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Structural aspects of HLA class I epitopes reacting with human monoclonal antibodies in Ig-binding, C1q-binding and lymphocytotoxicity assays

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ABSTRACT

This study addresses the reactivity patterns of human cytotoxic HLA class I epitope-specific monoclonal antibodies in Ig-binding and complement component C1q-binding Luminex assays in comparison with complement-dependent lymphocytotoxicity data reported at the 13th International HLA Workshop. Some monoclonal antibodies reacted similarly with epitope-carrying alleles in all three assays but others showed different reactivity patterns. These reactivity differences were analyzed with HLAMatchmaker and we incorporated the concept that eplets are essential parts of structural epitopes which can contact the six Complementarity Determining Regions (CDRs) of antibody. The data show that technique-dependent reactivity patterns are associated with distinct differences between polymorphic amino acid configurations on eplet-defined structural epitopes.

The findings have been viewed in context of antigen–antibody complex formation that results in the release of free energy necessary to stabilize binding and to induce conformational changes in the antibody molecule to expose the C1q binding site, the first step of complement activation. Moreover the amount of free energy should be sufficient to induce a conformational change of C1q thereby initiating the first stages of the classical complement cascade leading to lymphocytotoxicity. The complement-fixing properties of HLA antibodies require not only specific recognition of eplets but also depend on interactions of other CDRs with critical amino acid configurations within the structural epitope. Eplet-carrying alleles that lack such configurations may only bind with antibody. This concept is important to our understanding whether or not complement-fixing donor-specific HLA antibodies can initiate antibody-mediated rejection.

1. Introduction

Complement-fixing HLA antibodies play an important role in transplantation because they can initiate within the graft inflammatory processes that lead to rejection [1,2]. For this reason, the complement-dependent lymphocytotoxicity (CDC) test has traditionally been used to detect HLA antibodies but its sensitivity has often been questioned. Most histocompatibility testing laboratories are now using more sensitive Ig-binding assays such as Luminex (Lum-Ig) with single alleles for HLA antibody detection [3]. Because Lum-Ig cannot distinguish complement-fixing antibodies Tyan has introduced a novel Luminex test (Lum-C1q) based on the binding of C1q, the first component of the classical pathway of complement activation [4]. Several studies have demonstrated better correlations between transplant rejection and Lum-C1q detected than Lum-Ig detected HLA antibody reactivity [5–8]. About one-half of Lum-IgG positive sera reacted in Lum-C1q and it is possible that Lum-C1q negative reactions reflect antibodies with low avidity or have IgG subtypes that do not fix complement. About one quarter of Lum-C1q positive antibodies were CDC reactive and this might be due to the relative insensitivity of CDC. Our recent report has shown that the Lum-C1q reactivity of donor epitope-specific antibodies was associated with antibody-mediated rejection and that the efficacy of intervention corresponded with lower titers of such antibodies [9].

HLA epitopes can be characterized by molecular modelling and amino acid sequence comparisons [10,11]. In order to understand antibody specificity one must consider the concept of a structural epitope, i.e. that part of antigen that contacts the six Complementarity Determining Region (CDR) loops on the heavy and light chains of antibody. As reviewed elsewhere [12] stereochemical analyses of crystallized antigen–antibody complexes have shown

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that structural epitopes have about 15–25 contact residues in a surface area of 700–900 Å^2. Each structural epitope has a centrally located so-called functional epitope consisting of a few contact residues that play a dominant role in the specific binding with antibody.

HLAMatchmaker-defined eplets are considered equivalent to functional epitopes [12]. Based on a surface area of 700–900 Å^2, one can estimate that the contact residues of a corresponding structural HLA epitope would be within a radius of about 15 Å of a centrally located eplet. Many HLA antibodies are specific for single eplets but others recognize pairs of eplets that are 6–15 Å apart [13,14]. This means that different CDRs of antibody must interact with distinct configurations within structural HLA epitopes to develop stable complexes.

The structural epitope approach is useful in the interpretation of the reactivity patterns of cytotoxic typing sera against the HLA-A10 splits A25 and A26 [15]. Absorption/elution studies have shown that cytotoxic anti-A25 antibodies exhibit CYNAP (i.e. the Cytotoxicity-Negative, Adsorption-Positive) reactivity with A26 and that cytotoxic anti-A26 antibodies have CYNAP reactivity with A25 [16]. CYNAP means binding only. These antibodies had CYNAP specificity for the 150TAH eplet shared between A25 and A26 [17]. However, the cytotoxic reactivity with A25 required the presence of 80RIA and 82ALR and the cytotoxic reactivity with A26 required 76AN. Apparently, the complement-fixing reactivity of these antibodies involves at least two CDRs, one specific for 150TAH and the other reacting with a critical configuration in the 76–82 sequence location about 12 Å from 150TAH.

This report expands the concept that the complement-fixing abilities of HLA antibodies depend not only on the specific recognition of eplets but also corresponding structural HLA epitopes with certain configurations as critical contact sites. Our studies have compared the reactivity patterns of human anti-HLA class I epitope-specific monoclonal antibodies in all three assays.

2. Materials and methods

Human monoclonal antibodies (mAbs) were produced by cloned hybridomas generated from Epstein-Barr virus transformed B-cells derived from women who became sensitized during pregnancy [18–20]. These mAbs have local descriptions and numbers (e.g. ROU2D3 and HU-70) and 13th International Workshop numbers (e.g. W0025). They are either IgG or IgM type and all of them had been selected because of lymphocytotoxic reactivity with small cell panels. Because of their monoclonality, each recognizes a single epitope presented by an immunizing antigen and shared with antibody-reactive alleles. HLAMatchmaker comparisons of the HLA types of the immunizing antigen and antibody producer will determine all mismatched eplets but only one of them is associated with the epitope recognized by a given mAb.

We compared three methods to determine antibody reactivity patterns with HLA panels. Two binding assays were used: Lum-Ig and Lum-C1q with single allele panels in commercially available kits (One Lambda, Canoga Park, CA) and testing was done according to manufacturer's instructions. Lum-Ig testing used labeled anti-Ig reagents that distinguished between IgG and IgM type antibodies. Antibody reactivities with alleles in the Luminex assays were scored with Median Fluorescence Intensity (MFI) values; alleles were considered as nonreactive if they had similar MFI values as the self-alleles of the antibody producer.

