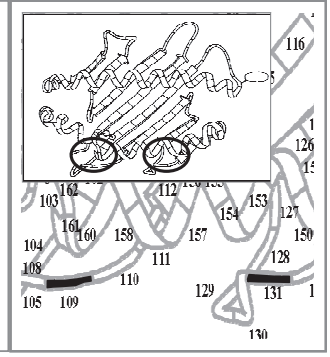


FIGURE 5 Single antigens A32, A74, B4005, B4101, B4102, B42, B48, B60, B61, B7, B73, B8, and B81 exhibit strong immunobinding with mAb F1119-9F4E7 and share unique amino acids (aa) leucine (L) at position 109 and arginine (R) at position 131 (epitope 205, Table 2). The two amino acids are approximately 15 Å apart and are located on the side loops of the HLA molecule.

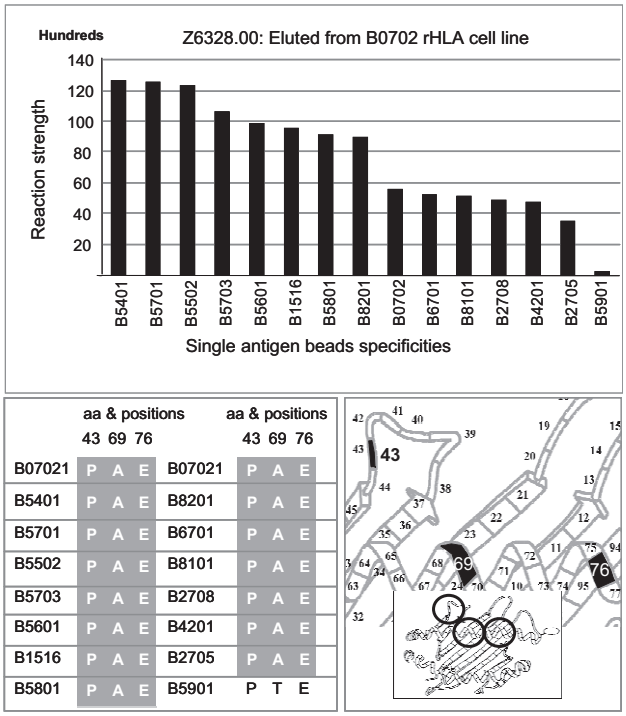


FIGURE 6 SA beads coated with B54, B5701, B55, B5703, B56, B63, B58, B82, B7, B67, B81, B2708, B42, and B2705 are positive to the eluate from B0702. All 14 antigens share the three amino acids proline (P), alanine (A), and glutamic acid (E) at positions 43, 69, and 76, respectively. The amino acid (aa) combination is exclusive to this group of antigens and within the binding span (19 × 26 Å) of a single antibody. The farthest residues, 43P and 76E, are ~18.9 Å apart (epitope 401, Table 3).

Spierings and Goulmy to be defined by peptides within the groove of the antigen [36]. It is quite likely that some antibodies react to an epitope on the peptide alone or to an epitope defined by amino acids present on both the peptide and the heavy chain of the HLA molecule.

ACKNOWLEDGMENTS

We thank Dr. Daniel Cook for helpful suggestions and Mike Chen, Mamie Lias, Rene Castro, Aaron Iddings, April Amonwatvorakul, and Nori Sasaki for their excellent technical assistance.

