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HLA-E monoclonal antibodies recognize shared peptide sequences on classical HLA class Ia: Relevance to human natural HLA antibodies

Mepur H. Ravindranath[a,*], Michiko Taniguchi[a], Chien-wei Chen[a], Miyuki Ozawa[b], Hugo Kaneku[a], Nadim El-Awar[b], Junchao Cai[a], Paul I. Terasaki[a,b]

[a] Terasaki Foundation Laboratory, 11570 W. Olympic Boulevard, Los Angeles, CA 90064, United States
[b] One Lambda Inc., 21001 Kittridge Street, Canoga Park, CA 91303-2801, United States

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ABSTRACT

The non-classical HLA-Ib molecule, HLA-E share several peptide sequence similarities with the heavy chains of classical HLA class Ia (-B and -C) molecules. Therefore, the antibodies to HLA-E, that recognize shared sequences, may bind to HLA-Ia alleles. This hypothesis is tested by examining the affinity of HLA-E monoclonal antibodies (HLA-E-MAbs) to HLA-Ia molecules and by inhibiting the antibody binding to both HLA-E and HLA-Ia with the shared peptide sequence(s). Single recombinant HLA molecule-coated beads are used for antibody binding. The antibody binding is evaluated by measuring mean fluorescence index [MFI] with Luminex multiplex flow-cytometric technology. The peptide-inhibition experiments are carried out with synthetic shared peptides, most prevalent to HLA-E and HLA-Ia alleles. The number of HLA-Ia alleles recognized by the HLA-E-MAbs varies with the density of the antigen (quantity of antigen-coated beads) and dilution of MAb. Binding of HLA-E-MAbs to β2 microglobulin (β_2m)-free HLA-Ia antigens confirms the location of the epitopes on the heavy chain (HC) of the antigens. Strikingly, the nature of alleles of HLA-Ia recognized by different HLA-E-MAbs is identical. The binding of HLA-E-MAbs to the HLA-Ia is inhibited dosimetrically by the adjacent peptides, ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}, but not by ^{126}LNEDLRSWTA^{135}, another closer shared peptide sequence. The inhibitory peptide sequences in HLA-E are at the α2-helix terminal facing β_2m. The HLA-Ia alleles recognized by HLA-E-MAb (e.g., MEM-E/02) are similar to those recognized by the natural anti-HLA antibodies found in the sera of healthy non-alloimmunized males. This study postulates that some, if not all, of the natural HLA-Ia antibodies seen in healthy males could be anti-HLA-E antibodies cross-reacting with HLA-Ia alleles.

1. Introduction

MHC class I molecules include highly polymorphic classical HLA class Ia (HLA-A, number of alleles: 767; HLA-B alleles: 1178; HLA-C alleles: 439) and least polymorphic non-classical HLA-Ib (HLA-E alleles: 9; HLA-F alleles: 21; HLA-G alleles: 43) (Geraghty et al., 1987, 1990; Koller et al., 1988; Shawar et al., 1994; O'Callaghan et al., 1998). Each of these molecules consists of a heavy chain (HC) (of about 346 amino acids) and β2 microglobulin ("β_2m") (99 amino acids), which is non-covalently linked to HC. HC consists of three extracellular domains (α1, α2 and α3), a transmembrane domain and a C-terminal cytoplasmic domain. HLA-Ia molecules are co-dominantly expressed on the cell membrane as pair of alleles for each of the three HLA-Ia molecules. The heavy chains of classical HLA class Ia (-A, -B and -C) and non-classical HLA-E share several peptide sequence similarities (Table 1).

Antibodies to allo-HLA-Ia occur in the sera after alloimmunization due to transfusions, pregnancies or after organ allograft. Interestingly, there are also several reports on the occurrence of natural antibodies reacting to allo-HLA-Ia in the sera of healthy non-immunized individuals (Collins et al., 1973; Lepage et al., 1976; Tongio et al., 1985; Ameglio et al., 1987; Májský, 1989; Luscher et al., 1988; Zhou et al., 2008; Morales-Buenrostro et al., 2008). These reports are perplexing since the allo-HLA antigens neither occur in these individuals nor are the nature of the immunogens responsible for the antibody production known. The HLA-Ia reactivity is attributed to antibodies produced against various exogenous antigens, including viral, bacterial, dietary meat proteins (Morales-Buenrostro et al., 2008) or to some common autoantigen (Tongio et al., 1985). The precise nature of the immunogen(s) responsible for induction of antibodies reacting to HLA-Ia alleles has not been identified. Since HLA-Ia share several peptide sequence similarities with a common autoantigen HLA-E, antibodies to HLA-E that recognize these shared sequences may also react with HLA-Ia alleles. The

* Corresponding author. Tel.: +1 310 479 6101x103; fax: +1 310 445 3381.
E-mail addresses: glycoimmune@terasakilab.org, mepurravi@yahoo.com (M.H. Ravindranath).

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Table 1
Peptide sequences shared between HLA-E and HLA class Ia alleles: monospecific versus polyspecific epitopes.

HLA-E peptide sequences [total number of amino acids]	HLA-alleles					Specificity
	Classical class Ia			Non-classical class Ib		
	A	B	Cw	F	G	
^{47}PRAPWMEQE^{55} [9]	1	0	0	0	0	A*3306
^{59}EYWDRETR^{65} [8]	5	0	0	0	0	A restricted
^{90}AGSHTLQW^{97} [8]	1	10	48	0	0	Polyspecific
^{108}RFLRGYE^{123} [7]	24	0	0	0	0	A restricted
^{115}QFAYDGKDY^{123} [9]	1	104	75	0	0	Polyspecific
^{117}AYDGKDY^{123} [7]	491	831	271	21	30	Polyspecific
^{126}LNEDLRSWTA^{135} [10]	239	219	261	21	30	Polyspecific
^{137}DTAAQI^{142} [6]	0	824	248	0	30	Polyspecific
^{137}DTAAQIS^{143} [7]	0	52	4	0	30	Polyspecific
^{157}RAYLED^{162} [6]	0	1	0	0	0	B*8201
^{163}TCVEWL^{168} [6]	282	206	200	0	30	Polyspecific
^{182}EPPKTHVT^{190} [8]	0	0	19	0	0	C restricted

hypothesis envisages that antibodies to HLA-E in human may be responsible for the observed natural anti-HLA-Ia antibodies, since they react with a variety of HLA-Ia alleles.

HLA-E is expressed by several cells including endothelial cells, immune cells (B, T lymphocytes, NK cells, monocytes and macrophages), trophoblasts, and some tumor cells including melanoma (Sullivan et al., 2008). Increased cellular expression of HLA-E induces the release of HLA-E in circulation (Coupel et al., 2007; Derré et al., 2006). The soluble HLA-E (sHLA-E) is found in the sera or plasma of patients with immune-mediated vascular diseases, Kawasaki Disease, a systemic pediatric vasculitis, as well as in normal individuals (Lin et al., 2009). The soluble HLA-E may occur with or without β_2m. In the intact HLA-E the presence of β_2m may mask some of the peptides sequences of the heavy chain, which upon exposure in sHLA-E that is free of β_2m, may become immunogenic. In other words, some of the immunogenic peptide sequences may be cryptic in the native state of sHLA. If these peptide sequences are shared between HLA-Ia and HLA-E, the antibodies produced against these peptides found in sHLA-E may bind to HLA-Ia molecules.

