Impact of Peptides on the Recognition of HLA Class I Molecules by Human HLA Antibodies

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MHC class I molecules expressed on cell surfaces are composed of H chain, β_2-microglobulin and any of a vast array of peptides. The role of peptide in the recognition of HLA class I by serum HLA Abs is unknown. In this study, the solid-phase assay of a series ($n = 11$) of HLA-A2-reactive, pregnancy-induced, human mAbs on a panel ($n = 12$) of recombinant monomeric HLA-A2 molecules, each containing a single peptide, revealed peptide selectivity of the mAbs. The flow cytometry membrane staining intensities on the HLA-A2-transduced cell line K562, caused by these mAbs, correlated with the number of monomer species detected by the mAbs. Flow cytometry staining on HLA-A2-bearing cell lines of a variety of lineages was indicative of tissue selectivity of these HLA-A2 mAbs. This tissue selectivity suggests that the deleterious effect on allografts is confined to alloantibodies recognizing only HLA class I loaded with peptides that are derived from tissue-specific and household proteins. Since Abs that are only reactive with HLA loaded with irrelevant peptides are expected to be harmless toward allografts, the practice of HLA Ab determination on lymphocyte-derived HLA deserves reconsideration. ***The Journal of Immunology,*** **2005, 175: 5950–5957.**

Human histocompatibility leukocyte Ag class I molecules play a pivotal role in the defense toward intracellular pathogens. The activation of $CD8^+$ T cells is initiated by recognition of pathogen-derived peptides bound in the HLA class I molecules of APCs, and this interaction requires at least partial MHC identity of T cells and APCs. In the case of MHC disparity, however, HLA molecules themselves are recognized as foreign, resulting in an adaptive alloimmune response to HLA class I. After blood transfusion, or transplantation of an incompatible graft, differences in the HLA type of donor and recipient can cause both formation of HLA-specific Abs (1) and T cell activation. These reactivities also occur during and after pregnancy (2) in a proportion of women. As a clinical consequence, preformed HLA Abs, detectable in a lymphocyte cross-match, are a contraindication to transplantation with an organ bearing the HLA Ags that are detected by these Abs.

Peptide embedded in alloantigen is a critical factor that determines the recognition of alloreactive, MHC class I-specific T cells (3). Crystallographic studies have shown that a role for peptide in this allorecognition is likely because subtle differences in conformational structure ensue when different peptides are incorporated in MHC class I (4, 5). This variability has also been detected serologically: the successful binding of some mouse monoclonal allo-MHC Abs to H2 class I Ags is governed by peptide, and, as these mAbs permit only a limited repertoire of peptides, they detect a limited number of H2 class I molecules on lymphocytic cell surfaces by flow cytometry (FCM) (6) and immunoprecipitation (7).

Whether serum HLA Abs are equally sensitive to peptide bound in the HLA class I groove has not been documented before. Monoclonal HLA Abs have been instrumental in determining epitopes that are responsible for alloantibody binding (8), but to what extent the shaping of the epitopes is peptide dependent is unknown. To determine whether peptide-induced changes of HLA class I conformation affect Ab binding in humans, we undertook the present study in which a series of recombinant HLA-A2 molecules, each containing a different peptide, served as ligands for human HLA mAbs. Differential binding characteristics indicated that the nature of the peptide bound in HLA class I is decisive for binding of HLA-A2 Abs and explained the limited binding patterns of some human mAbs to cell surface expressed HLA-A2.

Materials and Methods

HLA-A2 monomers

HLA-A2 monomers (based on the *HLA-A*0201* sequence) were synthesized as described previously (9), with minor modifications. Peptides used in HLA-A2 monomer synthesis were synthesized by solid-phase technology (10) and are listed in Table I.

Monoclonal Abs

Human hybridomas were established from B lymphocytes of seven HLA Ab-seropositive, multiparous women by EBV transformation, followed by electrofusion and hypoxanthine, aminopterin, thymidine, and ouabain selection of Ab-secreting EBV lines, and rigorously subcloning as described elsewhere (11). The Medical Ethics Committee of Leiden University Medical Center approved the use of human donors. Some individuals yielded multiple hybridomas. HLA specificities of human mAbs were determined by complement-dependent cytotoxicity (CDC)[3] against large ($n > 240$) panels of HLA-typed PBLs (Table II). mAbs were used as hybridoma

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[3] Abbreviations used in this paper: CDC, complement-dependent cytotoxicity; FCM, flow cytometry, LCL, lymphoblastoid cell line; NGFR, nerve growth factor receptor; PTEC, proximal tubular epithelial cell; RCC, renal cell carcinoma; SA, streptavidin; SAL, single Ag-expressing line.