Our analysis included lymphocytotoxicity data generated during the 13th International Histocompatibility Workshop whereby twelve laboratories worldwide had tested these mAbs with panels totaling more than 800 cells HLA-typed at the 4-digit level [21]. This very large panel offers opportunities to select informative cells with only one allele which carries the eplet specifically recognized by antibody. For each allele, we have calculated an average CDC score from traditionally determined reactivity grades 1 (negative), 2 (doubtful positive), 4 (weakly positive), 6 (positive) and 8 (strongly positive). CDC scores were generally determined with three or more informative cells. Average CDC scores of >5.0 were graded positive. We considered a 4–5 CDC score as weakly positive and a <4 CDC score as negative.

For each method, we conducted a HLAMatchmaker analysis of epitope specificity keeping in mind that antibody-reactive alleles carry the same mismatched eplet but may have amino acid differences within the corresponding structural epitopes. Considering the 700–900 Å^2 range of the structural epitope surface and a central location of the functional epitope (considered equivalent to eplet), one can calculate from circular surface = πr^2 that contact residues on the molecular HLA surface should be within about 15 Å from the eplet recognized by antibody. Such residues can be identified with the "select by distance" command of the Cn3D structure software program [22] using informative HLA models downloaded from Entrez Molecular Modeling Database on the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/Structure. This approach has also been used in mutational strategy to analyze epitopes on HLA-B7 [23]. The goal of our study was to determine if technique-dependent differences between antibody reactivity are associated with distinct amino acid configurations in corresponding structural epitopes of eplet-carrying alleles.

3. Results

This report describes six eplet-defined epitopes recognized by mAbs. In each case, we compared the reactivity of specific eplet-carrying alleles in the three assays and looked for residue differences in polymorphic sequence positions within 15 Å.

3.1. 62GE-defined epitope

This epitope is shared between HLA-A2, HLA-B57 and HLA-B58. Monoclonal ROU2D3 gave 62GE-specific positive reactions in all three assays and residue differences between alleles did not affect reactivity with antibody (Table 1a). B*58:01 exhibited a somewhat lower but still significantly positive MFI value in Lum-C1q but this allele was still strongly CDC reactive. The 62GE-specific SN230G6 showed strong reactivity in both Lum-Ig and Lum-C1q (Table 1b). All alleles except B*58:01 had positive CDC scores. We noted that B*58:02 for which only lymphocytoxicity data were available had a CDC score of only 3.3 with eight informative cells (data not shown). SN230G6 had a 3.3 ± 2.1 CDC score with 15 informative B58 cells significantly lower than the 7.7 ± 0.7 CDC score for ROU2D3 ($p < 0.001$). These B58 alleles have 45T rather than 45M present on the CDC-reactive alleles. Position 45 is below the molecular surface and cannot serve as a contact site for antibody but being away only about 4 Å from 62GE, it appears to have a negative conformational influence on the 62GE-defined eplet recognized by SN230G6. Although there was no effect on C1q binding it seems that this residue affected the activation of the classical complement pathway by the complex of SN230G6 with B*58:01. WK1D3 was specific for 62GE in Lum-Ig. A*02:01, A*02:03 and A*02:06 had weak Lum-C1q reactivity and this corresponded with negative CDC scores (Table 1c). In contrast, B*57:01, B*57:03 and B*58:01 reacted in both Lum-C1q and CDC; B*58:02 which was tested only in CDC was also positive (data not shown). The HLA-A2 alleles have multiple residues within 15 Å of 62GE that are different from those on HLA-B57 and HLA-B58. These residue positions are depicted on a structural model of 62GE on A*02:01

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Table 1
Reactivity patterns of three 62GE-specific human monoclonal antibodies.

(a) ROU2D3 (HU-70) W0025 (IgM)
Antibody producer: A1,25; B8,18; Cw7,- Immunizer: A2

Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	Sequence positions with residue differences within 15 Å: 9	11	43	45	46	66	67	70	71	74	156	163
A*02:01	62GE	13596	22954	7.7 (*N* = 211)	F	S	Q	M	E	K	V	H	S	H	L	T
A*02:03	62GE	14529	23869	7.3 (*N* = 6)	F	S	Q	M	E	K	V	H	S	H	W	T
A*02:06	62GE	13571	20588	8.0 (*N* = 27)	Y	S	Q	M	E	K	V	H	S	H	L	T
B*57:01	62GE	13670	23767	8.0 (*N* = 17)	Y	A	P	M	A	N	M	S	A	Y	L	L
B*57:03	62GE	13741	22101	8.0 (*N* = 6)	Y	A	P	M	A	N	M	S	A	Y	L	L
B*58:01	62GE	12256	4103	8.0 (*N* = 7)	Y	A	P	T	E	N	M	S	A	Y	L	L
62GE-negative alleles		9	32	1.3 (*N* = 402)												

(b) SN230G6 (HU-33) W0024 (IgG)
Antibody producer: A24,29; B7,44; Cw7,- Immunizer: A2/B57

Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	Sequence positions with residue differences within 15 Å: 9	11	43	45	46	66	67	70	71	74	156	163
A*02:01	62GE	17112	15679	6.8 (*N* = 215)	F	S	Q	M	E	K	V	H	S	H	L	T
A*02:03	62GE	20371	21265	6.1 (*N* = 7)	F	S	Q	M	E	K	V	H	S	H	W	T
A*02:06	62GE	17297	21981	6.8 (*N* = 23)	Y	S	Q	M	E	K	V	H	S	H	L	T
B*57:01	62GE	16396	14267	7.6 (*N* = 16)	Y	A	P	M	A	N	M	S	A	Y	L	L
B*57:03	62GE	14811	18374	7.7 (*N* = 6)	Y	A	P	M	A	N	M	S	A	Y	L	L
B*58:01	62GE	9084	11813	3.4 (*N* = 7)	Y	A	P	T	E	N	M	S	A	Y	L	L
62GE-negative alleles		163	25	1.3 (*N* = 399)												

(c) WK1D3 (HU-63) W0152 (IgM)
Antibody producer: A1,-; B8,-:Cw7,- Immunizer: A2

Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	Sequence positions with residue differences within 15 Å: 9	11	43	45	46	66	67	70	71	74	156	163
A*02:01	62GE	2751	1170	1.3 (*N* = 198)	F	S	Q	M	E	K	V	H	S	H	L	T
A*02:03	62GE	5058	1169	1.0 (*N* = 6)	F	S	Q	M	E	K	V	H	S	H	W	T
A*02:06	62GE	6821	635	1.1 (*N* = 21)	Y	S	Q	M	E	K	V	H	S	H	L	T
B*57:01	62GE	8622	13953	7.8 (*N* = 17)	Y	A	P	M	A	N	M	S	A	Y	L	L
B*57:03	62GE	11884	11770	8.0 (*N* = 4)	Y	A	P	M	A	N	M	S	A	Y	L	L
B*58:01	62GE	7165	8946	6.8 (*N* = 6)	Y	A	P	T	E	N	M	S	A	Y	L	L
62GE-negative alleles		7	16	1.3 (*N* = 403)												

(Fig. 1a). As discussed below, it should be noted that HLA-A2 was the immunizing antigen for WK1D3.

