Structural Relatedness of Distinct Determinants Recognized by Monoclonal Antibody TP25.99 on β_2-Microglobulin-Associated and β_2-Microglobulin-Free HLA Class I Heavy Chains[1]

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The association of HLA class I heavy chains with β_2-microglobulin (β_2m) changes their antigenic profile. As a result, Abs react with either β_2m-free or β_2m-associated HLA class I heavy chains. An exception to this rule is the mAb TP25.99, which reacts with both β_2m-associated and β_2m-free HLA class I heavy chains. The reactivity with β_2m-associated HLA class I heavy chains is mediated by a conformational determinant expressed on all HLA-A, -B, and -C Ags. This determinant has been mapped to amino acid residues 194–198 in the α3 domain. The reactivity with β_2m-free HLA class I heavy chains is mediated by a linear determinant expressed on all HLA-B Ags except the HLA-B73 allospecificity and on <50% of HLA-A allospecificities. The latter determinant has been mapped to amino acid residues 239–242, 245, and 246 in the α3 domain. The conformational and the linear determinants share several structural features, but have no homology in their amino acid sequence. mAb TP25.99 represents the first example of a mAb recognizing two distinct and spatially distant determinants on a protein. The structural homology of a linear and a conformational determinant on an antigenic entity provides a molecular mechanism for the sharing of specificity by B and TCRs. ***The Journal of Immunology,*** **2000, 165: 3275–3283.**

Like their counterparts in other animal species, HLA class I Ags are composed of two noncovalently associated subunits; the monomorphic polypeptide β_2-microglobulin (β_2m)[3] and a highly polymorphic transmembrane heavy chain encoded by the HLA-A, -B, and -C loci (1). The association of HLA class I heavy chains with β_2m drastically modifies their conformation. The resulting changes in their antigenic profile (2, 3) account for the selective reactivity of polyclonal and mAbs with either β_2m-free or β_2m-associated HLA class I heavy chains.

The mouse mAb TP25.99 (4) is among the few mAb (5, 6) reacting with both β_2m-associated and β_2m-free HLA class I heavy chains. Whether this unusual reactivity pattern reflects the sharing of an antigenic determinant between β_2m-associated and β_2m-free HLA class I heavy chains or the recognition by mAb TP25.99 of two distinct determinants is not known. A previous study has mapped the conformational determinant recognized by mAb TP25.99 on β_2m-associated HLA class I heavy chains to amino acid residues 184–199 in their α3 domain (7). In contrast, the linear determinant recognized by mAb TP25.99 on β_2m-free HLA class I heavy chains has not been mapped. In addition, the distribution of the determinant(s) recognized by mAb TP25.99 on HLA class I allospecificities has not been investigated.

In the present study, we have characterized the distribution of the determinant(s) recognized by mAb TP25.99 on β_2m-associated and β_2m-free HLA class I heavy chains. Furthermore, using immunochemical methods and phage display peptide libraries, we have mapped the conformational and the linear determinants recognized by mAb TP25.99 on HLA class I heavy chains. This information contributes to our understanding of the structural relatedness of distinct antigenic determinants defined by a mAb and of the conformational changes in HLA class I heavy chains induced by their association with β_2m.

Materials and Methods

mAbs and conventional antisera

mAb TP25.99 is secreted by a hybridoma generated by fusing the nonsecretor myeloma cells P3-X63-Ag8.653 with splenocytes from a BALB/c mouse immunized with multiple injections of IFN-γ-treated cultured human melanoma cells Colo 38. mAb W6/32 to a monomorphic determinant expressed on β_2m-associated HLA-A, -B, and -C heavy chains (8), mAb HC-10 to a determinant preferentially expressed on β_2m-free HLA-B and -C heavy chains (9), mAb CR11-351 to a conformational determinant shared by HLA-A2 and A28 Ags (10), anti-HLA-DR, DQ, DP mAb LGII-612.14 (11), rabbit anti-β_2m-free HLA class I heavy chain serum R 5996-4 (12), and rabbit anti-HLA class I heavy chain serum (13) were developed and characterized as previously described.

mAb were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulfate (14). The purity of the mAb preparations was assessed by SDS-PAGE. Biotinylation of mAb was performed using NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. mAb were radiolabeled with ^{125}I using the Iodogen method (15).

Affinity purified rabbit anti-mouse (3) IgG (heavy chain + light chain) Abs and HRP-conjugated goat anti-mouse (GAM-HRP) Abs were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell lines and Ag preparation

Cultured human B lymphoid cells, cultured human melanoma cells, and cultured human carcinoma cells were grown in RPMI 1640 medium (Life

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; 1D-IEF, one-dimensional isoelectric focusing; A_{490}, absorbance measured at 490 nm; GAM-HRP, HRP conjugated to goat anti-mouse Ab; SA-HRP, HRP conjugated to streptavidin.

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Technologies, Grand Island, NY) supplemented with 10% serum plus supplement (BioWhittaker, Walkersville, MD) and 2 mM L-glutamine (Life Technologies). All cell lines were cultured at 37°C in a 5% CO_2 atmosphere. Cell lysates were prepared as described (16).