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Table I. *Peptides used in HLA-A2 monomer synthesis*

Peptide	Gene	Amino Acid Sequence
HY	SMCY	FIDSYICQV
PRA	PRAME	SLYSFPEPEA
HA-1h	KIAA0223	VLHDDLLEA
HA-2	MYO1G	YIGEVLVSV
IB.54	Insulin	HLVEALYLV
HPV E7 12–20	HPV	MLDLQPETT
EII49	FASN	FLFDGSPTYV
EBV	BMLF-1	GLCTLVAML
Flue	IMP (matrix 58–66)	GILGFVFTL
hCMV	pp65	NLVPMVATV
HIV	Pol and RT2	ILKEPVHGV
PR-1	Proteinase-3	VLQELNVTV

supernatants at Ig concentrations ranging from 0.6 to 2.5 μg/ml. Murine mAb TP25.99, which binds a monomorphic epitope in the α3 domain of HLA class I (12), was provided by Dr. S. Ferrone (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY). Murine mAb 20.4 (anti-human nerve growth factor receptor; NGFR) was obtained from American Type Culture Collection.

ELISA

Microtiter plates were coated with 1 μg of streptavidin (SA; Pierce) in 10 mM Tris (pH 9.0), blocked with 2% BSA in PBS, and washed with 0.05% Tween 20 in PBS. HLA-A2 monomers were bound at 16 ng/well for 2 h and incubated for 4 h with human mAbs in the presence of Complete (protease inhibitor mixture; Roche Applied Science), followed by HRP-labeled goat anti-human Ig (Southern Biotechnology Associates) for 90 min. Upon ABTS addition, color was developed for 1 h and read at 450 nm. The efficacy of binding of monomers to SA was determined with mAb TP25.99 followed by HRP-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). All human mAb and TP25.99 incubations were done in duplicate. To correct for the various binding efficacies of monomers to SA (with the lowest binder, monomer-containing EBV peptide, and the best binder, monomer-containing HY peptide, yielding ODs of 0.65 and 1.42, respectively, in the TP25.99 assay), human mAb binding was reported as the ratio: (mean OD_{HuMAb} − mean OD_{blank})/(mean $OD_{TP25.99}$ − mean OD_{blank}). None of the mAbs reacted with SA alone.

Cells

A *s*ingle Ag-expressing line (SAL) was established as follows: a retroviral construct encoding HLA-A*0201 linked to the downstream internal ribosome entry sequence and the truncated form of NGFR (13) was transfected into φNX-A cells (provided by Dr. G. Nolan, Department of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA). Transfectants were selected with puromycin (BD Clontech). Supernatant of transfected φNX-A was used to transduce the HLA class I and class II-negative erythroleukemia cell line K562. Transduced cells were sorted by FACS on the basis of NGFR positivity with mAb20.4 and are called SAL-A2. Renal cell carcinoma (RCC) lines MZ1774 and MZ1257 were made available by Dr. A. Knuth (University Hospital Zurich, Zurich, Switzerland), lymphoblastoid cell lines (LCL) were obtained from the 12th International Histocompatibility Workshop and a proximal tubular epithelial cell (PTEC) line VDS was established locally (14).

Table III. *Reactivity of human mAbs*

mAb	Median fluorescence[a]	No. of Monomers Detected by ELISA[b]	CDC[c]
BVK1F9	9	0	0
JOK2C7	11	0	0
JOK3H4	11	0	0
Ha5C2	12	0	17
WIM1B3	81	1	99
WIM8E5	114	3	50
ROU2D3	286	7	89
WK4E3	309	10	86
WK3D10	339	8	100
SN66E3	392	11	87
SN607D8	419	12	74
SN230G6	429	12	100

[a] Determined on SAL-A2; mAbs sorted by these data.
[b] OD ratio (human mAb:TP25) of 0.100 was used as cutoff value for positivity.
[c] Percentage of SAL-A2 cells lysed in CDC.

Flow cytometry

Human mAbs were incubated with 350,000 cells in 50 μl for 1 h on ice, washed, incubated with FITC-conjugated rabbit $F(ab')_2$ of anti-human IgG or IgM conjugates (DakoCytomation) on ice, washed, fixed with paraformaldehyde (1%), and analyzed on a FACSCalibur (BD Biosciences) equipped with CellQuest. For quantification of fluorescence intensity, the medians of total histograms of gated cells were recorded. Gated cells constituted 85% or more of the total cell population.

Complement-dependent cytotoxicity

CDC on SAL-A2 was performed as described previously (15).

Immunoprecipitation

Aliquots of a precleared Nonidet P-40 lysate of ^{125}I-labeled (by lactoperoxidase) LCL CALOGERO (HLA-A2,-, B61,-, Cw2,-) were sequentially incubated with suspensions of protein A-Sepharose CL-4B beads (Pharmacia) that had been preloaded with mAbs WIM8E5 or SN230G6. Upon washing, bound Ags were released from the beads in reducing sample buffer, electrophoresed on a 12% polyacrylamide-SDS gel, visualized by autoradiography, and quantified on a PhosphoImager (Molecular Dynamics).