There are evidences in literature supporting the above hypothesis. For example, murine HLA-E-MAbs are found to bind to HLA-Ia molecules (Menier et al., 2003; Sibilio et al., 2003; Lo Monaco et al., 2008) although the reports are incomplete due to the absence of characterization of the affinity of HLA-E-MAbs to different alleles of HLA-Ia. Conversely, the murine HLA-Ia-MAbs W6/32 and HCA-2 bind to HLA-E (Braud et al., 1997; Seitz et al., 1998). Also, the rabbit antibodies to peptides derived from sequences of HLA-Ia (HLA-A, -B, -C) bound better to HLA-E rather than to HLA-A and -C (Chersi et al., 2002).

This investigation examines whether the murine MAbs generated against HC of recombinant human HLA-E prefer binding to any HLA-Ia alleles. If such preferences are observed, then the peptide sequence shared by the alleles commonly recognized by the anti-HLA antibodies can be mapped by assessing the dosimetric inhibition of the antibody binding to both HLA-E and HLA-Ia molecules, with shared peptide epitopes. Affinity of HLA-E-MAbs for HLA-Ia alleles and the dosimetric inhibition with purified synthetic peptides (of shared sequences) designed to block the binding of the antibodies to antigen-coated beads are evaluated by measuring mean fluorescent indices [MFI] using Luminex-based HLA-Ia specific screening with single recombinant HLA-Ia coated microbeads.

Indeed, the binding of HLA-E- MAbs to the HLA-Ia coated single beads is inhibited by the some, but not all, of the shared peptide sequences. The results provide evidence that antibodies to HLA-E preferentially recognized some of the HLA-Ia alleles. Comparison of the selective recognition of HLA-1a alleles by murine MAbs with those recognized by the human natural antibodies in healthy non-alloimmunized male expands the scope of this investigation.

2. Materials and methods

2.1. Monoclonal anti-HLA-E antibodies

This investigation is restricted to four murine MAbs (clone MEM-E/02, MEM-E/06, MEM-E/07 and MEM-E/08) to human HLA-E. These MAbs are characterized and are commercially available (Affinity Bioreagents (ABR, Golden, CO). The MEM-E/02 MAb (MA1-19309) is found to react with "the HLA-E denatured heavy chain by Western blot analysis but did not recognize native HLA-E molecule by flow cytometry" (Menier et al., 2003). Also, MEM-E/02 do not cross react "with the denatured form of the particular HLA-A, -B, -C alleles present on the M8-pc DNA cell lysate (p. 322; Menier et al., 2003)" or with cell lines expressing distinct sets of HLA-A, -B and -C alleles. MEM-E/06 (MA1-19356) recognized surface-expressed HLA-E but not β_2m-free heavy chain of HLA-E. It exhibited cross-reactivity with heavy chains of HLA-A3, -A29, -B27, -Cw3 and Cw7″ (p. 321, Menier et al., 2003). Workshop I Session on the 3rd International Conference on HLA-G (Paris, July 2003, Sibilio et al., 2003) confirms that the anti-HLA-E MAb exhibits much broader cross-reactivity to classical HLA-Ia molecules, namely with HLA-A24, -A32, -B8, -B15, -B27, -B35, -B44, -B54, -C3, -C4, -C5, -C7. Coupel et al. (2007) reported that E/07 also bind to HLA-B7 (strongly), HLA-B8 (moderately), and HLA-B27 and B44 (weakly) and E/08 cross-reacted weakly with HLA-A24, B7, B27, B51, B54 and C7. Clone MEM-E/07 (MA1-19360) recognized native surface-expressed HLA-E, but not denatured heavy chain of HLA-E. However, the testing results by Affinity Bioreagents (ABR, Golden, CO) revealed that the antibody cross-reacts with some classical HLA-Ia molecules: HLA-B7 (strongly), HLA-B8 (moderately), HLA-B27, -B44 (weakly). Clone MEM-E/08 (MA1-19362) also recognized native surface-expressed HLA-E, but not denatured heavy chain of HLA-E. Testing results by the same company (ABR) revealed that antibody is remarkably specific for HLA-E, but weakly reacts with HLA-A24, -B7, -B27, -B51, -B54, -C7. It is not known whether these MAbs bind to any specific domain or peptide sequence of the HLA-Ia molecules (Table 1).

In addition, we have also used murine MAb W6/32 (Barnstable et al., 1978; gift from One Lambda, Canoga Park, CA) which binds to HLA-Ia antigens at the site of β_2m-subunit (Parham et al., 1979) and HC10 (Hajek-Rosenmayr et al., 1989; gift from One Lambda, Canoga Park, CA) that binds to HC of HLA-Ia alleles even after removal of β_2m (Stam et al., 1990).

Table 2
Purity of the HLA-E peptides used for inhibition of binding of the anti-HLA-E murine MAbs to single recombinant HLA class Ia antigens coated beads in the immunoassay.

Peptide used and their position in HLA-E	Purity by HPLC (%)	Expected Mw (d)	Observed Mw (MS spectrum)	Hydrophobic index	
				pH 2.0	pH 7.0
^{115}QFAYDGKDY^{123}	95.205	1106.15	1106.55	146	124
^{126}LNEDLRSWTA^{135}	96.148	1204.29	1204.40	261	253
^{137}DTAAQI^{142}	97.389	617.65	618.30	171	129

2.2. Immunoassay with single antigen beads

To simultaneously detect the binding of minimal quantities of murine MAbs to HLA-E and other HLA-Ia antigens, multibead multiplex immunoassay is used. Using commercial dual-laser flow-cytometric principles of Luminex® xMAP® multiplex technology, the single antigen assays are carried out for data acquisition and analysis of HLA-Ia and HLA- E antibodies. The LABScreen® Single Antigen (One Lambda, Canoga Park, CA) assay, consists of a panel of color-coded microspheres (single antigen beads, SAB) coated with HLA antigens to identify antibody specificities. The array of HLA antigens representing various alleles on the beads are listed at one lambda website under Antibody detection products/Labscreen® Single Antigen Product sheet HLA-Ia combi-LS1A04-Lot 003 Worksheet Rev-1. The single recombinant HLA-Ia antigens in LS1A04-Lot 003 contain 31 HLA-A, 50 HLA-B and 16 HLA-C molecules. Recombinant HLA-E folded heavy chain (10 mg/ml in MES) is a gift from Professor Daniel Geraghty, Fred Hutchinson Cancer Center, University of Washington, Seattle, WA 98109. The recombinant HLA-E heavy chain, by a process of simple chemical coupling, is attached to 5.6 μm polystyrene microspheres, which are internally dyed with red and infrared flurophores, using different intensities of two dyes (xMAP microsphere number #005) at One Lambda.