Phage display peptide libraries

Phage display peptide libraries X_{15} and LX-8 (XCX_8CX) displaying 15 aa, random, linear peptides and 8 aa random, disulfide constrained peptides, respectively, were kindly provided by Dr. J. K. Scott (Simon Fraser University, Burnaby, British Columbia, Canada). The peptide libraries were constructed using bacteriophage vector f88.4 and had random peptide inserts at the N terminus of the synthetic pVIII major coat protein. Both peptide libraries were re-amplified in *Escherichia coli* host K91kan from an aliquot of phage stock provided. Phage particles were purified by two precipitations with polyethylene glycol/NaCl (16.7%/3.3 M) and resuspended in 10 ml TBS 50 (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) giving a titer of 5×10^{12} transducing U/ml.

Synthetic peptides

The amino acid sequence of the synthetic peptides used in the present study is shown in Table I. The cyclic LX-8 no. 1 peptide, the linear LX-8 no. 1 peptide, the linear LX-8 no. 1 C→S peptide, and the linear X_{19} no. 5 peptide were purchased from SynPep (Dublin, CA). The linear X_{15} no. 4 peptide, linear X_{17} no. 6 (X_{19}-EG) peptide, and the control no. 7 peptide were synthesized using standard Fmoc solid phase peptide synthesis in an automated peptide synthesizer (9050 Plus; PerSeptive Biosystems, Cambridge, MA). The cyclic LX-8 no. 1 peptide was cyclized using 5% DMSO and purified by reverse-phase HPLC. Cyclization of peptides was confirmed by mass spectroscopy. The purity of the cyclic LX-8 no. 1 peptide, the linear LX-8 no. 1 peptide, the linear LX-8 no. 1 C→S peptide, and the linear X_{19} no. 5 peptide was >96%, as assessed by HPLC. The linear X_{15} no. 4 peptide, the linear X_{17} no. 6 (X_{19}-EG) peptide, and the control no. 7 peptide were used as crude preparations at ~75% purity. Peptides were reconstituted in distilled water at the concentration of 5 mM, aliquoted and stored at −20°C.

Coating of plates with Ags

Polyvinylchloride, U-bottom, 96-well microtiter plates (Titertek, Huntsville, AL) coated with HLA class I Ags captured from cell lysates by anti-HLA class I mAb were prepared as described elsewhere (16). Plates were coated with synthetic peptides by adding to each well 100 μl of a 0.25% glutaraldehyde cross-linked synthetic peptide solution at concentrations varying from 1 to 50 μM. Following a 2-h incubation at 37°C and washing of the plate with TBS 10 (10 mM Tris-HCl (pH 7.5), 150 mM NaCl), wells were blocked for 1 h at 37°C with TBS 10 containing 2% BSA.

Binding assays

The assay to measure the immunoreactive fraction of radiolabeled mAb (17), the binding assay with ^{125}I-labeled mAb (18), and the Scatchard plot analysis of the binding of radiolabeled mAb to cells (19) were performed as described elsewhere.

The inhibition assay to determine the ability of synthetic peptides to inhibit the binding of mAb to HLA class I Ags or to a synthetic peptide was performed by mixing varying amounts of synthetic peptide solution with unconjugated or biotinylated mAb. mAb were used at concentrations giving absorbance measured at 490 nm (A_{490}) = 1.0 in the absence of inhibitors. Following an overnight incubation at 4°C, the mixture was transferred to plates coated with HLA class I Ags or a synthetic peptide, and incubation was continued for an additional hour at room temperature. Following washing of the plates with TBS-T (TBS 10 containing 0.05% Tween 20), 100 μl of a 1:2,500 dilution of HRP-conjugated streptavidin (SA-HRP; Pierce) or of a 1:10,000 dilution of a GAM-HRP solution were added to each well and incubation was continued for an additional hour at room temperature. The reaction was developed using an *o*-phenylenediamine-H_2O_2 substrate solution and terminated by the addition of 4 N H_2SO_4. Results are expressed as the percent of inhibition calculated using the following formula: % inhibition = ((A_{490} in the absence of inhibitor-A_{490} in the presence of inhibitor)/A_{490} in the absence of inhibitor) × 100.

Immunochemical methods

Following labeling of cells with ^{125}I (20) or with [^{35}S]methionine (Trans-^{35}S-label; ICN Pharmaceuticals, Costa Mesa, CA; Ref. 4), cells were solubilized with lysis buffer supplemented with 1% Nonidet P-40. Indirect immunoprecipitation with monoclonal and polyclonal Abs was performed essentially as described (21). Immunoprecipitates were analyzed on 12% SDS-PAGE. Gels were fixed, dried under vacuum, and exposed at −70°C to Hyperfilm-MP (Amersham, Arlington Heights, IL) with intensifying screens. Gels containing [^{35}S]methionine labeled proteins were incubated for 30 min in Enlightening (DuPont, Wilmington, DE) before drying.