Table II. *Human monoclonal HLA Abs*

mAb[a]	HLA Specificity by CDC	r^b	Epitope[c]	Isotype
JOK3H4	A2	0.952	107W	IgM,λ
ROU2D3	A2/B17	0.944	62G	IgM,λ
SN230G6	A2/B17	0.904	62G	IgG1,λ
WIM1B3	A2/B17	0.815	62G	IgM,λ
HA5C2	A2/A28	0.711	149A 152V	IgM,κ
JOK2C7	A2/A28	0.533	149A 152V	IgM,λ
SN607D8	A2/A28	0.933	149A 152V	IgG1,κ
SN66E3	A2/A28	0.902	149A 152V	IgM,κ
WK4E3	A locus not A1/A24	0.826	166EW	IgM,λ
WK3D10	A2/A3/A23/A31/B7/B17/B13/B40/B21/B62	0.745	nd	IgM,κ
WIM8E5	A1/A10/A11/A9/A29/A30/A31/A33/A28[d]	0.578	nd	IgG1,κ
BVK1F9	B8	0.979	n/a[e]	IgG1,κ

[a] mAbs with the same two- or three-letter prefix are from a single individual.
[b] Correlation coefficient.
[c] The epitopes involved in human mAb binding as determined by analysis of amino acid sequences of HLA class I molecules (5).
[d] Plus HLA-A2 on cells homozygous for HLA-A2 only (15 of 16 individuals tested).
[e] n/a, Not applicable.

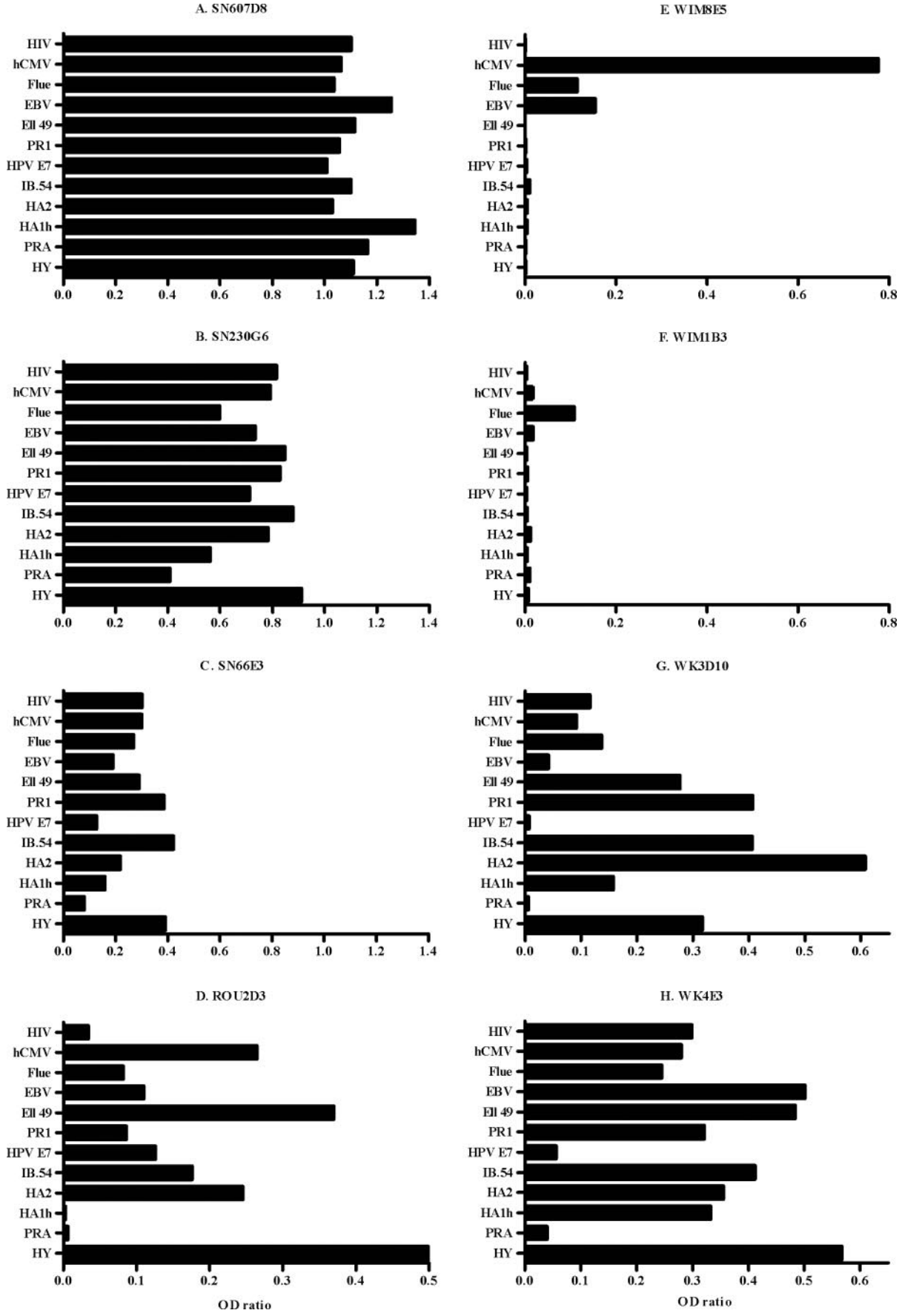

FIGURE 1. Reactivity of human mAbs with HLA-A monomers. Monomer-embedded peptides are given on the *y*-axis. Results are expressed as OD ratio (see *Materials and Methods*). Only mAbs causing an OD ratio >0.1 for one or more monomers are shown.