3.2. 144TKR-defined epitope

This epitope is shared by a group of HLA-A alleles. OK5A3 reacted specifically with all 144TKR-carrying alleles in Lum-Ig and Lum-C1q (Table 2a). All alleles except A*80:01 reacted in CDC. Within 15 Å of 144TKR, there are 15 sequence positions with residue differences between 144TKR-carrying alleles. The CDC nonreactive A*80:01 has one unique residue 151R whereas the CDC-reactive alleles share 151H. Although the nearby 152R might also have affected the non-CDC reactivity of A*80:01 it seems that OK5A3 is specific for 144TKR in Lum-Ig and Lum-C1q but recognizes 144TKR + 151H in CDC. The distance between 144TKR and 151H is about 7 Å far enough for two CDRs that contact the epitope (Fig. 1b).

BRO11F6 (HU-16) is also specific for 144TKR but with a different reactivity pattern than OK5A3 (Table 2b). This mAb does not recognize the 151R-carrying A*80:01 in any assay. However, only A*11:01 and A*11:02 had positive CDC scores. Their sequences 150–152 have a unique residue composition not present in the CDC-nonreactive alleles. This suggests that 151H is a critical contact site for the Ig-and C1q-binding with BRO11F6 but this residue alone is not enough for CDC because nearby residues 150A on the surface and 152A below the surface seem also necessary. The locations of 144TKR and 150AHA on a structural model of A*11:01 suggest that these configurations can be contacted by two different CDRs (Fig. 1b).

3.3. 219W-defined epitope

This epitope is located on the α3-domain of HLA-C molecules. The HLA-Cw9-induced TRA2G9 reacted specifically with 219W-carrying HLA-C alleles in all three assays (Table 3). C*04:01 was at best weakly reactive in Lum-C1q (MFI = 678) but had a strongly positive CDC score (7.7; *N* = 114). This allele has 275K and the other 219W-carrying alleles have 275E.

3.4. 163LW-defined epitope

This epitope is shared by a large group of HLA-B alleles and HLA-Cw3. VDE1F11 reacted specifically with all 163LW-carrying alleles in all three assays (Table 4a). The CDC data had insufficient numbers of reactions (*N* < 3) for B*15:10, B*57:03, B*78:01 and C*03:02; each of these alleles appeared to be CDC reactive as indicated by a combined CDC score of 7.1 for seven informative cells. B*51:01 and B*58:01 had rather low MFI values in Lum-C1q but their CDC scores were positive and weakly positive, respectively). No distinct residue differences were associated with the low Lum-C1q reactivity of B*51:01 and B*58:01. Altogether, the Lum-Ig and CDC reactivities correlated well and appeared unaffected by any residue difference in the 17 polymorphic sequence positions within 15 Å of 163LW.

The B*15:03-induced OK6H10 had Lum-Ig specificity for all 163LW alleles except B*46:01, C*03:02, C*03:03 and C*03:04. They share the 66K and 69R residues not found on the OK6H10-reactive alleles (Table 4b). These residues are about 6 and 14 Å from 163LW (Fig. 1d). The Cw3 alleles have also other unique residues 52V, 131R and 173K. The Lum-C1q data showed a wide range of

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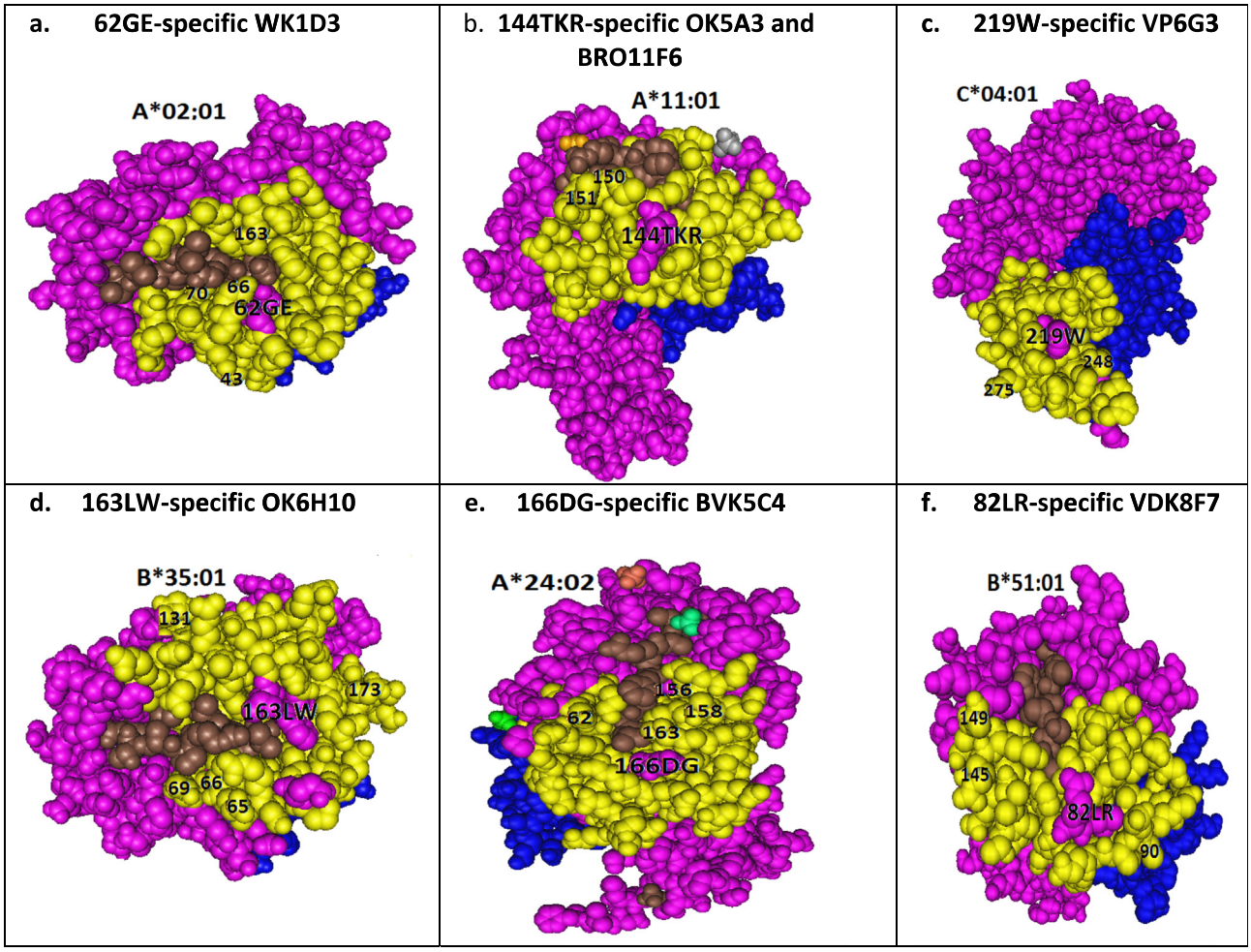