Western blot analysis was performed as described by Towbin et al. (22) with minor modifications. Briefly, a 1% Nonidet P-40 extract of cultured cells was electrophoresed on a 12% polyacrylamide gel in the presence of SDS. Separated proteins were then transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Following blocking of the membrane for 1 h at room temperature with PBS supplemented with 5% skimmed milk powder, filters were incubated with 2×10^5 cpm/ml ^{125}I-labeled mAb for 4 h at room temperature. After three washes with PBS containing 0.05% Tween 20, filters were dried and autoradiographed at −70°C using Hyperfilm-MP (Amersham). One-dimensional isoelectric focusing (1D-IEF) of HLA class I Ags using detergent-solubilized B lymphoblastoid cell line lysates was performed as described by Neefjes et al. (13), followed by immunoblotting with mAb TP25.99 or with rabbit anti-HLA class I heavy chain antiserum (23).

Panning of peptide libraries with mAb TP25.99

Micropanning of amplified peptide libraries X_{15} and LX-8 with biotinylated mAb TP25.99 was performed in 96-well microtiter plates (Falcon; Becton Dickinson, Lincoln Park, NJ) as described (24). The first round of panning was performed using 1×10^{12} phage particles in TBS 50 and 1 μg biotinylated mAb TP25.99 per well. The subsequent three rounds of selection were conducted using 1×10^{10} phage particles and 0.1 μg biotinylated mAb per well. Eluted phages from each round of panning were amplified in K91kan cells prepared as described by Smith and Scott (25) and used as input for the next round of panning. Phage enrichment, (i.e., percent yield, defined as the percent of eluted phages/input phages) at the end of panning was determined by spot titering on NZY plates containing 20 μg/ml tetracycline, as described by Smith and Scott (25).

Immunoscreening of peptide libraries with mAb

The immunoscreening assay to test the reactivity of random phage clones with mAb was performed as described by Valadon and Scharff (26) with minor modifications. Briefly, nitrocellulose filter (Protran; Schleicher and Schuell, Keene, NH) lifts from plates containing colonies were probed with 5 μg/ml mAb and 2 μg/ml biotinylated rabbit anti-mouse IgG (heavy chain + light chain) Abs in TNT buffer (TBS-T containing 20% FBS). Following an overnight incubation at 4°C, filters were washed with TBS 10 containing 1 mg/ml BSA, and incubated with a 1:2500 dilution of a SA-HRP solution in TNT buffer. Following three washes with TBS 10 containing 1 mg/ml BSA, filters were developed using enhanced chemiluminescence (Amersham).

Nucleotide sequence analysis of phage inserts

Nucleotide sequence of peptide inserts was determined by the dideoxynucleotide chain termination method (27) as described by Bonnycastle et al. (24) with the following modifications. Purified phages were prepared from the supernatant of individual clones by polyethylene glycol/NaCl precipitation. Sequencing reactions were performed with 2×10^{11} phage particles in microtiter wells (GeNunc; Nunc, Roskilde, Denmark) using the SEQUENASE kit (version 2.0, United State Biochemicals, Cleveland, OH) and ^{32}P-end-labeled f88.4 sequencing primer, 5′-CTGAAGAGAGTCAAAAGC-3′.

Results

Reactivity of mAb TP25.99 with β_2m-associated and β_2m-free HLA class I heavy chains

In binding assays, ^{125}I-labeled mAb TP25.99 reacted with a large panel of human cell lines expressing HLA class I Ags irrespective of their HLA phenotype, but did not react with cell lines which do not express HLA class I Ags. The latter include B lymphoid cells Daudi (28) and melanoma cells FO-1 (4) and SK-MEL-33 (29). The association constant of mAb TP25.99, as determined by Scatchard plot analysis of the binding of ^{125}I-labeled mAb TP25.99 to cells was at least 1.93×10^9 M^{-1} (data not shown).

mAb TP25.99 immunoprecipitated components with the characteristic electrophoretic profile of the two subunits of HLA class I Ags from radiolabeled human cells (Fig. 1). Furthermore, mAb TP25.99 completely immunodepleted cell extracts of β_2m-associated HLA class I heavy chain complexes recognized by anti-β_2m

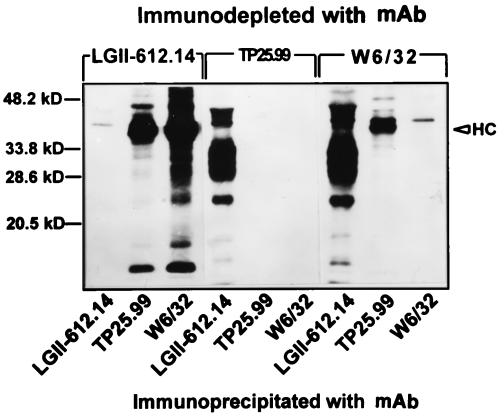

FIGURE 1. Structural relationship among the molecules recognized in a cultured human LKT13 B lymphoid cell extract by anti-HLA class I mAb TP25.99 and by mAb W6/32. A 1% Nonidet P-40 extract of [^{35}S]methionine-labeled LKT13 cells was immunodepleted with mAb TP25.99 and anti-HLA class I mAb W6/32. Each immunodepleted extract was immunoprecipitated with insolubilized mAb TP25.99 and mAb W6/32. Ags were then eluted and analyzed by SDS-PAGE in a 12% polyacrylamide gel in the presence of 2-ME. Gels were then processed for fluorography. Immunodepletion and immunoprecipitation with anti-HLA class II mAb LGII-612.14 was used as a specificity control.