Statistics

Rank correlations were determined using Spearman's rank test; Ab specificity correlations were determined using Pearson's test with Yates correction (both from GraphPad InStat).

Results

Reactivity of mAbs with cell surface-expressed HLA-A2

We produced a SAL (SAL-A2) by transduction of the HLA class I and II-negative cell line K562 with the *HLA-A*0201* gene. FCM on this cell line, that expresses HLA-A2 with the K562-encoded repertoire of endogenously produced peptides, permits the relative quantification of HLA-A2 Ag recognized by various HLA-A2 mAbs. Eight human mAbs with HLA-A2 reactivity (Table II) showed reactivity by FCM with SAL-A2, but with a vast range of staining intensities (Table III). Differences in staining intensity were observed even among mAbs of identical specificity and isotype (compare mAbs ROU2D3 and WIM1B3). Titration experiments demonstrated that mAbs were used at saturating concentrations (data not shown). Six mAbs (ROUD2D3, SN230G6, SN607D8, SN66E3, WK4E3, and WK3D10) showed high staining intensity, exceeding a median channel value of 280, and 2 mAbs (WIM1B3 and WIM8E5) showed intermediate intensity. Three other mAbs (JOK3H4, Ha5C2, and JOK2C7) did not stain. Comparisons of staining intensities between isotype-matched mAbs revealed a ratio for WIM1B3:ROU2D3 (both IgM,λ) of 0.25 and likewise for WIM8E5:SN607D8 (both IgG1,κ) of 0.25. This suggests that mAbs WIM1B3 and WIM8E5 react with subsets of HLA-A2 molecules that contain a limited repertoire of bound peptides. In addition, we used CDC to test the lytic capacity of human mAbs on the SAL-A2 (Table III). The CDC reactivity correlated well with FCM staining intensity for all mAbs, except for two mAbs (HA5C2 and WIM1B3) that deviated considerably. Taken together, HLA-A2-reactive mAbs, even of identical isotype, react to varying degrees with HLA-A2 expressed on whole cells.

Impact of peptides on humoral HLA-A2 recognition

The availability of a panel of HLA-A2 monomers, each containing a different peptide, permitted analysis of the peptide selectivity of HLA-A2-specific human mAbs by ELISA. These 12 peptides (Table I), all containing HLA-A2 binding motifs, were based on viral, bacterial, and autoantigen-derived sequences. The monomer reactivity of human mAbs, expressed as an OD ratio is given in Fig. 1. We considered a monomeric HLA-A2/human mAb combination that showed an OD ratio of 0.100 or higher as positive. With this criterion, eight human mAbs showed reactivity with the monomers, but with vastly different reactivity patterns. These ranged from reactivity with the complete monomer panel, as exemplified in Fig. 1, *A–C*, by the three mAbs derived from donor SN (with mAb SN66E3 reacting with 11 of12 monomers), to reactivity with only one or three monomers as shown by the mAbs WIM1B3 and WIM8E5, respectively (Fig. 1, *E* and *F*). Intermediate degrees of selectivity were shown by 3 mAbs: the mAb ROU2D3 reactive with 7 of 12 monomers (Fig. 1*D*), the mAb WK3D10 (8 monomers, Fig. 1*G*), and WK4E3 (10 monomers, Fig. 1*H*). The three remaining CDC defined, HLA-A2-reactive mAbs JOK2C7, JOK3H4, and Ha5C2 and the HLA-B8-reactive mAb BVK1F9 did not bind to any monomer (data not shown). The peptide selectivity shown by some mAbs is likely due to critical contributions of certain amino acids in the HLA-A2-bound peptides. This is illustrated for mAb WK4E3 that was intolerant for a unique combination of amino acids P and E in positions 6 and 7 in the two nonreactive HLA-A2-bound peptides (Table IV). The peptide selectivity was not confined to these positions for the other selective mAbs (data not shown).