Data generated from the LABScan 100 are analyzed using computer software. The protocol is exactly similar to that reported earlier (El-Awar et al., 2007, 2009). Trimmed mean fluorescence values for the SAB reactions are obtained from the output (.csv is converted to .xls) file generated by the flow analyzer, and are adjusted for blank and background signal using the formula described below.

2.3. Determination of normalized trimmed Mean Fluorescent Index (MFI)

To express the values of anti-HLA antibodies at different dilutions, the sample specific fluorescent values (trimmed MFI) for each bead are taken into consideration. Different kinds of Sample # Number (S # N) of beads are obtained as described elsewhere (El-Awar et al., 2009). Number #1 bead refers to the negative control. The Number #2 bead refers to the positive control. The other number of beads refers to the different alleles of HLA-Ia. In essence, the following four different kinds of values are obtained. They are (1) trimmed MFI for MAbs obtained with HLA-Ia coated beads (other bead numbers), (2) trimmed MFI for the negative control beads (bead # 1) used for each MAb; (3) trimmed MFI for HLA-Ia coated beads (PE-conjugated 2nd antibody only). (4) Trimmed MFI for the negative control beads (with PE-conjugated 2nd antibody only). Normalized trimmed MFI is calculated as follows: (*S # N value of* (1) – *S # N value of* (2)) – (*S # N value* (3) – *S # N value of* (4). The HLA-Ia microbeads have inbuilt control beads, which included those coated with human IgG (considered positive control but not applicable as positive control for murine MAbs) and HSA (negative control), respectively. For HLA-E, we have separately included identical control beads (both positive and negative controls). Each experiment is done in duplicate. For each analysis, at least 100 beads are counted. Trimmed MFI is calculated from these beads. Origin Graphics Software® is used to plot the data. Basic statistical analyses are carried out with Excel software.

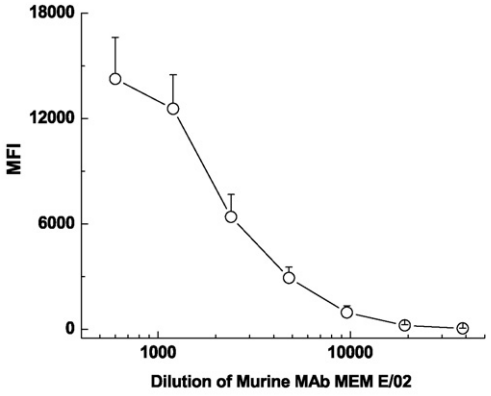
2.4. Acid treatment of HLA-Ia single beads

The acid treatment of beads (HLA class I combi-LS1A04-Lot 003) is performed modifying a procedure described earlier (Poláková et al., 2003, 2004). HLA-E beads contain HC of β_2m-free HLA-E and hence are not acid treated. The beads (30 μl) on ice are resuspended either in 150 μl of PBS-2% BSA, pH 7.0 (batch # 1) or in 120 μl (two lots, batches # 2 and 3), citric acid (1 M)–Na_2PO_4 buffer, pH 3.2. All batches are placed on a shaker for 20 min, centrifuged and then the supernatants are discarded. For batches # 1 and # 2, 150 μl of PBS–2% BSA, pH 7.0, and for batch # 3, 50 μl of Tris-buffered saline–2% BSA, pH 9.5 are added. All buffers are tested for initial and final pH. All batches are incubated on the shaker for 20 min followed by three washes. Batch # 1 always remained at neutral pH and it served as the control. The pH of Batch # 2 changed to pH of 4.0 after adding buffer at pH 7.0. The pH of Batch # 3 changed to 6.5, due to neutralization with pH 9.5. All batches were again treated with 150 μl of PBS–2% BSA, pH 7.0 and placed on a shaker for 40 min, and then washed. The pellets are suspended in 30 μl of PBS–2% BSA, pH 7.0. 2 μl of beads are used for each test. The following MAbs (7 μl) are used: HLA-E MEM-E/02, -E/06, -E/07, -E/08 (diluted 1/400), and HLA class Ia antigens, W6/32 (1/250) and HC10 (diluted 1/50). In all the above experiments, these MAbs are added to wells and diluted with 14 μl of PBS–2% BSA, pH 7.0. PE-conjugated anti-mouse IgG antibodies (1/100) are used for Labscreen Assay. Each experiment is done in duplicate. The MFI obtained with batch # 2 (pH 4.0) batch # 3 (pH 6.5) are compared with that obtained with the control (batch # 1) and percentage decrease or increase from the control is presented in the results (see Fig. 5).

2.5. Peptide-inhibition assays

All the peptides used in this investigation are synthesized by GenScript Corporation (Piscataway, NJ). Peptides are purified by reversed-phase HPLC (Shimadzu VP-ODS, 4.6 mm × 150 mm) column with Pump A (0.065% trifluoroacetic acid (TFA) in 100% water) and Pump B (0.05% trifluoroacetic acid in 100% acetylnitrile) at a flow rate of 1.0 ml/min and measured at 214 nm (detector A, Ch1). Details of the purity of the peptides are provided in Table 2. The molecular mass of the peptides is measured by mass spectrometry and electrospray ionization (ESI) is used. Nebulizing gas flow is adjusted to 1.50 l/min and Total flow 0.2 ml/min. ESI-MS *m/z* spectrum is presented in Table 2. Various concentrations of peptides (serial diluted peptides from the initial concentration of 100 μg/100 μl) are added to each of the diluted (1/400) MAbs. Diluted MAbs (7 μl) are further diluted by adding 14 μl of PBS-BSA pH 7.0 (final dilution 1/1200) and exposed to 2 μl of beads. PE-conjugated anti-mouse IgG is used for LabScreen Assay. Each experiment is done in triplicate. Normalized trimmed mean is determined with appropriate controls described above.

(A)



(B)

Bead Volume μL	MEM E/02 [1/600]	MEM E/02 [1/1800]	MEM E/06 [1/600]	MEM E/07 [1/600]	MEM E/08 [1/600]
2	16978 ± 2291	13916 ± 2289	10973 ± 2194	9778 ± 1935	12608 ± 2527
3	17200 ± 2906	13760 ± 2181	10839 ± 2050	9597 ± 1730	12722 ± 1851
4	16423 ± 2454	13107 ± 1744	10617 ± 2122	9335 ± 1741	12229 ± 1869
5	15888 ± 2220	11544 ± 2194	10678 + 2338	6985 ± 1279	10867 ± 1583

Fig. 1. (A) Titration of binding of murine MAb MEM-E/02 with HLA-E (heavy chain only). 7 μl of MAb diluted 1/200 to 1/12800 is further diluted to 3-fold in well with 14 ml of PBS. After titrating the different MAbs with different concentrations of antigen-coated beads (see table in B), 2 μl of bead/well is added to wells containing diluted antibody and after incubation, the binding of the MAb to beads is determined with a PE-conjugated secondary antibody using a Luminex platform. All MFI values are mean and SD of triplicates. (B) Reactivity of different MAbs to different concentrations of beads coated with HLA-E (Heavy chain only). The protocol of dilution is same as in (A).