Fig. 1. Structural models of monoclonal antibody-specific HLA class I epitopes and sequence positions on the molecular surface with polymorphic residue differences associated with altered reactivity in Lum-Ig, Lum-C1q and CDC assays. Residues within 15 Å of the eplet are colored yellow and the numbers indicate sequence positions associated with differences in antibody reactivity in the various assays. Residues of the bound peptide which are colored green and β2-microglobulin residues are colored blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Reactivity patterns of two 144TKR-specific human monoclonal antibodies.

(a) OK5A3 (HU-12) W0042 (IgM)
Antibody producer: A2,68; B7,27; Cw2,7 Immunizer: A3

Allele	Eplet	Lum-Ig	Lum-C1q	CDC score		Sequence positions with residue differences within 15 Å											
						76	77	79	80	82	83	116	127	150	151	152	156	158
A*03:01	144TKR	10073	23870	7.8	(*N* = 87)	V	D	G	T	R	G	D	N	A	H	E	L	A
A*01:01	144TKR	7462	23994	6.8	(*N* = 89)	A	N	G	T	R	G	D	N	V	H	A	R	V
A*11:01	144TKR	10261	23522	7.7	(*N* = 77)	V	D	G	T	R	G	D	N	A	H	A	Q	A
A*11:02	144TKR	11520	24443	8.0	(*N* = 3)	V	D	G	T	R	G	D	N	A	H	A	Q	A
A*24:02	144TKR	9049	21579	7.4	(*N* = 112)	E	N	R	I	L	R	Y	K	A	H	V	Q	A
A*24:03	144TKR	9541	24294	8.0	(*N* = 4)	E	N	R	I	L	R	Y	K	A	H	V	Q	A
A*36:01	144TKR	7679	24073	7.7	(*N* = 7)	A	N	G	T	R	G	D	N	V	H	A	R	V
A*80:01	144TKR	9803	23992	1.0	(*N* = 5)	A	N	G	T	R	G	D	N	A	R	R	L	A
144TKR-negative alleles		12	289	1.7	(*N* = 282)													

(b) BRO11F6 (HU-16) W0060 (IgG)
Antibody producer: A26,68; B38,44; Cw7,- Immunizer A11?

Allele	Eplet	Lum-Ig	Lum-C1q	CDC score		Sequence positions with residue differences within 15 Å											
						76	77	79	80	82	83	116	127	150	151	152	156	158
A*11:01	144TKR	16907	8929	7.0	(*N* = 66)	V	D	G	T	R	G	D	N	A	H	A	Q	A
A*11:02	144TKR	20290	15159	7.5	(*N* = 4)	V	D	G	T	R	G	D	N	A	H	A	Q	A
A*01:01	144TKR	20375	14587	1.2	(*N* = 78)	A	N	G	T	R	G	D	N	V	H	A	R	V
A*03:01	144TKR	22162	16717	1.7	(*N* = 86)	V	D	G	T	R	G	D	N	A	H	E	L	A
A*24:02	144TKR	21608	16188	1.8	(*N* = 110)	E	N	R	I	L	R	Y	K	A	H	V	Q	A
A*24:03	144TKR	20406	19156	1.0	(*N* = 5)	E	N	R	I	L	R	Y	K	A	H	V	Q	A
A*36:01	144TKR	14045	9113	1.0	(*N* = 7)	A	N	G	T	R	G	D	N	V	H	A	R	V
A*80:01	144TKR	13	22	1.0	(*N* = 6)	A	N	G	T	R	G	D	N	A	R	R	L	A
144TKR-negative alleles		31	44	1.1	(*N* = 288)													

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Table 3
Reactivity pattern of a 219W-specific human monoclonal antibody.

TRA2G9 (HU-56) W0143 (IgM)					Sequence differences
Antibody producer: A2,24; B14,38; Cw7,8			Immunizer: Cw9		within 15 Å
Allele	Eplet	LUM-OL	LUM-C1q	CDC Score	248 275
C*01:02	219W	13483	3070	7.7 (*N* = 77)	M E
C*03:02	219W	11733	2992	6.8 (*N* = 9)	V E
C*03:03	219W	13204	7088	7.4 (*N* = 68)	V E
C*03:04	219W	14135	3547	7.4 (*N* = 45)	V E
C*04:01	219W	7073	678	7.7 (*N* = 114)	V K
C*14:02	219W	12176	5777	8.0 (*N* = 26)	V E
219W-negative alleles		9	23	1.9 (*N* = 319)	

Table 4
Reactivity patterns of two 163LW-specific human monoclonal antibodies.

(a) VDE1F11 (HU-29) W0147 (IgM)