Table IV. *Analysis of aminoacids critical in binding of mAb WK4E3 to monomers*[a]

Peptide	WK4E3 OD Ratio	Amino Acid Position 1	2	3	4	5	6	7	8	9	10
HY	0.567	F	I	D	S	Y	I	C	Q	V	
EBV	0.501	G	L	C	T	L	V	A	M	L	
EII49	0.484	F	L	F	D	G	S	P	T	Y	V
IB.54	0.412	H	L	V	E	A	L	Y	L	V	
HA-2	0.355	Y	I	G	E	V	L	V	S	V	
HA-1h	0.333	V	L	H	D	D	L	L	E	A	
PR-1	0.321	V	L	Q	E	L	N	V	T	V	
HIV	0.299	I	L	K	E	P	V	H	G	V	
hCMV	0.280	N	L	V	P	M	V	A	T	V	
Flue	0.245	G	I	L	G	F	V	F	T	L	
HPV E7	0.056	M	L	D	L	Q	**P**	**E**	T	T	
PRA	0.040	S	L	Y	S	F	**P**	**E**	P	E	A

[a] Amino acids unique for preventing mAb binding are in bold. Monomers were sorted by mAb reactivity.

The number of monomers detected by each mAb in ELISA is included in Table III. When fluorescence intensity on SAL-A2 and the number of reactive monomers were compared for each HLA-A2 mAb, these values showed extremely good correlation ($r = 0.800$, $p = 0.003$ by Spearman's rank test, Fig. 2). These data show that the amino acid sequence of the peptide bound in HLA-A2 dictates binding of the human mAbs to these molecules.

Biochemical identification of HLA-A2 molecules recognized by HLA-A2 mAbs

To probe the relationship of HLA-A2 molecules that are recognizable by various HLA-A2 mAbs on cell surfaces, sequential immunoprecipitation was performed with two human mAbs of the IgG isotype on the HLA-A2 homozygous LCL CALOGERO. Exposing the CALOGERO lysate to three rounds of mAb SN230G6 resulted in precipitation of all available HLA-A2 molecules, as evidenced by bands visible at M_r 46,000 (H chain) and 13,000 (β_2-microglobulin), but no bands visible upon three subsequent rounds of mAb WIM8E5 (Fig. 3*A*). When the order of mAbs was reversed (Fig. 3*B*), three rounds of WIM8E5 precipitated only a fraction of HLA-A2 visible at M_r 46,000 and 13,000 because three subsequent rounds of SN230G6 precipitated the preponderance of material at M_r 46,000 and 13,000. Quantification of bands in the latter sequential experiment showed that WIM8E5 precipitated 13% of the total H chain precipitated by WIM8E5 and SN230G6

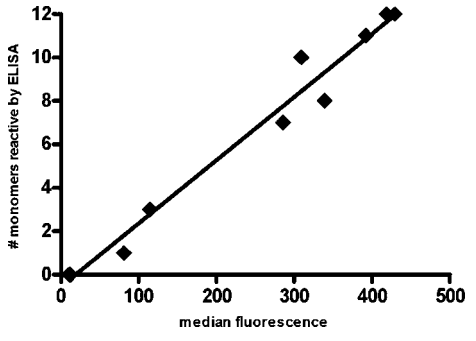

FIGURE 2. Correlation of fluorescence intensity caused by mAbs on SAL-A2 and monomer panel binding of mAbs. (mAb BVK1F9 not included in this analysis).

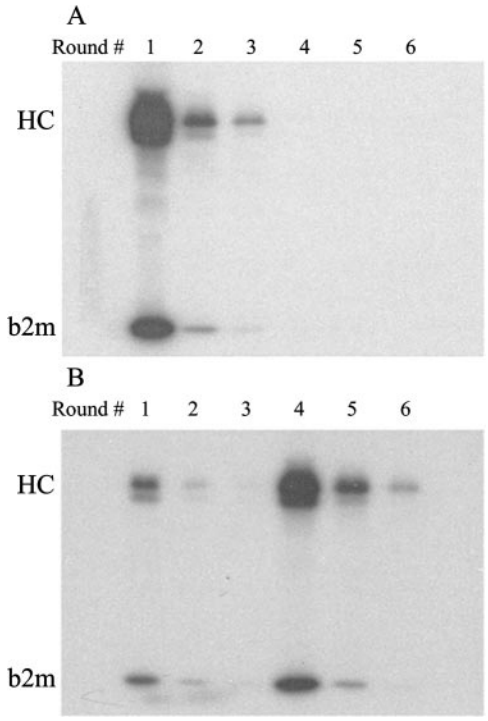

FIGURE 3. Immunoprecipitation on lysate of ^{125}I-labeled CALOGERO, with two human mAbs analyzed by PAGE. *A,* Three rounds of immobilized SN230G6 (*lanes 1–3*) followed by three rounds of WIM8E5 (*lanes 4–6*). *B,* Three rounds of WIM8E5 (*lanes 1–3*) followed by three rounds of SN230G6 (*lanes 4–6*). Positions of H chain (HC, M_r 46,000) and β_2-microglobulin (β2M; M_r 13,000) are interpolated from the positions of M_r markers in a separate lane.

together. Likewise, WIM8E5 precipitated 25% of the total β_2-microglobulin. Thus, the WIM8E5 recognizable HLA-A2 molecules are a subset of the SN230G6 recognizable molecules.