mAb NAMB-1 (data not shown) and by mAb W6/32 (Fig. 1). The latter mAb recognizes a framework determinant expressed on β_2m-associated HLA-A, -B, and -C heavy chains (8). In contrast, mAb TP25.99 immunoprecipitated HLA class I heavy chains from cell extracts immunodepleted of β_2m-associated HLA class I heavy chains with mAb NAMB-1 or with mAb W6/32. These results suggest that besides recognizing a monomorphic determinant expressed on β_2m-associated HLA class I heavy chains, mAb TP25.99 recognizes a determinant expressed on β_2m-free HLA class I heavy chains. Two additional lines of evidence corroborate the latter possibility. First, mAb TP25.99 reacts with HLA class I heavy chains when tested in Western blotting with cell extracts (Fig. 2). Second, mAb TP25.99 immunoprecipitates HLA class I heavy chains from [^{35}S]methionine-labeled B lymphoid cells Daudi (Fig. 3) and melanoma cells FO-1 and SK-MEL-33 (data not shown), all of which do not express β_2m (4, 28, 29). Only a subpopulation of β_2m-free HLA class I heavy chains expresses the determinant recognized by mAb TP25.99, because immunodepletion of a cell extract with mAb TP25.99 did not completely remove the HLA class I heavy chains recognized by rabbit serum R 5996-4 (Fig. 3). In contrast, mAb TP25.99 did not immunoprecipitate any components from a cell extract immunodepleted with rabbit serum R 5996-4. These results indicate that mAb TP25.99 recognizes determinant(s) on β_2m-associated and β_2m-free HLA class I heavy chains.

Differential distribution of the determinants recognized by mAb TP25.99 on β_2m-associated and β_2m-free HLA class I allospecificities

The distribution of the conformational determinant recognized by mAb TP25.99 on β_2m-associated HLA class I allospecificities was analyzed by 1D-IEF analysis of HLA class I Ags immunoprecipitated from Triton X-114 detergent-solubilized lysates of B lymphoid cell lines (data not shown). All the known HLA-A and HLA-B allospecificities were detected in the immunoprecipitates with mAb TP25.99. The intensity of bands corresponding to HLA-A2, A10, A19, and A28 alleles were much weaker than those of the other HLA class I alleles.

The distribution of the determinant recognized by mAb TP25.99 on β_2m-free HLA class I heavy chains encoded by A and B loci was investigated by testing its reactivity in Western blotting with HLA class I allospecificities solubilized from B lymphoid cell lines and separated by 1D-IEF (Fig. 4). mAb TP25.99 reacted with all the known β_2m-free HLA-B allospecificities except HLA-B73 and with $<$50% of all the known β_2m-free HLA-A allospecificities. The latter include HLA-A1, A3, A9, A11, and A30 alleles. Correlation between the differential reactivity with mAb TP25.99 of HLA class I allospecificities and the amino acid sequence of their heavy chain α3 domain is shown in Table I. This data suggests that changes in amino acid residues P193, I194, G207, A245, A246, and E253 are associated with lack of reactivity of mAb TP25.99 with β_2m-free HLA class I heavy chains. This information together with mapping of the linear determinant recognized by

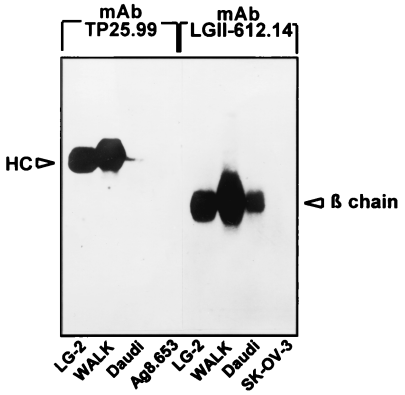

FIGURE 2. Western blot analysis of the reactivity of mAb TP25.99 with HLA class I heavy chains. A 1% Nonidet P-40 extract of human B lymphoid cells LG-2, WALK, and Daudi was separated by SDS-PAGE on a 14% polyacrylamide gel in the presence of 2-ME and transferred to Immobilon-P membranes. Following blocking, filters were incubated with 2×10^5 cpm/ml ^{125}I-labeled mAb, washed, dried, and autoradiographed at -70°C using Hyperfilm-MP (Amersham). Mouse myeloma cells Ag 8.653, human ovarian carcinoma cells SK-OV-3, and mAb LGII-612.14 which reacts with the β-chain of HLA class II Ags (Temponi et al., Ref. 11) were used as controls.