Antibody producer: A1,3; B7,8; Cw7			Immunizer: unknown		Sequence positions with residue differences within 15 Å															
Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	9	45	52	62	63	65	66	67	69	70	103	113	131	152	156	171	173
B*15:01	163LW	8362	4959	7.1 (*N* = 8)	Y	M	I	R	E	Q	I	S	T	N	V	H	S	E	W	Y	E
B*15:02	163LW	7226	1677	8.0 (*N* = 4)	Y	M	I	R	N	Q	I	S	T	N	V	Y	S	E	L	Y	E
B*15:03	163LW	6474	2714	8.0 (*N* = 14)	Y	E	I	R	E	Q	I	S	T	N	V	H	S	E	L	Y	E
B*15:10	163LW	8361	3676	Insufficient data	Y	E	I	R	N	Q	I	C	T	N	V	H	S	E	L	Y	E
B*15:13	163LW	5416	1281	7.0 (*N* = 4)	Y	M	I	R	N	Q	I	S	T	N	V	Y	S	E	L	Y	E
B*15:16	163LW	5480	3501	8.0 (*N* = 4)	Y	M	I	R	E	R	N	M	A	S	L	H	S	E	L	Y	E
B*35:01	163LW	7244	5017	7.7 (*N* = 31)	Y	T	I	R	N	Q	I	F	T	N	L	H	S	V	L	Y	E
B*46:01	163LW	5172	4788	8.0 (*N* = 8)	Y	M	I	R	E	Q	K	Y	R	Q	V	H	S	E	W	Y	E
B*49:01	163LW	4577	2342	7.1 (*N* = 10)	H	K	I	R	E	Q	I	S	T	N	L	Y	S	E	L	Y	E
B*50:01	163LW	7812	4706	6.6 (*N* = 13)	H	K	I	R	E	Q	I	S	T	N	L	Y	S	E	L	Y	E
B*51:01	163LW	5586	422	6.3 (*N* = 50)	Y	T	I	R	N	Q	I	F	T	N	V	H	S	E	L	H	E
B*51:02	163LW	7879	5474	8.0 (*N* = 3)	Y	T	I	R	N	Q	I	F	T	N	V	H	S	E	L	Y	E
B*52:01	163LW	2049	1909	6.1 (*N*=14)	Y	T	I	R	E	Q	I	S	T	N	V	H	S	E	L	H	E
B*53:01	163LW	6010	2372	7.0 (*N* = 4)	Y	T	I	R	N	Q	I	F	T	N	L	H	S	V	L	Y	E
B*56:01	163LW	5275	2232	7.6 (*N* = 10)	Y	E	I	R	N	Q	I	Y	A	Q	L	H	S	V	L	Y	E
B*57:01	163LW	3749	3536	7.4 (*N* = 18)	Y	M	I	G	E	R	N	M	A	S	V	H	S	V	L	Y	E
B*57:03	163LW	3749	3257	Insufficient data	Y	M	I	G	E	R	N	M	A	S	V	H	S	V	L	Y	E
B*58:01	163LW	2783	561	4.2 (*N* = 5)	Y	T	I	G	E	R	N	M	A	S	L	H	S	V	L	Y	E
B*78:01	163LW	5547	675	Insufficient data	Y	T	I	R	N	Q	I	F	T	N	V	H	S	E	L	H	E
C*03:02	163LW	8056	1237	Insufficient data	Y	G	V	R	E	Q	K	Y	R	Q	V	Y	R	E	L	Y	K
C*03:03	163LW	7453	5640	6.3 (*N* = 16)	Y	G	V	R	E	Q	K	Y	R	Q	V	Y	R	E	L	Y	K
C*03:04	163LW	7532	3438	6.3 (*N* = 28)	Y	G	V	R	E	Q	K	Y	R	Q	V	Y	R	E	L	Y	K
163LW-negative alleles		95	38	1.5 (*N* = 209)																	

(b) OK6H10 (HU-11) W0144 (IgM)

Anibody producer: A2,68; B7,27; Cw2			Immunizer: B70		Sequence positions with residue differences within 15 Å															
Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	9	45	52	62	63	65	66	67	69	70	103	113	131	152	156	171	173
B*15:03	163LW	7803	7071	7.9 (*N* = 16)	Y	E	I	R	E	Q	I	S	T	N	V	H	S	E	L	Y	E
B*15:01	163LW	8273	2229	6.1 (*N* = 47)	Y	M	I	R	E	Q	I	S	T	N	V	H	S	E	W	Y	E
B*15:02	163LW	9194	11070	8.0 (*N* = 8)	Y	M	I	R	N	Q	I	S	T	N	V	Y	S	E	L	Y	E
B*15:10	163LW	8666	4750	7.5 (*N* = 4)	Y	E	I	R	N	Q	I	C	T	N	V	H	S	E	L	Y	E
B*35:01	163LW	8811	8950	6.7 (*N* = 49)	Y	T	I	R	N	Q	I	F	T	N	L	H	S	V	L	Y	E
B*49:01	163LW	6758	13726	6.9 (*N* = 14)	H	K	I	R	E	Q	I	S	T	N	L	Y	S	E	L	Y	E
B*50:01	163LW	9946	15184	8.0 (*N* = 14)	H	K	I	R	E	Q	I	S	T	N	L	Y	S	E	L	Y	E
B*51:02	163LW	8695	5440	5.6 (*N* = 5)	Y	T	I	R	N	Q	I	F	T	N	V	H	S	E	L	Y	E
B*53:01	163LW	7491	5968	6.6 (*N* = 7)	Y	T	I	R	N	Q	I	F	T	N	L	H	S	V	L	Y	E
B*15:13	163LW	7498	1015	7.2 (*N* = 6)	Y	M	I	R	N	Q	I	S	T	N	V	Y	S	E	L	Y	E
B*56:01	163LW	6582	585	6.9 (*N* = 17)	Y	E	I	R	N	Q	I	Y	A	Q	L	H	S	V	L	Y	E
B*51:01	163LW	2105	341	1.4 (*N* = 113)	Y	T	I	R	N	Q	I	F	T	N	V	H	S	E	L	H	E
B*52:01	163LW	4188	1080	2.7 (*N* = 29)	Y	T	I	R	E	Q	I	S	T	N	V	H	S	E	L	H	E
B*78:01	163LW	3847	292	1.5 (*N* = 8)	Y	T	I	R	N	Q	I	F	T	N	V	H	S	E	L	H	E
B*15:16	163LW	2179	697	2.3 (*N* = 7)	Y	M	I	R	E	R	N	M	A	S	L	H	S	E	L	Y	E
B*57:01	163LW	3930	389	5.3 (*N* = 25)	Y	M	I	G	E	R	N	M	A	S	V	H	S	V	L	Y	E
B*57:03	163LW	3428	346	4.8 (*N* = 4)	Y	M	I	G	E	R	N	M	A	S	V	H	S	V	L	Y	E
B*58:01	163LW	3729	310	2.7 (*N* = 19)	Y	T	I	G	E	R	N	M	A	S	L	H	S	V	L	Y	E
B*46:01	163LW	112	328	1.0 (*N* = 34)	Y	M	I	R	E	Q	K	Y	R	Q	V	H	S	E	W	Y	E
C*03:02	163LW	837	228	1.3 (*N* = 8)	Y	G	V	R	E	Q	K	Y	R	Q	V	Y	R	E	L	Y	K
C*03:03	163LW	451	105	1.3 (*N* = 64)	Y	G	V	R	E	Q	K	Y	R	Q	V	Y	R	E	L	Y	K
C*03:04	163LW	585	102	1.5 (*N* = 65)	Y	G	V	R	E	Q	K	Y	R	Q	V	Y	R	E	L	Y	K
163LW-negative alleles		10	101																		

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Table 5
Reactivity patterns of a 166DG-specific human monoclonal antibody.