3. Results

3.1. Murine HLA-E-MAbs bind to β_2m-free HLA-E and the binding is inhibited by the shared peptides

Fig. 1A (*x*-axis dilutions/*y*-axis MFI) shows the differential binding of E/02 at different dilutions. Fig. 1B (in tabular form) shows the binding of MEM-E/02, -E/06, -E/07 and -E/08 murine MAbs to heavy chain (HC) of HLA-E coated on to the beads. E/02 (1/600) binds more strongly than other MAbs. The binding intensity of other MAbs ranged as follows: E/02 > E/08 > E/06 > E/07. We have selected 1/1200 as appropriate dilution to investigate peptide binding. The most commonly shared three peptides (Table 1) found in HC of HLA-E and HLA-Ia alleles, namely ^{115}QFAYDGKDY^{123}, ^{137}DTAAQI^{142} and ^{126}LNEDLRSWTA^{135} are selected for peptide inhibition of antibody binding. Fig. 2 shows that ^{137}DTAAQI^{142} and ^{115}QFAYDGKDY^{123} potentially inhibited the binding of MAb-E/02 to HLA-E. The peptide ^{137}DTAAQI^{142} inhibited 48% of the binding of E/02 to HLA-E, whereas the inhibition by ^{115}QFAYDGKDY^{123} is considerably low (24%). Interestingly, ^{126}LNEDLRSWTA^{135} did not inhibit the binding of E/02 to the HLA-E and served as a potential control representing a non-inhibitory peptide.

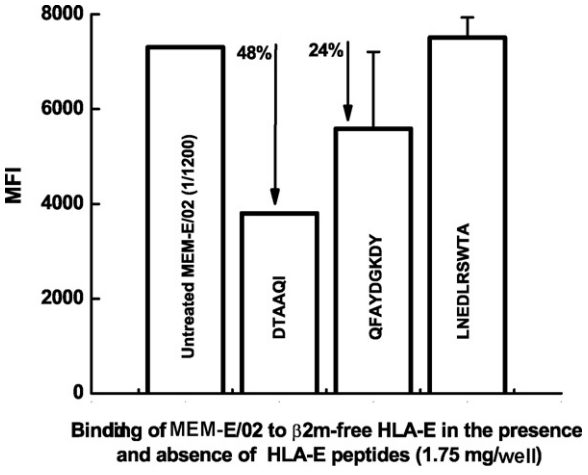


Fig. 2. Murine monoclonal anti-HLA-E MEM-E/02 binds to heavy chain of HLA-E free of β_2m and the binding is selectively inhibited by two of the shared HLA-E peptides (^{137}DTAAQI^{142} [48%] > ^{115}QFAYDGKDY^{123} [24%]) but not inhibited by another closely associated HLA-E peptide (^{126}LNEDLRSWTA^{135}). Mean and standard deviation of triplicate values are presented.

3.2. Murine HLA-E-MAbs bind to a variety of HLA-Ia antigens

All the HLA-E-MAbs (E/02, E/06, E/07 and E/08) at different dilutions 1/300, 1/600 and 1/1200 bound to a wide variety of HLA-Ia antigens. The binding of the HLA-E-MAbs to alleles are considered to be positive if the MFI is >500, based on the MFI cut-off recommended in previous literature (El-Awar et al., 2007, 2009; Morales-Buenrostro et al., 2008). E/06 bound to more HLA-Ia alleles, in addition to those recognized by other MEM-MAbs used in this study (data not presented). All HLA-E-MAbs recognized essentially the somewhat identical HLA-Ia alleles but at varying intensities (Fig. 3A, C and E). The list of HLA-Ia alleles included B*1301, B*3501, B*4006, B*4101(except for MAb-E/08), B*7301, B*8201 (except for MAb-E/08), Cw*0501, Cw*0701 and Cw*1801. The binding affinity of the three MAbs to the above alleles declined as follows: E/06 (data not presented) > E/02 > E/07 > E/08. In addition to the above alleles, E/02 (and also E/06) bound to B*1513, B*1801, B*3701, B*5601 and B*7801 (Fig. 3B). While binding of the HLA-E-MAbs to A-alleles (at MFI > 500) is very rare, E/07 bound well to A*2402 (Fig. 3D) and E/06 bound to some A* alleles. The number of alleles recognized by a MAb (e.g. new lot of E/02) is 48 at 1/300 dilution, 15 at 1/600 dilution and only 9 at 1/1200 dilution (Fig. 3F). Table 3 (data from the old and new lots of E/02) provides a detailed list of specificity of E/02 for a variety of HLA-Ia alleles and the effect of decreasing the concentration (by dilution) resulted in reduction in the number of alleles recognized by the antibody. Similarly, when the bead volume is increased from 3 μl to 5 μl for the immunoassay, the number of alleles recognized by E/02 (new lot) declined from 40 to 20 (Fig. 3F). Obviously decrease in antibody concentration and increase in antigen density affect the number of HLA-Ia alleles recognized by anti-HLA-E MAbs.

Table 3
Ranking of the recognition of various alleles of HLA class Ia antigens by murine monoclonal antibody MEM-E/02, which varied with the concentration or final dilution of the anti-HLA-E antibody in the immunoassay. Dilutions of antibody and normalized trimmed mean values expressed as MFI are presented here. Only MFI values > 1000 are presented, while *n* presented in parenthesis includes those above MFI 500.

HLA-Ia allele specificity	MAb MEM-E/02 [Lot #1]			HLA-Ia alleles	MAb MEM-E/02 [Lot # 2]		
	[1/300]	[1/600]	[1/1200]	Specificity	1/300	1/600	1/1200
B*4006	5420	2609	1381	B*4006	4432	3012	2206
Cw*0501	4400	1807	<1000	Cw*0501	3131	2150	1488
Cw*1802	3862	1584	<1000	Cw*0701	3010	2058	1486
B*3501	3789	1527	<1000	Cw*1802	2724	1762	1258
Cw*0701	3501	1855	1006	B*3501	2469	1560	1171
B*1301	2787	1215		B*1301	2323	1446	1030
B*4101	2362	<1000		B*5601	2035	1305	
B*5601	2325	1026		B*4101	1901	1305	
B*8201	2230			B*8201	1592	1076	
B*7301	1936			B*1801	1519		
B*1801	1884			B*4001	1190		
B*7801	1518			B*7301	1274		
B*4001	1430			B*7801	1025		
B*3701	1325						
B*1513	1260						
Cw*0102	1186						
A*2402	1159						
B*1502	1104						
B*4601	1088						
B*1401	1068						
B*4501	1067						
Cw*0602	1044						
Cw*0304	1001						
B*4403	1000						
n with MFI > 500	[*n* = 48]	[*n* = 14]	[*n* = 7]		[*n* = 36]	[*n* = 20]	[*n* = 13]