Differential expression of mAb recognizable HLA-A2 on cells of different lineage

Cell surface-expressed HLA-A2 contains peptides derived from household proteins, virus-encoded proteins, and proteins uniquely expressed in cell lineages. HLA-A2 mAbs that show peptide selectivity also may harbor selectivity for certain tissues. To test this hypothesis, we quantified the expression of HLA-A2 by FCM on cell lines from various lineages. Ratios of expression of HLA-A2 calculated from peptide-selective mAbs are given in Table V. Although mAb WIM8E5 detects a small fraction of SN230G6-detectable HLA-A2 molecules on SAL-A2 (Fig. 4*A*) and HLA-A2 homozygous LCL CALOGERO (Fig. 4*B*), it detects a larger proportion of the HLA-A2 molecules expressed on the PTEC line VDS (Fig. 4*C*). In contrast, WIM1B3 detects a larger fraction of ROU2D3-detectable HLA-A2, both on RCC lines (MZ1774 shown in Fig. 5*A*) and on LCL (CALOGERO shown in Fig. 5*B*), than on SAL-A2 (Fig. 5*C*). The latter indicates that WIM1B3 and WIM8E5 exhibit diverse preferences for peptides. In contrast, the ratios of expression of HLA-A2 as detected by the two mAbs SN230G6 and SN607D8 were around 1.0 throughout this range of cell lineages (Table V), which is in line with the finding that these mAbs were not peptide selective on the monomer HLA-A2 panel.

Discussion

Cell surface-expressed HLA class I encoded by a single allele is a heterogeneous set of molecules presenting an array of peptides generated from intracellularly processed proteins. HLA class I molecules of a single allele, but from different organs, contain different arrays of peptides and these differences are detectable by CTL (16). In this study, we present evidence that human HLA Abs have also have the capacity to distinguish peptide-induced alterations. Although the set of monomers used in the ELISA was limited, the human mAbs show a range of peptides selectivities that has been found in allo-CTL clones also (3). The combined use of human mAbs, HLA-A2 monomers, and the single Ag transductant was instrumental in demonstrating the decisive role of peptides in the recognition of MHC molecules by human alloantibodies. We observed differential binding of six HLA-A2-reactive mAbs with the HLA-A2 monomer panel. There was no relationship between the monomer reaction patterns and the HLA-A2 epitope specificity or the Ig isotype of the human mAbs. When these mAbs were confronted with HLA-A2 bearing a multitude of peptides, various subsets of HLA-A2 molecules reacted, yielding a range of staining intensities by FCM on the SAL-A2. Ratios of staining intensities were calculated for mAbs of identical isotype. Strikingly, biochemical analysis and FCM with two IgG mAbs, WIM8E5 and SN230G6, showed that in these divergent techniques, ratios of reactivity were in the same range. Furthermore, HLA-A2 molecules recognized by one mAb are nested within the larger set of HLA-A2 molecules seen by the other mAb. In this respect it is remarkable that the rank order of Ag densities reactive with mAbs as detected by FCM on SAL-A2, almost completely concurs with the rank order of the number of monomers recognized in the ELISA by the mAbs (Fig. 2). Three mAbs that react with HLA-A2+ lymphocytes by CDC failed to react with SAL-A2 by FCM and CDC, suggesting their preference for peptides that are not sufficiently provided in the erythroid precursor environment of

Table V. *Differential binding of mAbs to cell lines of various lineages*

					Ratio[a]			
				mAb1→	WIM8E5	WIM1B3	WIM1B3	SN230G6
Designation	Cell Type	HLA Type		mAb2→	SN230G6	ROU2D3	SN66E3	SN607D8
MZ1774	RCC	A2,A30	B13,B18		ni[b]	0.73	0.60	0.87
MZ1257	RCC	A2,A3	B7,B44		0.26	0.42	0.29	1.13
VDS	PTEC	*A*02,A*03*	*B*0702,B*4402*		0.77	0.15	0.10	0.84
AWELLS	LCL	A2,-	B44,-		0.26	0.69	0.58	0.97
CALOGERO	LCL	A2,-	B61,-		0.18	0.83	0.48	1.00
WT24	LCL	A2,-	B27,-		0.26	0.92	0.57	1.08
SAL-A2	Erythroid	*A*0201*			0.25	0.26	0.19	1.03

[a] Ratios of reactivity were determined by calculating (median mAb1 − median (negative control)/median mAb2 − median negative control) in which negative control was cells with conjugate only.
[b] ni, Not informative.