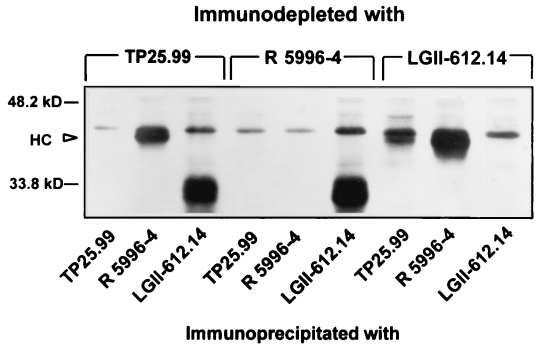

FIGURE 3. Structural relationship between the molecules recognized in a cultured human Daudi B lymphoid cell extract by mAb TP25.99 and by rabbit anti-β_2m-free HLA class I heavy chain serum R 5996-4. A 1% Nonidet P-40 extract of [^{35}S]methionine-labeled Daudi cells was immunodepleted with mAb TP25.99 (*lanes 1–3*) and with rabbit serum R 5996-4 (*lanes 4–6*). Each immunodepleted extract was immunoprecipitated with insolubilized mAb TP25.99 (*lanes 1, 4,* and *7*) and rabbit serum R 5996-4 (*lanes 2, 5,* and *8*). Ags were eluted and analyzed by SDS-PAGE in a 12% polyacrylamide gel in the presence of 2-ME. Gels were then processed for fluorography. Immunodepletion (*lanes 7–9*) and immunoprecipitation (*lanes 3, 6,* and *9*) with anti-HLA class II mAb LGII-612.14 was used as a specificity control.

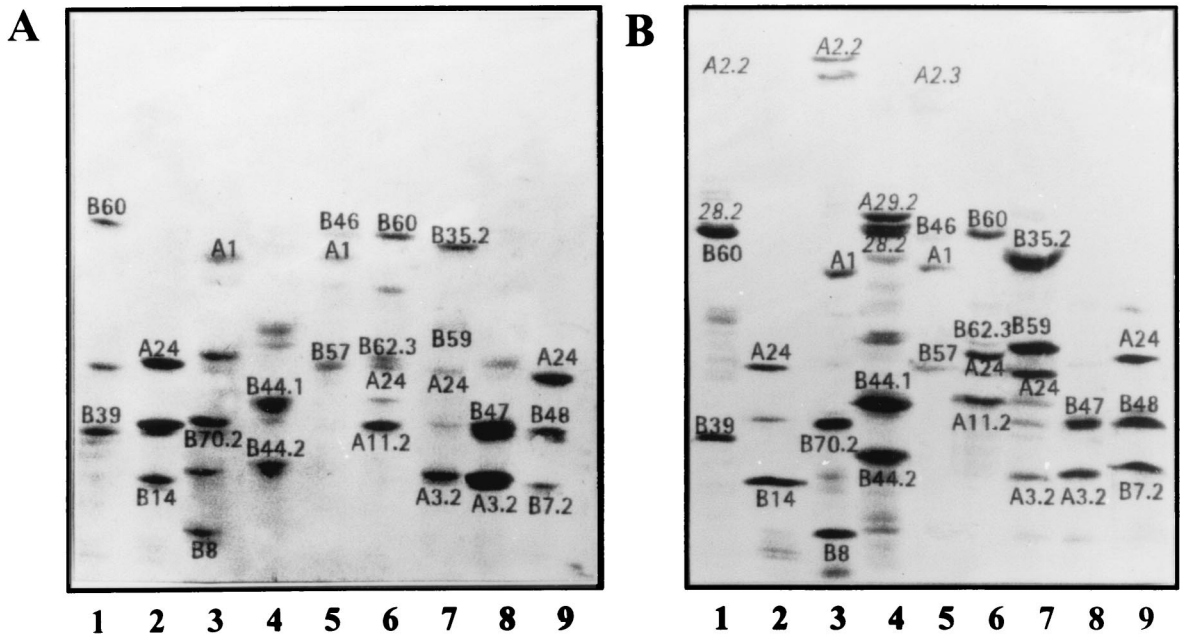

FIGURE 4. Analysis by 1D-IEF and Western blotting of the reactivity of mAb TP25.99 with β_2m-free HLA class I alleles. *A*, Triton X-114 detergent lysates of cultured B lymphoid cells BRU (HLA-A2.2, A28; B39, B60; *lane 1*), MZ (HLA-A24; B14; *lane 2*), ENID (HLA-A1, A2.2; B8, B70.2; *lane 3*), WET (HLA-A28.2, A29.2; B44.1, B44.2; *lane 4*), DCHO$_2$4 (HLA-A1, A2.3; B46, B57; *lane 5*), DCHO38 (HLA-A11.2, A24; B60, B62.3; *lane 6*), YIL (HLA-A3.2, A24; B35.2, B59; *lane 7*), PLH (HLA-A3.2, B47; *lane 8*), and DSCH (HLA-A24; B48, B7.2; *lane 9*) were separated by 1D-IEF followed by Western blotting with mAb TP25.99. *B*, Western blot probed with rabbit anti-HLA class I heavy chain Abs as the primary Ab was used as a positive control.

mAb TP25.99 on β_2m-free HLA class I heavy chains (to be described in a later section) suggests that the two alanine residues at positions 245 and 246 and especially the glutamic acid residue at position 253 play a crucial role in the expression of the determinant recognized by mAb TP25.99 on β_2m-free HLA class I heavy chains. Thus the substitution of A and E by V and Q at positions 245 and 253, respectively, as in the HLA-A68 allospecificity; the substitution of A and E by S and Q at positions 246 and 253, respectively, as in the HLA-A10, A29, A31, A32, A33, A34, A66, and A74 allospecificities; or the substitution of E by Q at position 253 as in the HLA-A2, A69, and B73 allospecificities results in loss of the expression of the determinant recognized by mAb TP25.99.