3.3. Peptide inhibition of the binding of HLA-E MAbs to HLA-Ia alleles

The results of peptide inhibition on the binding affinity of the MAb-E/02 to HLA-Ia antigens are presented in Fig. 4A. The inhibition experiments involved exposure of the peptides to antibodies for an hour on a shaker. Such exposure *per se*, of the diluted antibodies without inhibitory peptides, may affect the binding. To ascertain that possibility, experiments were carried out and the results are presented in Fig. 4A. The results confirmed that exposure of the diluted MAb to physical stress such as shaking at higher temperatures may affect binding of the antibodies. Fig. 4B and C reveal that two of the shared peptide sequences, ^{137}DTAAQI^{142} and ^{115}QFAYDGKDY^{123}, inhibited the binding of MAb-E/02 to several HLA-Ia alleles in a dose-dependent manner. Interestingly, the peptide ^{126}LNEDLRSWTA^{135} enhanced the binding of the MAb-E/02 to HLA-Ia antigens (Fig. 4D). Both ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142} also strongly inhibited the binding of E/07 (Fig. 4E). Only 15% of the binding of E/08 is inhibited by ^{137}DTAAQI^{142} (Fig. 4F). The peptide ^{115}QFAYDGKDY^{123} failed to inhibit the binding of E/08, whereas the peptide ^{126}LNEDLRSWTA^{135} enhanced the binding of E/08 (Fig. 4F). None of the three peptides inhibited the binding of MAb-E/06 with any of the HLA-Ia antigens at any of the concentrations of the peptides tested or dilution of MAb (data not shown), suggesting that this MAb-E-06 *may be directed against either some other peptide sequence* or to the same peptide sequence with additional sequence or at some other conformation.

3.4. Effect of acid treatment on the binding of the HLA-E-MAbs with HLA-Ia antigens

Both classical (HLA-Ia) and non-classical (HLA-I b) molecules are released on to the cell surface as a heterodimer with β_2m-subunit. However, on the cell surface they may occur with or without β_2m (Barnstable et al., 1978; Poláková et al., 2004; Marozzi et al., 1993; Demaria et al., 1994; Tsai et al., 2002). Acid treatment dissociates β_2m-subunit from the heavy chain of HLA molecule. We have tested whether the antibodies differ in their binding of HLA-Ia antigens, before and after acid treatment that dissociates β_2m-subunit. We have also used mouse MAb W6/32, which do not recognize HLA without β_2m-subunit and the MAb HC10 which binds HLA-Ia heavy chains to confirm the dissociation of β_2m. Fig. 5A–D clarifies the experimental results. The *x*-axis shows the alleles of class Ia and MFI (mean ± SD) and the *y*-axis represents percentage (%) decrease in reactivity after treatment (based comparing batches # 2 (pH 4.0) and # 3 (pH 6.5) with control or batch # 1). The reactivity of MEM-E/02 and other MAbs were decreased after acid treatment. The reactivity of the HLA-E-MAb MEM-E/08 is affected strongly by the acid treatment and based on the percentage decrease observed in binding after acid treatment, the antibodies can be ranked as follows: W6/32 ⋙ MEM-E/08 (Fig. 5C) > MEM-E/06 (Fig. 5B) ≫ MEM-E/02 (Fig. 5A) > HC10 (Fig. 5D). As seen in Fig. 5D, after acid treatment of the beads, W3/62 failed to bind to any of the HLA-Ia alleles, whereas HC10 continued to bind, though to a lesser degree to the alleles recognized by HLA-E MAbs. Essentially all the MAbs tested with exception of W6/32 bound to the heavy chain of HLA-Ia molecules. Based on the results presented in Fig. 5A–D, the affinity of the MAbs to β_2m-free heavy chain can be ranked as follows: HC10 > E/02 > E/06 > E/08. MAb MEM-E/08 seems to more affected by acid treatment suggesting that it recognizes intact (with β_2m) HLA better than other MAbs.

4. Discussion

The results reveal that the MAbs developed against purified recombinant heavy chain of HLA-E bound to several HLA-Ia molecules. Two factors limit the binding of HLA-E-MAbs to HLA-Ia molecules (Fig. 3F); (1) the concentration or density of the antibody (as evidenced from antibody dilution experiments), and (2) the density of the target HLA-Ia molecules (as evidenced from varying the density of antigen-carriers or beads).

Binding of HLA-E-MAbs to acid treated HLA-Ia coated beads establish that the HLA-E- MAbs bind to the peptide epitopes located on the heavy chain (HC) of HLA-Ia alleles (Fig. 5A–D). In contrast,