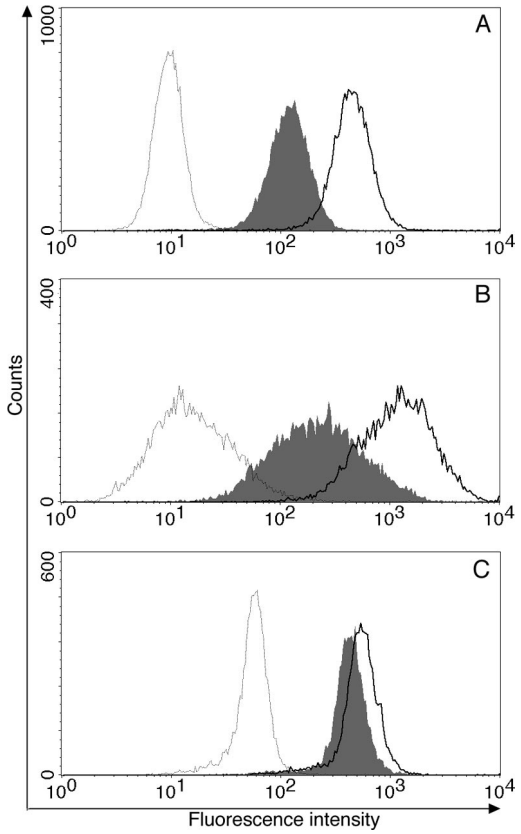

FIGURE 4. Flow cytometric characteristics of IgG isotype human mAbs. Solid line, SN230G6; gray area, WIM8E5; stippled, control. *A,* SAL-A2; *B,* CALOGERO; *C,* VDS.

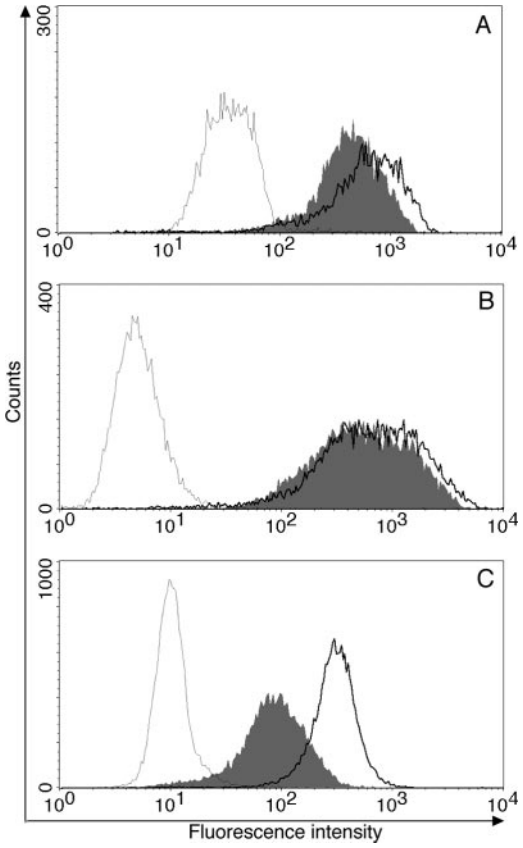

FIGURE 5. Flow cytometric characteristics of IgM isotype human mAbs. Solid line, ROU2D3; gray area, WIM1B3; stippled, control. *A,* MZ1774; *B,* CALOGERO; *C,* SAL-A2.

K562. In contrast, the degree of susceptibility of SAL-A2 to complement-dependent lysis is only partially reflected in the number of monomers reactive with a given mAb. The notable exception here is mAb WIM1B3 which causes complete lysis of SAL-A2, yet binds only one monomer of the panel (Table III). Similar to CTL-induced lysis, complement-dependent lysis may require only a limited density of Ab-reactive Ags on the cell surface. That this threshold is low is underpinned by the fact that one mAb, Ha5C2, is able to cause a low degree of lysis by CDC on SAL-A2, despite lack of FCM staining.

The differentially reactive mAbs clearly see different subsets of cell surface-expressed HLA-A2. Several mechanisms may underlie differential mAb binding to HLA-A2 and these include peptide-induced conformational changes causing epitope disruption, steric hindrance by large side chains of peptides resulting in epitope blocking, and requirement for mAb to interact with Ag by two CDRs at distinct antigenic sites. Our exploratory study does not allow favoring any of these possibilities. The influence of peptide-induced conformational changes in MHC class I molecules on alloantibody binding has been shown with murine K^d-reactive mAbs (7). Another study with K^b-specific mAbs showed that by increasing the number of peptides, a larger number of K^b-reactive mAbs appear to be peptide dependent. Peptide pools eluted from K^b molecules that were immunoprecipitated with a peptide-selective mAb were successfully used to reconstitute empty K^b molecules on T2-K^b targets that were lysable by some allo-CTL clones (17). This suggests a relationship between alloreactive CTL and alloantibody recognition of MHC class I. Even deliberate allo-immunization and boosting with the SIINFEKL peptide-loaded tumor RMA-S yielded a peptide-MHC class I complex mAb (18) that nonetheless turned out to be tolerant to single amino acid substitutions (19). Peptide selectivity by mAbs has also been demonstrated in xenogeneic (mouse anti-HLA-B27) combinations (20, 21).