BVK5C4 (HU-32) W047 (IgM)																			
Antibody producer: A2,-; B18,44; Cw5,- Immunizer: A1					Sequence positions with residue differences within 15 Ångstroms														
Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	9	56	62	65	66	67	69	99	105	109	113	131	152	156	158	163
A*01:01	166DG	10728	967	1.3 (*N* = 82)	F	G	Q	R	N	M	A	Y	P	F	Y	R	A	R	V	R
A*23:01	166DG	14354	10530	7.9 (*N* = 34)	S	G	E	G	K	V	A	F	S	F	Y	R	V	L	A	T
A*24:02	166DG	13489	3321	7.5 (*N* = 135)	S	G	E	G	K	V	A	F	S	F	Y	R	V	Q	A	T
A*80:01	166DG	12911	13013	8.0 (*N* = 6)	F	E	E	R	N	V	A	Y	S	F	Y	R	R	L	A	E
B*15:12	166DG	14272	4114	8.0 (*N* = 2)	Y	G	R	Q	I	S	T	Y	P	L	H	S	E	W	A	L
166DG-negative alleles		19	26	1.5 (*N* = 434)																

Table 6
Reactivity patterns of an 82LR-specific human monoclonal antibody.

VDK8F7 (HU-66) W0191 (IgM)																				
Produced by A3,31; B35,-; Cw4 Immunizer: B37					Sequence positions with residue differences within 15 Ångstroms															
Allele	Eplet	Lum-Ig	Lum-C1q	CDC score	11	12	70	71	74	77	80	81	90	94	95	97	116	138	144	145	149
B*37:01	82LR	8901	8809	7.1 (*N* = 12)	S	V	N	T	Y	D	T	L	A	T	I	R	F	T	Q	R	A
A*23:01	82LR	11599	8036	6.2 (*N* = 6)	S	V	H	S	D	N	I	A	A	T	L	M	Y	M	Q	R	A
A*24:02	82LR	10797	3389	5.7 (*N* = 66)	S	V	H	S	D	N	I	A	A	T	L	M	Y	M	K	R	A
A*32:01	82LR	11474	14652	7.1 (*N* = 14)	S	V	H	S	D	S	I	A	A	T	I	M	D	M	Q	R	A
B*27:05	82LR	11153	11360	8.0 (*N* = 5)	S	V	K	A	D	D	T	L	A	T	L	N	D	T	Q	R	A
B*38:01	82LR	10297	8933	7.2 (*N* = 11)	S	V	N	T	Y	N	I	A	A	T	L	R	F	T	Q	R	A
B*44:02	82LR	9540	830	7.4 (*N* = 27)	A	M	N	T	Y	N	T	A	A	I	I	R	D	T	Q	R	A
B*44:03	82LR	8735	3918	6.7 (*N* = 35)	A	M	N	T	Y	N	T	A	A	I	I	R	D	T	Q	R	A
B*47:01	82LR	6787	965	7.3 (*N* = 6)	A	M	N	T	Y	D	T	L	A	T	L	R	D	T	Q	R	A
B*49:01	82LR	2415	2216	5.8 (*N* = 6)	A	M	N	T	Y	N	I	A	A	T	W	R	L	T	Q	R	A
B*51:01	82LR	5661	657	2.7 (*N* = 31)	A	M	N	T	Y	N	I	A	A	T	W	T	Y	T	Q	R	A
B*52:01	82LR	2827	895	2.4 (*N* = 10)	A	M	N	T	Y	N	I	A	A	T	W	T	Y	T	Q	R	A
B*53:01	82LR	13134	8787	6.4 (*N* = 9)	A	M	N	T	Y	N	I	A	A	I	I	R	S	T	Q	R	A
B*57:01	82LR	6465	15497	7.3 (*N* = 12)	A	M	S	A	Y	N	I	A	A	I	I	V	S	T	Q	R	A
B*58:01	82LR	5204	3517	7.3 (*N* = 9)	A	M	S	A	Y	N	I	A	A	I	I	R	S	T	Q	R	A
A*25:01	82LR	2	7	1.0 (*N* = 7)	S	V	H	S	D	S	I	A	D	T	I	R	D	M	Q	R	T
B*13:01	82LR	7	34	1.0 (*N* = 3)	A	M	N	T	Y	N	T	A	A	I	I	R	L	T	Q	L	A
B*13:02	82LR	4	18	1.0 (*N* = 6)	A	M	N	T	Y	N	T	A	A	T	W	T	L	T	Q	L	A
82LR-negative alleles		144	24	1.5 (*N* = 194)																	

reactivity. High MFI values corresponded to positive CDC scores. Low MFI values were associated with negative or weak CDC reactivity, but B*15:13 and B*56:01 were exceptions. The weakly Lum-C1q reactive and CDC-negative B*51:01, B*52:01 and B*78:01 have a distinct residue difference (H versus Y) in sequence position 171 which is below the molecular surface but in close proximity to 163LW. This suggests that the CDC specificity of OK6H10 requires the 163LW + 171Y combination. The residue configuration in the 65–70 sequence may also play a role in the CDC reactivity of OK6H10. B*15:16, B*57:01, B*57:03 and B*58:01 had very low Lum-C1q reactivity (MFI < 700) and they shared 65R, 66N, 67M, 69A and 70S. Their CDC scores ranged from negative to weakly positive. The CDC reactive alleles have similar configurations in the 65–70 sequence and their residue differences had no effect. Positions 65, 66 and 69 are well exposed on the molecular surface and it seems that the presence of 66K and 69R prevents antibody binding altogether and that especially 65R and 66N inhibits C1q binding and CDC. We could not identify distinct residues that may explain why B*56:01 had very low Lum-C1q reactivity but had a positive CDC score. The pictured surface locations of residues affecting reactivity with OK6H10 suggest contact with several CDRs (Fig. 1d).

3.5. 166DG-defined epitope

This epitope is shared between A*01:01, A*23:01, A*24:02 (but not A*24:03), A*80:01 and B*15:12. BVK5C4 was specific for 166DG in Lum-Ig; only two informative cells were available for B*15:12 but both were strongly positive in CDC (Table 5). This allele panel reacted also in Lum-C1q and CDC except A*01:01 which was weakly reactive in Lum-C1q and negative in CDC. The lack of lymphocytotoxicity for this allele might be due to an adverse influence of 62Q on the α1 domain surface and/or a cluster of 156R, 158V and 163R residues on the α2 domain surface as depicted in Fig. 1e. A*01:01 has also distinct residues 67M and 152A below the molecular surface. As discussed below, it should be noted that HLA-A1 was the immunizing antigen for BVK5C4.