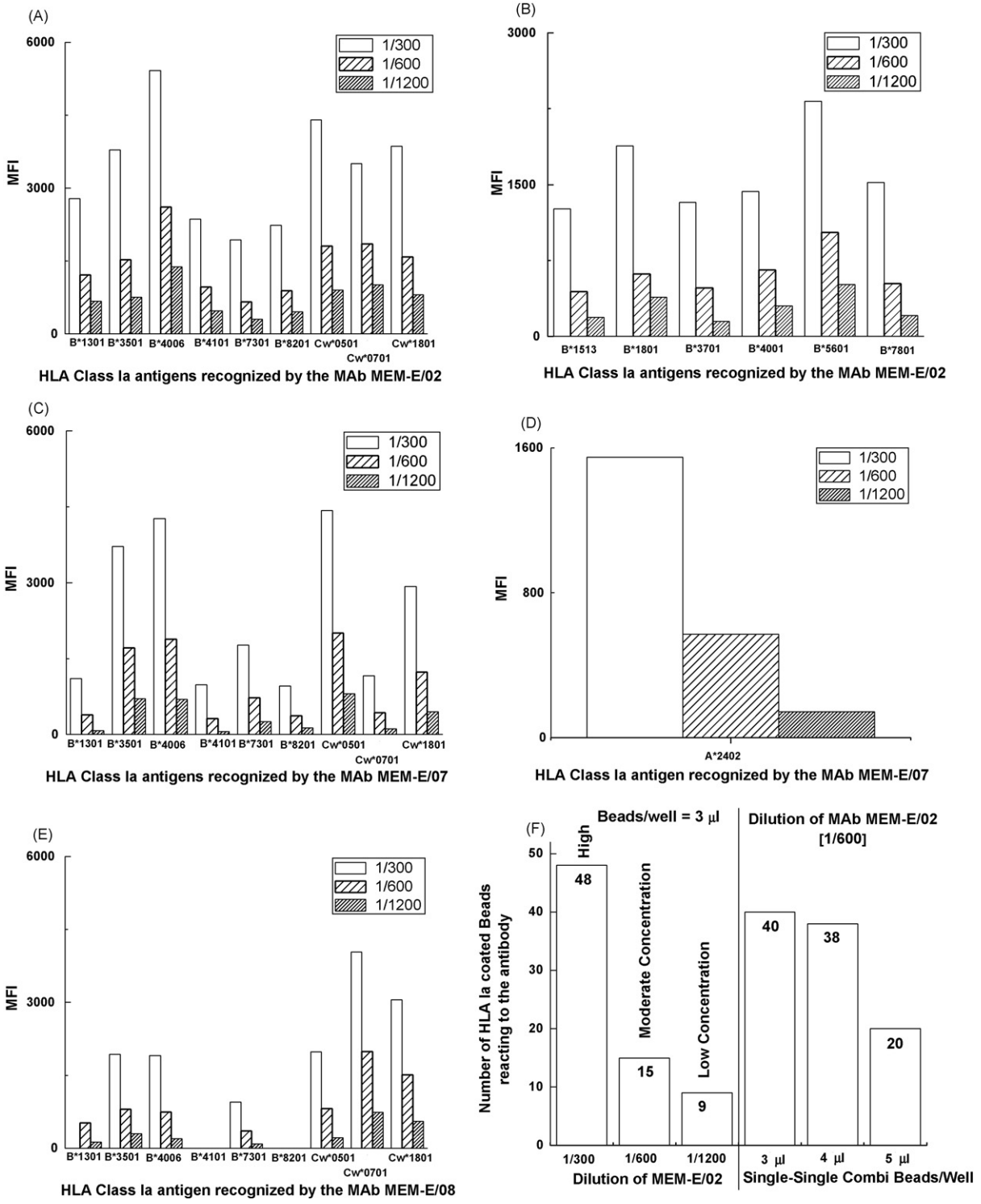


Fig. 3. Murine MAbs MEM-E/02, MEM-E/07 and MEM-E/08 binds to intact recombinant HLA-Ia molecules attached to beads. These molecules on beads could be heavy chain only or intact with β_2m or both. A, C and E show that all three antibodies bind to identical or the same HLA class Ia alleles (B*1301, B*3501, B*4006, B*4101, B*7301, B*8201, Cw*0501, Cw*0701, Cw*1801). The intensity of MFI of different alleles may differ with different MAbs. For example, E/02 and E/07 bind strongly with B*4006 and Cw*0501 but E/08 binds strongly with Cw*0701. B and D show that E/02 and E/07 also bind to other class Ia alleles in addition to the common antigens. (F) Histograms showing the differences in the number of HLA-Ia alleles bound by MAb MEM-E/02 differs with the concentration of the antibody and the number of beads used in the assay. With increasing dilution of antibodies the number of HLA-Ia alleles recognized by MAb-E/02 decrease. Similarly, when antigen concentration is increased by increasing the number of beads, the number of HLA-Ia alleles recognized by MAb-E/02 decreases. In all wells, 7 µl of MAbs diluted 1/100 to 1/400 is further diluted to 3-fold in well with 14 ml of PBS. 2 µl of bead/well is added to wells containing diluted antibody and after incubation, the binding of the MAb to beads is determined with a PE-conjugated secondary antibody using a Luminex platform. The cut-off used for MFI is > 500.

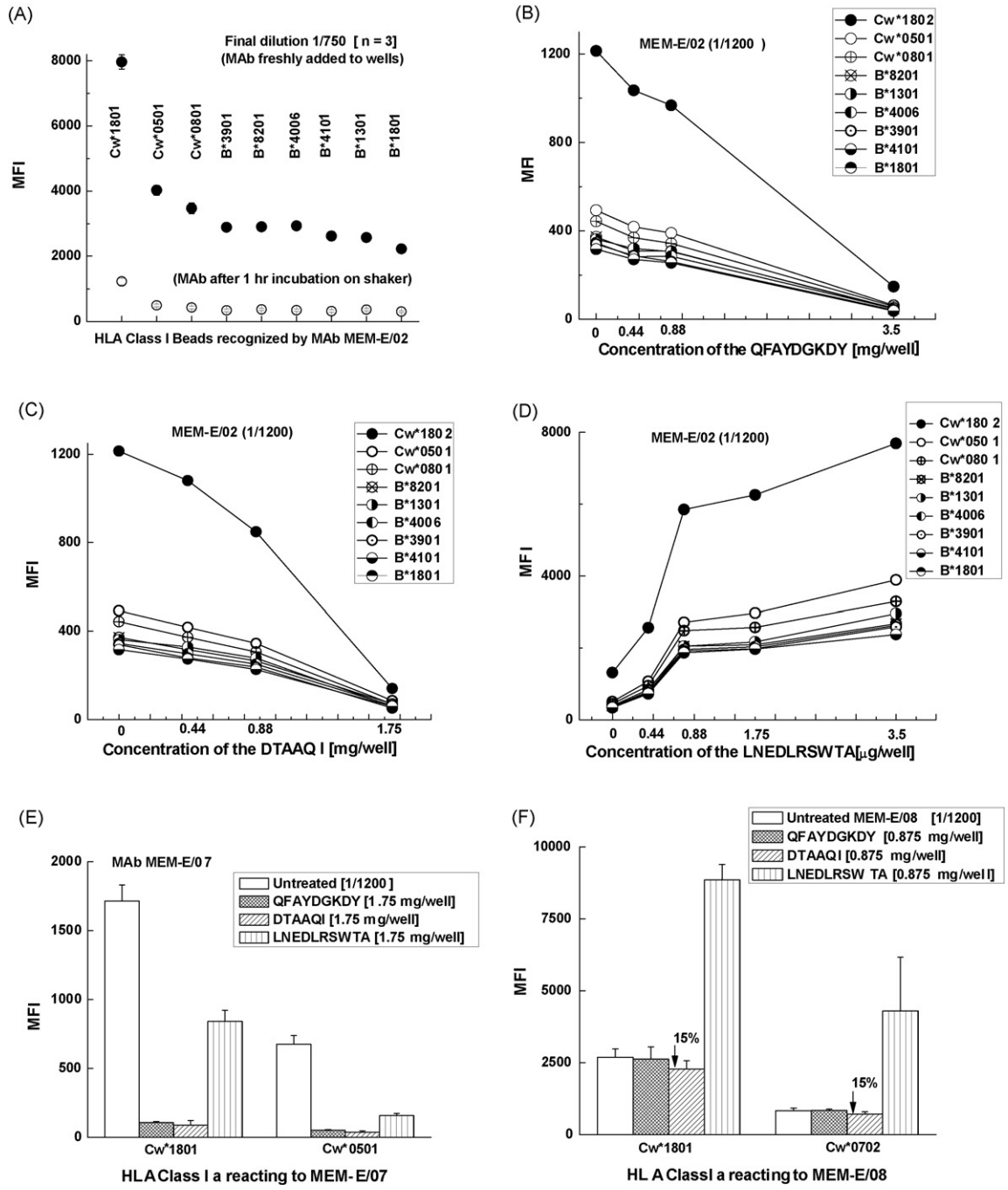


Fig. 4. Peptide-inhibition studies on the binding of murine anti-HLA-E MAbs MEM-E/02, E/07 and E/08 to several HLA class Ia antigens. (A) The binding of murine anti-HLA-E MAb MEM-E/02 to class Ia antigens differs with exposure of the antibodies to room temperature at experimental conditions. These experiments serve as a control to document that the difference in the binding capabilities of MAb-E/02 between freshly diluted, and diluted and exposed to 1 h incubation under shaking (physical stress). All inhibition values are adjusted to stress exposure. (B) Dosimetry of inhibition of E/02 binding to HLA class Ia antigens by ^{115}QFAYDGKDY^{123}. (C) Dosimetry of inhibition of E/02 binding to HLA class Ia antigens by ^{137}DTAAQI^{142}. (D) The peptide ^{126}LNEDLRSWTA^{135} not only failed to inhibit the binding but enhanced the binding. (E) Similar inhibition or enhancement was observed with MAb-E/07. Peptides ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142} effectively inhibit binding of MEM-E/07 to class Ia antigens. The peptide ^{126}LNEDLRSWTA^{135} not only failed to inhibit the binding but enhanced the binding. (F) Similar inhibition or enhancement was observed with MAb-E/08. Peptide ^{137}DTAAQI^{142} effectively inhibits binding of MEM-E/08, whereas ^{126}LNEDLRSWTA^{135} enhanced binding. Mean and/or standard deviation of triplicate values are presented.