To our knowledge, the present study is the first demonstration of peptide selectivity of human HLA-alloreactive Abs and corroborates an earlier study (22) that showed peptide (des-Tyr^1-γ-endorphin) blocking of HLA allosera. In this study, we took advantage of availability of recombinant monomeric HLA-A2 which provides a more convenient platform for peptide studies, compared with peptide reconstitution of TAP-deficient cell lines. Our results are at variance with data published by others. Using a similar ELISA method for screening sera for HLA Abs, Barnardo et al. (23) failed to observe a difference in serum Ab binding with HLA-A11 monomers (two peptides tested). Likewise, an anti HLA-A11 human mAb (24) detected no differences in cell surface-expressed, empty HLA-A11 molecules that were reconstituted with an array of peptides, while in the same study mouse mAbs of identical specificity were discriminatory. The findings of these two groups may have been fortuitous, as our present observation of five differentially HLA-A2-reactive human mAbs, which were derived from three different immune donors, indicates that peptide selectivity of HLA Abs is no isolated incident. This is further supported by preliminary data demonstrating that more than one-half of the HLA Abs present in supernatants of monoclonally seeded B cells of donor ROU, cultured after tetrameric HLA-A2-guided isolation (25), were peptide selective. (C. Eijsink and A. Mulder, unpublished observations). The human mAbs described here were derived from women who had been immunized by pregnancy. The

fact that human mAbs with identical specificity (e.g., for the HLA-A2/B17 shared epitope) and isotype, but derived from different donors, show different monomer panel reactivity and different staining intensity by FCM suggests that in each donor a different set of peptides embedded in the allogeneic HLA-A2 molecule was responsible for the immunization that caused the clonal B cell expansion that we subsequently immortalized. Curiously, one individual, SN, yielded three HLA-A2 mAbs that were tolerant to peptide exchange, whereas the HLA-A2 mAbs generated from three other individuals were peptide selective. HLA Abs require one CDR for serological epitope recognition, with a critical contribution of a second CDR to stabilize Ag binding (26). An attractive hypothesis is that the SN-derived mAbs contain a second CDR that contacts a nonpolymorphic determinant exposed on the HLA backbone, but that the second CDR of the other mAbs interact with solvent-exposed amino acids of the embedded peptide. We expect Abs with even greater peptide-induced stringency to be present in the sera of women after HLA-A2 syngeneic pregnancies. The existence of such MHC class I-restricted humoral response was demonstrated in a serum Ab with HY specificity after bone marrow transplantation (27). More recently, phage display technology has enabled the isolation of recombinant Abs specific for the gp100 melanoma peptide-HLA-A2 complex from a nonimmune library (28), supporting the notion that Abs with TCR-like specificity are part of the normal humoral repertoire. The increased affinity of these TCR-like Abs (as compared with TCR themselves) makes them attractive tools for immunotherapy. The ELISA based on monomeric HLA class I loaded with peptides relevant to the purpose will be a useful tool for additional specificity testing of these novel Abs.

Besides advancing the knowledge of molecular interactions in humoral HLA recognition, a clinically relevant notion emerges from the present study. The peptide dependence of some human HLA-A2 mAbs implies that HLA Abs may harbor a certain degree of tissue specificity. Our FCM data on cells of various lineages confirm this concept. Having established that mAbs WIM1B3 and WIM8E5 recognize only fractions of total available HLA-A2 molecules on the erythroid lineage cell SAL-A2, we demonstrated that these two selective mAbs show increases in reactive fractions of HLA-A2 on cells of other lineages. However, among these two mAbs, the patterns of cell lineage preference were different from each other. The monomer panel reactivity patterns (Fig. 1, *E* and *F*) of these mAbs confirm this difference. Apparently, these two mAbs have preference for peptides that are expressed with greater abundance on renal epithelial cells from malignant (RCC) and primary origin (PTEC) than on the SAL-A2.

The expression of lineage-specific and/or virally encoded peptides in HLA-A2 is therefore the likely cause of this selectivity. Biochemical studies, with peptides that are derived from proteins uniquely expressed in relevant cell types and incorporated in HLA-A2 monomers should prove or negate this concept. If such tissue specificity of Abs is shown to exist, the implications for solid organ transplantation are far-reaching. Although Ab screening and cross-matching is performed on lymphocytes, the organ to be transplanted likely contains a set of peptides embedded in its HLA class I molecules that is partially different from the set of lymphocyte-expressed peptides. It is at least theoretically possible that patients who have been transplanted with a positive serological cross-match on lymphocytes, nonetheless have a well-functioning graft. By reanalyzing their pretransplant sera for the presence of Ab reactivity on relevant monomers, one would gain insight in the issue of tissue-specific Abs. In this light, it is important to note that tissue specificity has been observed in alloreactive CTLs (29, 30).

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Disclosures

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