These results suggest that the conformational and linear determinants are located on distinct regions of the HLA class I heavy chain α3 domain. Furthermore the determinant recognized by mAb TP25.99 on β_2m-free HLA class I heavy chains has a differential distribution on HLA class I allospecificities.

Selection and screening of phage-displayed peptides binding to mAb TP25.99

To identify the determinant(s) recognized by mAb TP25.99 on β_2m-associated and β_2m-free HLA class I heavy chains, phage-displayed peptide clones were isolated by panning the random phage-displayed peptide libraries, LX-8 and X_{15} (24) with mAb TP25.99. Four rounds of panning of the LX-8 and X_{15} libraries

Table I. *Association of amino acid changes in HLA class I heavy chain α3 domains with differential reactivity with mAb TP25.99*

α3 Domain Position	184	189	193	194	207	239	245	246	253	267	268	270	1D-IEF Reactivity
Consensus	P	V	P	I	G	R	A	A	E	P	K	L	
HLA-A1		M				G							+
HLA-A2	A	M	A	V	S	G			Q				−
HLA-A3		M				G							+
HLA-A9	A	M				G							+
HLA-A10	A	M	A	V	S	G		S	Q				−
HLA-A11		M				G							+
HLA-A29	A	M	A	V	S	G		S	Q				−
HLA-A30		M				G							+
HLA-A31		M	A	V	S	G		S	Q				−
HLA-A32	A	M	A	V	S	G		S	Q				−
HLA-A33		M	A	V	S	G		S	Q				−
HLA-A34	A	M	A	V	S	G		S	Q				−
HLA-A66	A	M	A	V	S	G		S	Q				−
HLA-A68	A	M	A	V	S	G	V		Q				−
HLA-A69	A	M	A	V	S	G			Q				−
HLA-A74	A	M	A	V	S	G		S	Q				−
HLA-B73						G			Q	Q	E	C	−
HLA-B7													+

resulted in an enrichment of 0.44% and 0.92%, respectively. Immunological screening of colonies at the end of the fourth round of panning detected reactivity of mAb TP25.99 with 35% and 70% of the colonies isolated from the LX-8 and X_{15} libraries, respectively.

Amino acid sequence homology of peptides identified by mAb TP25.99 with HLA class I heavy chains

Nucleotide sequence analysis identified three distinct sequences in the peptides expressed by seven randomly selected clones among those isolated from the LX-8 library. Four, two, and one clone had peptide inserts with the sequences QCTNFISDHECH, SCDG FYTGPACM, and QCVETWNRIECK, respectively (Table II). In contrast, only the sequence IDPVGWGNERTFQVP was found in the peptides expressed by eight randomly selected clones sequenced from the X_{15} library.

The peptides expressed by the clones isolated from the two libraries display homology in their amino acid sequence with HLA-A, -B, and -C heavy chain α3 domain. The ISDHE sequence in the peptide most frequently expressed in clones isolated from the LX-8 library is present at positions 194–198 in HLA-B and -C heavy chain α3 domains (Fig. 5). These amino acids with the exception of I are also present at positions 195–198 in HLA-A heavy chain α3 domains. Furthermore, the GFY sequence in the peptides expressed by two clones isolated from the LX-8 library is present at positions 207–209 in the HLA-B and -C heavy chain α3 domains and the amino acids P and A are present at positions 210 and 211 in the HLA-A, -B, and -C heavy chain α3 domain. Additionally, the VET sequence in the peptide expressed by one clone isolated from the LX-8 library is present at positions 231–233 in the HLA-A, -B, and -C heavy chain α3 domains.

Among the three phage-displayed peptides isolated from the LX-8 library, the phage expressing the peptide insert SCDGFYT GPACM reacted to a lower extent with mAb TP25.99 than the phages expressing the peptide inserts QCTNFISDHECH and QCVETWNRIECK. The latter two did not differ in their reactivity with mAb TP25.99 (Table II). The residues ISDHE are likely to mediate the binding of mAb TP25.99 to β_2m-associated HLA class I heavy chains, because the three dimensional structure of the HLA class I Ag reveals that these residues are exposed on the β_2m-associated HLA class I heavy chain complex. In contrast, the residues GFYPA and VET are not accessible for binding to mAb TP25.99 when HLA class I heavy chains are associated with β_2m. Thus, these residues are less likely to be involved in the binding of mAb TP25.99 to β_2m-associated HLA class I heavy chains.