3.6. 82LR-defined epitope

This eplet is shared between Bw4-positive HLA-B antigens and HLA-A23, -A24, -A25, and -A32. The HLA-B37-induced VDK8F7 reacted in Lum-Ig with all 82LR-carrying antigens except HLA-A25 and HLA-B13. We have previously reported this epitope is defined by 82LR paired with 145RA whereby the latter is a self-configuration in the HLA type of the antibody producer [14]. Three sequence positions within 15 Å of 82LR have residue differences associated with a lack of Lum-Ig binding namely 90D versus 90A, 145L versus 145R and 149T versus 149A (Table 6). The Lum-C1q results showed a wide reactivity range. High MFI values corresponded to positive CDC scores. B*51:01 and B*52:01 had weak Lum-C1q reactivity (MFI < 1000) and were CDC-negative; these alleles shared a unique 97T located below the molecular surface. B*44:02 and B*47:01 reacted also weakly in Lum-C1q but they had positive CDC scores;

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there were no distinct residue differences for these alleles. In conclusion, there was a good correlation between the Lum-Ig results and CDC scores for all alleles except for B*51:01 and B*52:01 and residue differences did not have a significant impact.

4. Discussion

HLA antibodies can be considered clinically relevant if they initiate inflammatory or other pathologic processes leading to transplant failure and decreased survival. Complement-induced damage to the transplant is an important mechanism of antibody-mediated rejection. Our studies were designed to gain an understanding why complement-fixing monoclonal antibodies are lymphocytotoxic with some HLA antigens but can only bind to other epitope-carrying antigens. These differences followed two readily recognizable patterns. First, a given allele reacted only in Lum-Ig but not in Lum-C1q or CDC. This means that the interaction between antibody and that allele was limited to the formation of a stable immune complex but without subsequent C1q binding and the activation of complement. In the second pattern, a given allele reacted with antibody in both Lum-Ig and Lum-C1q but not in CDC. In this case, the binding of C1q, the first required step in the classical complement pathway, was apparently insufficient for the activation of the complete cascade leading to lymphocytotoxicity. This suggests that a positive Lum-C1q assay does not always predict a positive CDC and one might consider binding assays with factors such as C3d that participate in the later steps of the complement pathway [24–26].

Technique-dependent differences between antibody reactivity can be explained in context of energetic interactions on the paratope–epitope interface. Paratopes are the loops of three CDRs in antibody heavy chains and three CDRs in antibody light chains that interact with epitopes. The contacted amino acid residues on antigenic proteins are collectively referred to as structural epitopes and they cover a molecular surface of 700–900 $Å^2$ [27–30]. The binding of the CDRs of antibody to amino acid configurations within structural epitopes leads to the release of free energy which stabilizes the antigen–antibody complex and induces conformational changes in the antibody molecule to activate functions such as complement fixation [31]. Each antibody has a CDR that dominates specificity and binding strength to a functional epitope which represents a distinct amino acid residue configuration centrally located within a structural epitope. The binding of other CDRs will augment the release of free energy but this will depend on appropriate amino acid configurations required for making efficient contact. Accordingly, certain configurations may serve as critical contact sites for CDR and others may have more flexibility although in some cases, they contain certain residues that prevent or inhibit CDR binding. Altogether, the total amount of free energy released upon binding of most if not all CDRs to structural epitopes will determine the stability of the immune complex and the biological function of antibody.

These free energy concepts are useful to our understanding of technique-dependent differences in antibody reactivity with HLA alleles (Table 7). Alleles that lack the specifically recognized epitope will not have any significant free energy with antibody in any assay. Specific epitope-carrying alleles might have different free energy levels ranging from low (+: only Ig-binding) to intermediate (++: only Ig-binding and C1q-binding) to high (+++: Ig-binding, C1q-binding and CDC reactivity). The free energy release would operate at three sequential levels: (1) stabilization of the antigen–antibody complex, (2) conformational change in the antibody molecule exposing the C1q-binding site which leads to complex formation with C1q and, (3) conformational change in C1q bound to Ig to convert the C1qrs complex to activate C4 and C2 and the rest of the complement cascade leading to lymphocytotoxicity.

This report describes examples how the antibody reactivity of specific eplet-carrying alleles is associated with amino acid differences within a 15 Å radius, the presumed dimensions of corresponding structural epitopes. Certain residues appear to play critical roles in Ig-binding and/or C1q-binding and/or CDC reactivity. These residues are away far enough from specifically recognized eplets to be contacted by separate CDRs of antibody and this would lead to the release of additional free energy necessary for a positive reaction in a given assay. Fig. 2 represents a molecular model to explain how structural epitope configuration affects free energy and the outcome of a technique-dependent antibody testing. It shows four different alleles that share the same centrally located eplet that binds to CDR-H3 of the depicted antibody. For each allele, the corresponding structural epitope has additional configurations that can serve as contact sites for the other CDRs of antibody and the sizes of the circles reflect the amount of free energy released upon binding. Some configurations might have residue differences between alleles whereas others could have the same or similar residue compositions. This scenario considers a complement-fixing antibody that reacts with the immunizing allele in all three assays. Such allele carries the specifically recognized eplet and critical contact sites needed for free energy release sufficient for stable binding and activation of the complement cascade.

Allele 1 shares the same eplet with the immunizing allele but within the structural epitope it has significant residue differences that inhibit CDR binding and free energy release so that there is only Ig-binding. Allele 2 is structurally more similar to the immunizing allele but still lacks certain critical residues that bind CDRs

Table 7
Concept of antibody binding energy and the interpretation of technique-dependent HLA antibody reactivity with epitope-carrying alleles.