the binding of MAb W6/32 to HLA-Ia is totally abolished by the acid treatment revealing that the HLA-E-MAbs may not be binding to the exact site on HLA-Ia as that recognized by MAb W6/32. All HLA-E-MAbs bound to the several HCs of HLA-B* and HLA-Cw* alleles. MEM-E/08 seems to prefer the HLA heterodimer conjugated with β_2m compared to other HLA-E-MAbs, suggesting that E/08 may be specific and useful for studying intact (β_2m-bound) HLA-E on the cell surface. Better affinity of MEM-E/02 and MEM-E/06 for HC of HLA validates the observations of the earlier investigators on the suitability of these antibodies for detecting β_2m-free HLA-E antigens on Western blots (Coupel et al., 2007; Derre et al., 2006).

The results of the peptide-inhibition experiments (Fig. 4B–D) revealed that the HLA-E-MAbs recognize peptide sequences shared by HLA-E and specific HLA-Ia alleles. Since we have used monoclonal antibodies, we could identify the plausible epitope(s) recognized by the HLA-E-MAbs, as ^{115}QFAYDGKDY^{123}

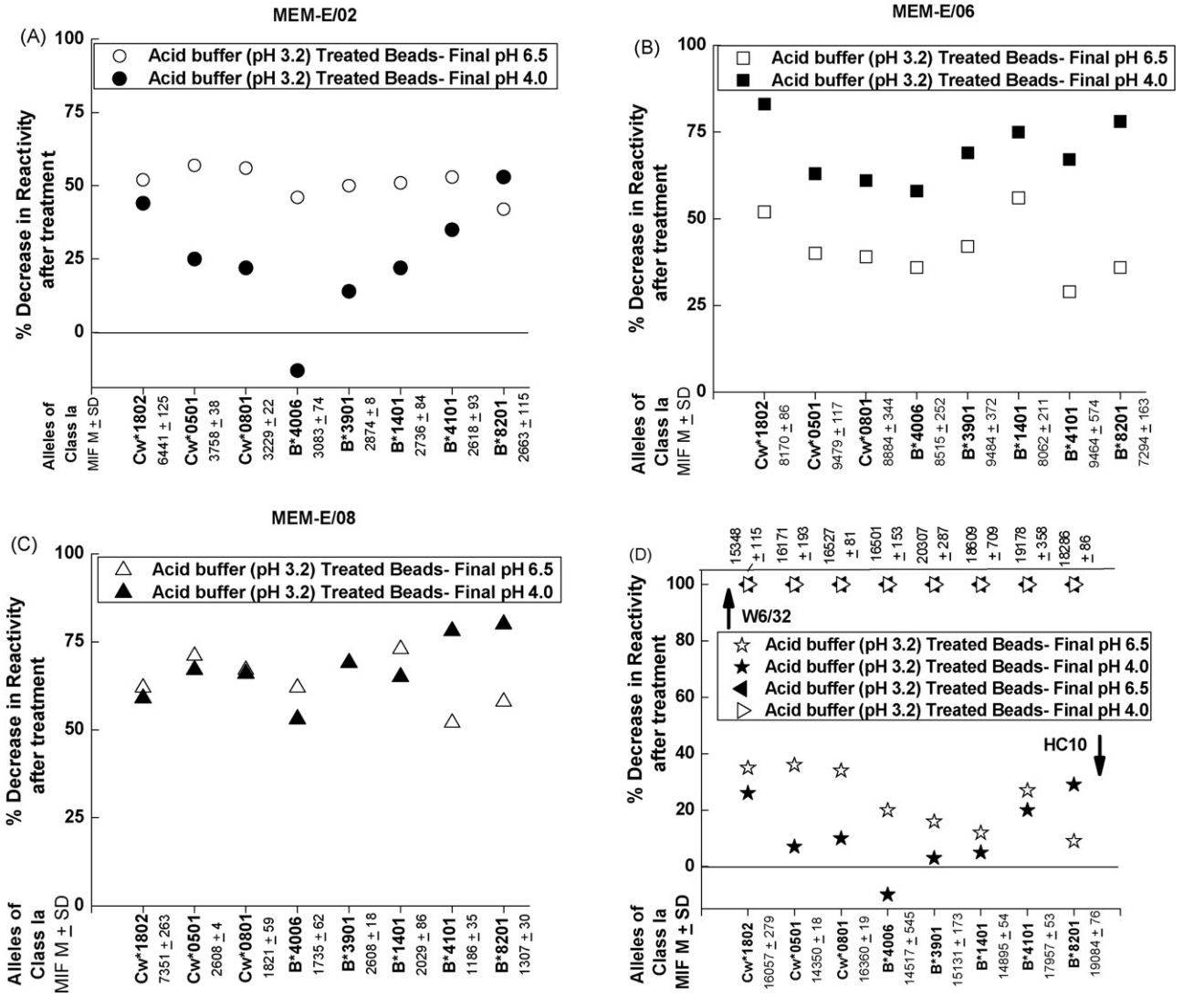


Fig. 5. Percentage decrease in reactivity of various murine MAbs [anti-HLA-E mouse MAbs (E/02 [A], E/06 [B], E/08[C]) and anti-HLA class Ia MAbs W6/32 and HC10] after treating with acid buffer. The antibody reactivity is tested (in triplicate) on HLA class Ia beads before and after treating with acid buffer (pH 3.2) and measured at specified final pH. Often in literature (Polakova et al., 2004), the beads are "neutralized" with buffer pH at 7.0 after treating with acid buffer, which often maintains the final pH acidic and it is at this pH antibodies are added, which may affect binding. However, if neutralized with alkaline buffer at pH higher than 9.5, the final pH is restored to neutral pH. The figures show that the final pH affects the binding, possibly depending of ionized or non-ionized reactive groups of amino acids in the conformational epitopes of the HLA molecules. The *x*-axis shows the alleles of class Ia and MFI (mean + SD) and the *y*-axis represents percentage (%) decrease in reactivity after treatment (based comparing batches # 2 (pH 4.0) and # 3 (pH 6.5) with control or batch #1). The reactivity of the HLA-E-MAb MEM-E/08 is affected strongly by the acid treatment and based on the percentage decrease observed in binding after acid treatment, the antibodies can be ranked as follows: W6/32 ⋙ MEM-E/08 (C) > MEM-E/06 (B) ≫ MEM-E/02 (A) > HC10 (D). D shows that after acid treatment of the beads, W3/62 failed to bind to any of the HLA-Ia alleles, whereas HC10 continued to bind, though to a lesser degree to the alleles recognized by HLA-E MAbs.

and ^{137}DTAAQI^{142}. The dose-dependent inhibition confirms the inhibitory potential of these two peptides on HLA-E-MAbs. These peptides inhibit antibody binding to HLA-E as well as the HLA-Ia molecules confirming further that these peptides may not only be immunogenic site in HLA-E molecules but also serve as target sites on HLA-Ia molecules. Table 5 shows the presence or absence of the sequences on the heavy chains of different HLA-Ia alleles. The location of the inhibitory peptides is illustrated diagrammatically in a structural model of HLA (Fig. 6). The peptide DTAAQIS is located at the terminal end of α2-helix and extends at an easily accessible site for antibody. The synthetic ^{137}DTAAQI^{142} inhibited 45% of the binding of E/02 to HLA-E while ^{115}QFAYDGKDY^{123} inhibited 24%. Failure to observe higher inhibition with the peptides could be due to recognition of both the peptides simultaneously by the antibody. The antibody may be recognizing a *discontinuous epitope* of both the peptides. Both the peptides may provide "conformational antibody-binding sites".