The RTFQ sequence in the peptide expressed by the clones isolated from the X_{15} library is present at positions 239–242 in the HLA-B heavy chain α3 domain. These residues, with the exception of R, are also present at the same positions in the HLA-A and -C heavy chain α3 domains (Fig. 6). Furthermore, for reasons which will be discussed in the next sections, it is noteworthy that the two alanine residues present at the N terminus of the pVIII major coat protein of the bacteriophage vector f88.4 (24) are also present at positions 245 and 246 in HLA-A, -B, and -C heavy chain α3 domains.

Table II. *Phage-displayed peptide sequences isolated by panning the LX-8 and X_{15} phage display peptide libraries with mAb TP25.99*

Peptide Library	Peptide Insert	Frequency[a]	A_{490}[b]
LX-8	QCTNFISDHECH	4/7 (57%)	1.428
	SCDGFYTGPACM	2/7 (29%)	0.181
	QCVETWNRIECK	1/7 (14%)	1.531
X_{15}	IDPVGWGNERTFQVP	8/8 (100%)	1.826

[a] Percent of phages displaying the indicated peptide sequences.
[b] Reactivity of phage-displayed peptides (1×10^{12} particles/ml) with mAb TP25.99 as measured by ELISA.

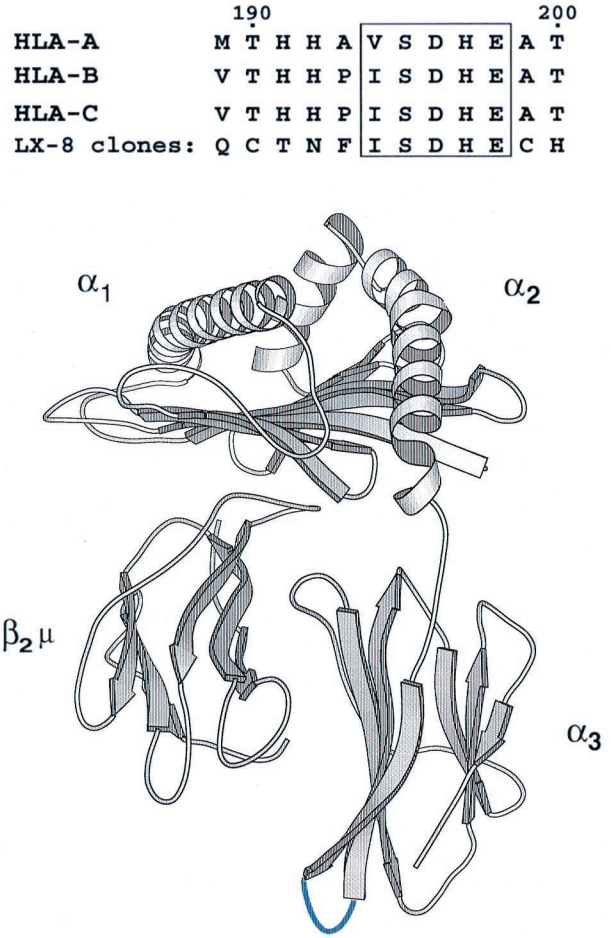

FIGURE 5. Amino acid sequence homology of HLA-A, -B, and -C heavy chain α3 domains with peptides expressed by clones isolated by panning the LX-8 phage display peptide library with mAb TP25.99. The amino acids identical in the HLA-A, -B, and -C heavy chain α3 domains and in the LX-8 phage-displayed peptides are shown in box. The lower panel shows the region of homology (highlighted in blue) in the three-dimensional structure of β_2m-associated HLA class I heavy chains.

Reactivity of mAb TP25.99 with the cyclic LX-8 peptides

Glutaraldehyde cross-linked cyclic LX-8 no. 1 peptide with the sequence corresponding to that present in the phage-displayed LX-8 clone reacted in binding assays with mAb TP25.99 (data not shown). Furthermore, the cyclic LX-8 no. 1 peptide inhibited the reactivity of mAb TP25.99 with β_2m-associated and β_2m-free HLA class I heavy chains in a concentration-dependent fashion with an IC_{50} of ~70 μM (Fig. 7). The inhibition is specific, as the cyclic LX-8 no. 1 peptide did not inhibit the binding of mAb CR11-351 and mAb HC-10 to HLA-A2 Ags and to β_2m-free HLA class I heavy chains, respectively. Furthermore, the control no. 7 peptide did not inhibit the binding of mAb TP25.99 to HLA class I Ags (Table III).

The reactivity of mAb TP25.99 with the cyclic LX-8 no. 1 peptide requires the presence of disulfide bonds, because the corresponding linear LX-8 no. 1 peptide, in the presence of the reducing agent, DTT did not inhibit the binding of mAb TP25.99 to HLA class I Ags (data not shown). Furthermore mAb TP25.99 did not

	230								240											250
HLA-A	L	V	E	T	R	P	A	G	D	G	T	F	Q	K	W	A	A	V	V	V	P
HLA-B	L	V	E	T	R	P	A	G	D	R	T	F	Q	K	W	A	A	V	V	V	P
HLA-C	L	V	E	T	R	P	A	G	D	G	T	F	Q	K	W	A	A	V	V	V	P
X15 clones:	I	D	P	V	G	W	G	N	E	R	T	F	Q	V	P	A	A	E	G		