Reactivity	Binding Energy	Interpretation
Negative in all 3 assays	0	No specific epitope recognition
Only Lum-Ig positive	+	Epitope is recognized but insufficient conformational change in antibody
Lum-Ig positive Lum-C1q positive CDC negative	++	Epitope is recognized + conformational change exposes C1q-binding site on antibody but no complement activation
Positive in all 3 assays	+++	Epitope is recognized + exposed C1q-binding site + conformational change in C1q to activate C1qrs complex and classical complement cascade

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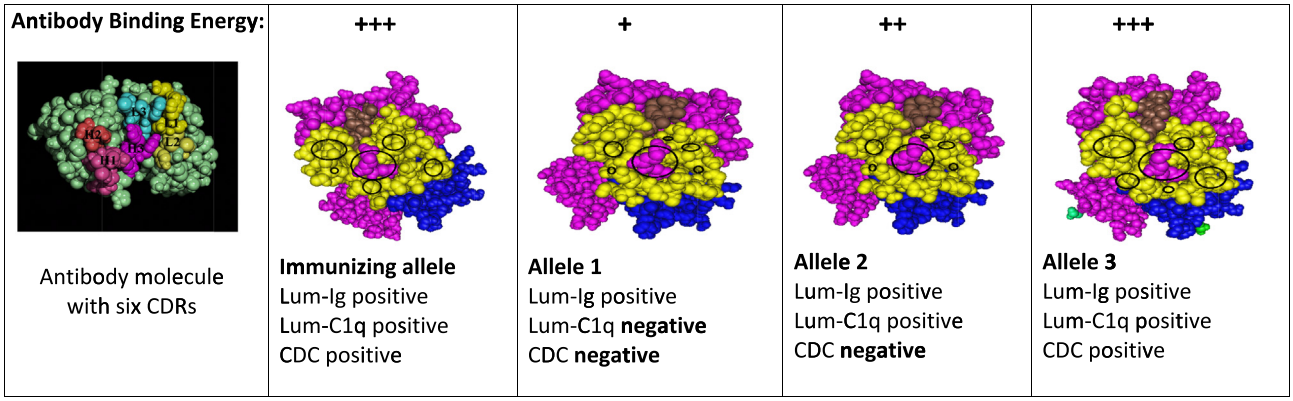

Fig. 2. Structural modeling of HLA epitope-carrying alleles reacting with complement-fixing antibodies in Ig-binding, C1q-binding and CDC assays. The sizes of the circles reflect the amount of affinity between the different parts of the structural epitopes and the CDRs of antibody.

so that less than optimal amounts of free energy are released. Such allele would react in Ig-binding and C1q-binding but will be negative in lymphocytotoxicity. Allele 3 has comparable structural configurations with the immunizing allele and its binding with antibody will release sufficient free energy for complement activation.

This model offers a new understanding how certain specific epitope-carrying alleles react in Ig-binding but not in C1q-binding and/or CDC. It provides also opportunities to evaluate the clinical relevance of HLA antibodies in transplantation and to develop criteria for HLA mismatch acceptability for sensitized patients.

It should be noted that peptides bound to the groove may also reside within a 15 Å radius of certain eplets recognized by antibody. Certain residues in such peptides might serve as critical contact sites with antibody. Indeed, peptide-dependent anti-MHC antibodies have been reported in mouse and human models [32–39]. Mulder and co-workers demonstrated that the reactivity of some mAbs specific for epitopes on HLA-A2 was inhibited by certain peptides loaded onto the grooves of HLA-A2 molecules [40]. One might expect that peptides will influence the reactivity patterns of certain eplet-specific antibodies in a technique-dependent manner. Each allele can be expected to have its own repertoire of bound peptides a proportion of which might have residues that interfere with antigen–antibody binding and the release of free energy necessary for complement activation. Moreover, the same allele used in Luminex kits from the different vendors might have different peptide repertoires and similar differences might affect cytotoxic reactivity of lymphocytes from different individuals. These technique-related differences of antibody reactivity might especially apply to eplets located on α-helices adjacent to peptides in the groove. Altogether, these peptide-related effects may lead to complex antibody reactivity patterns which cannot be readily explained with the structural epitope concept.

This structural model to explain technique-dependent differences of HLA antibody reactivity patterns should be viewed in context with the nonself-self paradigm of epitope immunogenicity which considers the concept that antibodies originate from B-lymphocytes with immunoglobulin receptors for self-HLA epitopes [41,42]. Accordingly, the activation of such cells by non-self eplets can only occur if the remainder of the structural epitope of the immunizing antigen has considerable amino acid similarity with one of the antibody producer's alleles. Two additional studies have provided further experimental support of the nonself-self paradigm [43,44]. Once a B-cell has been activated by antigen, the coding sequences of the Ig variable regions undergo further diversification through so-called somatic hypermutation [45–47]. These point mutations will alter the structures of most if not all CDRs as to increase the affinity of antibody towards antigen and this process of affinity maturation continues during the immune response. It seems likely that the critical role of certain self-configurations in structural HLA epitopes in the binding with antibody is due to affinity maturation of CDRs making contact.

The model in Fig. 2 describes the immunizing allele as antibody-reactive in all three assays. We have noted however, some exceptions as exemplified by the A*02:01-induced 62GE-specific WK1D3 (Table 1c) and the A*01:01-induced 166DG-specific BVK5C4 (Table 5). In contrast to the other epitope-carrying alleles, the immunizing antigens reacted only in Lum-Ig but not in Lum-C1q and CDC. Apparently, their affinity with antibody was insufficient for complement activation and in both cases there were certain residue configurations that might have played a role. This behavior resembles that of so-called heteroclitic antibodies which by definition exhibit higher affinity with cross-reacting antigens than with the immunizing antigen [48].

As an example, anti-hen egg lysozyme antibody (D11.15) cross-reacts with a fourfold higher affinity with pheasant and guinea fowl egg lysozyme than with the original immunogen [49]. D11.15 recognizes an epitope shared with other cross-reacting lysozymes and includes 10 residues in sequence positions 21–23, 102–106, and 112–119 as determined by three-dimensional modeling of crystallized immune complexes with pheasant and hen egg lysozymes. The heteroclitic pheasant and guinea fowl egg lysozymes have a single residue difference within the structural epitope: 113K but not 113N interacts directly with CDR-H2. These interactions increased the affinity of D11.15 with pheasant and quail egg lysozymes [49]. The less reactive Japanese quail egg lysozyme had less affinity due to residue differences in positions 102 and 103. In our study, the lack of complement-fixing activity of WK1D3 and BVK5C4 with the immunizing antigens suggests that this decreased affinity reflects heterocliticity due to residue differences within the structural epitopes.

In summary, the complement-fixing property of HLA-specific antibodies requires not only the specific recognition of eplets but also depends on the interactions of their CDRs with critical amino acid configurations within corresponding structural HLA epitopes. It might just be a matter of sufficient release of free energy upon antigen–antibody complex formation that determines whether or not complement-dependent donor-specific HLA antibodies can initiate antibody-mediated rejection.

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