The failure of most of the anti-HLA-E MAbs to bind to HLA-A-alleles could be due to the following unique characteristics of HLA-A. In HLA-A-alleles, threonine (T) in ^{137}DTAAQI^{142} is replaced with methionine (M) and phenylalanine (F) in ^{115}QFAYDGKDY^{123} or asparagines (N) in ^{126}LNEDLRSWTA^{135} is replaced with aspartic acid (D) or lysine (K) in addition to other conformation changers are observed at positions 114, 143 and 144.

The closer proximity of these two peptides (^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}) strongly supports the contention that they could be the potential immunogen(s) that would have resulted in the production of the murine MAbs used in this study. At present, we cannot rule out the involvement of other peptide epitopes listed in Table 4, acting as immunogen to generate anti-HLA-E antibodies.

Table 4
Ranking of epitopes shared between HLA-E and various alleles of HLA class Ia antigens recognized by the murine monoclonal antibody MEM-E/02. Data shown for MFI > 600 but *n* in parenthesis refers of MFI > 500; shaded box refers to presence of peptide and empty box refers to absence of peptide in the alleles.

Table 5
Correlation between anti-HLA class Ia reactivity of murine anti-HLA-E MAb (MEM-E/02) and HLA class Ia reactivity of natural anti-HLA antibody found in the sera of healthy non-alloimmunized (*) males.

HLA-Ia reactivity of murine MAb-E/02 (Lot # 1) raised against human HLA-E heavy chain (present study)						Frequency of HLA-Ia reactivity of natural antibodies in the sera of healthy males* (report of Morales-Buenrostro et al., 2008)					
A locus (3 of 4 reported)	MFI	B locus (10 of 30 reported)	MFI	C locus (9 of 14 reported)	MFI	A locus (3 of 19 reported)	%	B locus (10 of 16 reported)	%	C locus (9 of 10 reported)	%
A*2402	1159	B*5601	2325	Cw*0501	4400	A*2402	4.0	B*5601	3.8	Cw*0501	4.0
A*3301	923	B*8201	2230	Cw*1802	3862	A*3301	5.2	B*8201	10.4	Cw*1802	3.1
A*2403	531	B*3701	1325	Cw*0102	1186	A*2403	3.3	B*3701	7.8	Cw*0102	3.8
		B*1502	1104	Cw*0602	1044			B*1502	3.1	Cw*0602	4.2
		B*4501	1067	Cw*0303	923			B*4501	5.9	Cw*0303	4.0
		B*4402	917	Cw*0202	900			B*4402	6.1	Cw*0202	5.0
		B*0702	685	Cw*1502	737			B*0702	3.3	Cw*1502	3.5
		B*5401	653	Cw*0302	538			B*5401	4.2	Cw*0302	4.7
		B*1512	554	Cw*1701	500			B*1512	11.1	Cw*1701	11.3
		B*5703	537					B*5703	3.3		

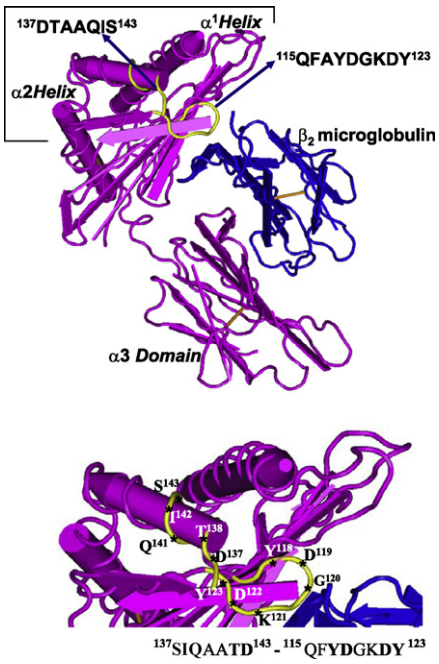


Fig. 6. Structure of HLA-E with α1 and α2 helices and the position of the inhibitory peptides [DTAAQIS and QFAYDGKDY] involved in antibody recognition and binding to HLA. The insert clarifies the molecular basis of antibody recognition of HLA-E. Insert shows the arrangement of peptide sequences involved in antibody recognition. HLA-E-MAbs may recognize discontinuous peptide sequences. Six to nine amino acids may be involved. The amino acids involved in antibody recognition are shown in bold. Hidden amino acids are shown in small letters.

Inability of ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142} to inhibit MEM-E/08 favors the contention that other peptide epitopes in HLA-E can be immunogenic. Interestingly, the peptide ^{126}LNEDLRSWTA^{135} not only failed to inhibit the binding but protected or prevented denaturation of the monoclonal antibody, as evidenced in Fig. 4D.

The significance of the findings can be appreciated by the reports on the occurrence of antibodies to HLA-Ia alleles in the sera of healthy non-alloimmunized individuals as "natural" HLA antibodies (Collins et al., 1973; Lepage et al., 1976; Tongio et al., 1985, Ameglio et al., 1987, Májský, 1989; Luscher et al., 1988; Zhou et al., 2008; Morales-Buenrostro et al., 2008) and in the sera of organ transplant–recipients as non-donor specific allo-HLA antibodies (Terasaki and Cai, 2005, 2008; Wu et al., 2008; Briggs et al., 2009; Seveso et al., 2009). Neither the origin nor the significance of these class Ia antibodies in normal individuals or in transplant–recipients is known. Comparing the present findings with the report of Morales-Buenrostro et al. (2008) on the natural HLA antibodies found in non-alloimmunized healthy males (Table 5), a striking similarity is observed between anti-HLA class Ia reactivity of anti-HLA-E MAb (MEM-E/02) and class Ia reactivity of natural anti-HLA antibody in the sera of healthy non-alloimmunized males. These similarities together with the reports on the occurrence of soluble HLA-E in circulation (Derré et al., 2006; Coupel et al., 2007; Lin et al., 2009) suggest the possibility that the antibodies against HLA-E exist in human sera with similar allo-HLA class Ia specificities as exhibited by the anti-HLA-E murine MAbs. A manuscript incorporating the experimental results demonstrating similar HLA-Ia reactivity of human HLA-E antibodies is in preparation and the results would provide an understanding on the immunogenicity of HLA-E in human.

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