REFERENCES

1. Svejgaard A, Kissmeyer-Nielsen F: Cross-reactive human HL-A isoantibodies. Nature 219:868, 1968.
2. Colombani J, Colombani M, Dausset J: Cross-reactions in the HL-A System with Special Reference to Da6 Cross-reacting Group. In Terasaki PI (ed): Histocompatibility Testing. Los Angeles, Munksgaard, 1970.
3. Mittal KK, Terasaki PI: Cross-reactivity in the HL-A system. Tissue Antigens 2:94, 1972.
4. Rodey GE, Neylan JF, Whelchel JD, Revels KW, Bray RA: Epitope specificity of HLA class I alloantibodies. I. Frequency analysis of antibodies to private versus public specificities in potential transplant recipients. Hum Immunol 39:272, 1994.
5. Fuller AA, Trevithick JE, Rodey GE, Parham P, Fuller TC: Topographic map of the HLA-A2 CREG epitopes using human alloantibody probes. Hum Immunol 28: 284, 1990.
6. van Rood JJ, van Leeuwen A: Leukocyte grouping. A method and its application. J Clin Invest 42:1382, 1963.
7. Konoeda Y, Terasaki PI, Wakisaka A, Park MS, Mickey MR: Public determinants of HLA indicated by pregnancy antibodies. Transplantation 41:253, 1986.
8. Lopez de Castro JA, Strominger JL, Strong DM, Orr HT: Structure of crossreactive human histocompatibility antigens HLA-A28 and HLA-A2: possible implications for the generation of HLA polymorphism. Proc Natl Acad Sci U S A 79:3813, 1982.
9. Bjorkman PJ, Strominger JL, Wiley DC: Crystallization and X-ray diffraction studies on the histocompatibility antigens HLA-A2 and HLA-A28 from human cell membranes. J Mol Biol 186:205, 1985.
10. Parham P: Antigenic determinants of the HLA-B7 molecule; Bw6- and B7-specific determinants are spatially separate. Immunogenetics 18:1, 1983.
11. Salter RD, Parham P: Mutually exclusive public epitopes of HLA-A,B,C molecules. Hum Immunol 26:85, 1989.
12. Wan AM, Ennis P, Parham P, Holmes N: The primary structure of HLA-A32 suggests a region involved in formation of the Bw4/Bw6 epitopes. J Immunol 137:3671, 1986.
13. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC: Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329:506, 1987.
14. Fuller AA, Rodey GE, Parham P, Fuller TC: Epitope map of the HLA-B7 CREG using affinity-purified human alloantibody probes. Hum Immunol 28:306, 1990.
15. van der Horst AR, Lardy NM, de Waal LP: Analysis of the HLA-A10-crossreacting group of antigens by one-dimensional isoelectric focusing. Immunogenetics 29:1, 1989.
16. Duquesnoy RJ, Marrari M: HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. II. Verification of the algorithm and determination of the relative immunogenicity of amino acid triplet-defined epitopes. Hum Immunol 63:353, 2002.
17. Duquesnoy RJ, Mulder A, Askar M, Fernandez-Vina M, Claas FH: HLAMatchmaker-based analysis of human monoclonal antibody reactivity demonstrates the importance of an additional contact site for specific recognition of triplet-defined epitopes. Hum Immunol 66:749, 2005.

18. McMichael AJ, Parham P, Rust N, Brodsky F: A monoclonal antibody that recognizes an antigenic determinant shared by HLA A2 and B17. Hum Immunol 1:121, 1980.

19. Claas F, Castelli-Visser R, Schreuder I, van Rood J: Alloantibodies to an antigenic determinant shared by HLA-A2 and B17. Tissue Antigens 19:388, 1982.

20. Ways JP, Rothbard JB, Parham P: Amino acid residues 56 to 69 of HLA-A2 specify an antigenic determinant shared by HLA-A2 and HLA-B17. J Immunol 137:217, 1986.

21. Brodsky FM, Parham P, Barnstable CJ, Crumpton MJ, Bodmer WF: Monoclonal antibodies for analysis of the HLA system. Immunol Rev 47:3, 1979.

22. Rodey GE, Fuller TC: Public epitopes and the antigenic structure of the HLA molecules. Crit Rev Immunol 7:229, 1987.

23. Pei R, Lee JH, Shih NJ, Chen M, Terasaki PI: Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. Transplantation 75:43, 2003.

24. El-Awar N, Lee J, Terasaki PI: HLA antibody identification with single antigen beads compared to conventional methods. Hum Immunol 66:989, 2005.

25. Terasaki PI: Humoral theory of transplantation. Am J Transplant 3:665, 2003.

26. Terasaki PI, Cai J: Humoral theory of transplantation: further evidence. Curr Opin Immunol 17:541, 2005.

27. Anthony Nolan Research Institute Web site: Available at: http://www.anthonynolan.org.uk/HIG/data.html. Accessed May 30, 2003.

28. Sheriff S, Silverton EW, Padlan EA, Cohen GH, Smith-Gill SJ, Finzel BC, Davies DR: Three-dimensional structure of an antibody-antigen complex. Proc Natl Acad Sci USA 84:8075, 1987.

29. Amit AG, Mariuzza RA, Phillips SE, Poljak RJ: Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution. Science 233:747, 1986.

30. National Center for Biotechnology Information Web site: Available at: http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml. Accessed November 11, 2005.

31. National Center for Biotechnology Information Web site: Available at: http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=25415. Accessed November 11, 2005.

32. Terasaki PI, Takemoto S, Park MS, Cecka JM, Mickey MR: Molecular HLA matching. Transplant Proc 23:365, 1991.

33. Terasaki PI, Takemoto S, Park MS, Clark B: Landsteiner Award. HLA epitope matching. Transfusion 32:775, 1992.

34. Cai JTP, Mao Q, Pham T, El-Awar N, Lee J: Development of nondonor specific HLA-DR antibodies in allograft recipients is associated with shared epitopes with mismatched donor DR antigens. Am J Transplant 6:2947, 2006.

35. Maruya E, Takemoto S, Terasaki PI: HLA matching: identification of permissible HLA mismatches. In: Terasaki, Cecka (eds): Clinical Transplants 1993, UCLA Tissue Typing Laboratory, Los Angeles.

36. Spierings E, Goulmy E. Expanding the immunotherapeutic potential of minor histocompatibility antigens. J Clin Invest 115:3397, 2005.