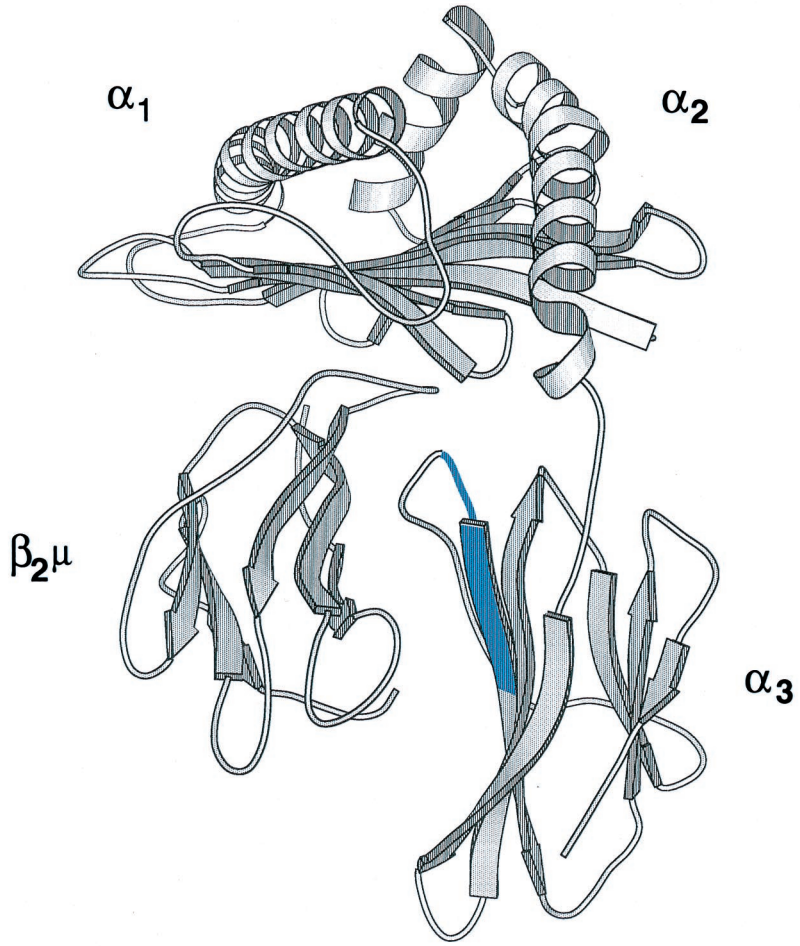

FIGURE 6. Amino acid sequence homology of HLA-A, -B, and -C heavy chain α3 domains with peptides expressed by clones isolated by panning the X_{15} phage display peptide library with mAb TP25.99. The amino acids identical in the HLA-A, -B, and -C heavy chain α3 domains and the X_{15} phage-displayed peptides are shown in box. The lower panel shows the region of homology (highlighted in blue) in the three-dimensional structure of β_2m-associated HLA class I heavy chains.

react with the linear LX-8 no. 1, C→S peptide (Fig. 7), where the cysteine residues were substituted with serine residues, precluding the formation of a disulfide bond. These results strongly suggest that mAb TP25.99 recognizes a structural motif.

Reactivity of mAb TP25.99 with the linear X_{15} peptide

The synthetic linear X_{15} no. 4 peptide with a sequence corresponding to the X_{15} phage-displayed peptide had no detectable effect on the reactivity of mAb TP25.99 with β_2m-free and β_2m-associated HLA class I heavy chains, because the amino acids AA present at the N terminus of the pVIII major coat protein of bacteriophage vector f88.4 contribute to the expression of the determinant recognized by mAb TP25.99. The two alanine residues are also present in the HLA class I heavy chain α3 domain at positions 245 and 246. Therefore, a peptide was synthesized with a sequence corresponding to the linear X_{15} no. 4 peptide with the addition of the amino acid residues AAEG at the C terminus. This peptide, referred to as linear X_{19} no. 5 peptide, inhibited the reactivity of mAb TP25.99 with both β_2m-associated and β_2m-free HLA class I heavy chains with an IC_{50} of ~30 μM (Fig. 7). The inhibition is specific, as the linear X_{19} no. 5 peptide did not inhibit the binding of mAb CR11-351 and mAb HC-10 to β_2m-associated HLA-A2 heavy chains and to β_2m-free HLA class I heavy chains, respectively. It is noteworthy that the linear X_{17} no. 6 (X_{19}-EG) peptide inhibited the binding of mAb TP25.99 to HLA class I Ags to the same extent as the linear X_{19} no. 5 peptide (data not shown). Thus, the amino acid residues EG present in the pVIII major coat protein, at positions adjacent to the two A residues do not contribute to the expression of the determinant recognized by mAb TP25.99.

Simultaneous interaction of mAb TP25.99 with both the cyclic LX-8 no. 1 peptide and the linear X_{19} no. 5 peptide

The cyclic LX-8 no. 1 peptide and the linear X_{19} no. 5 peptide, which correspond to the conformational and linear determinants

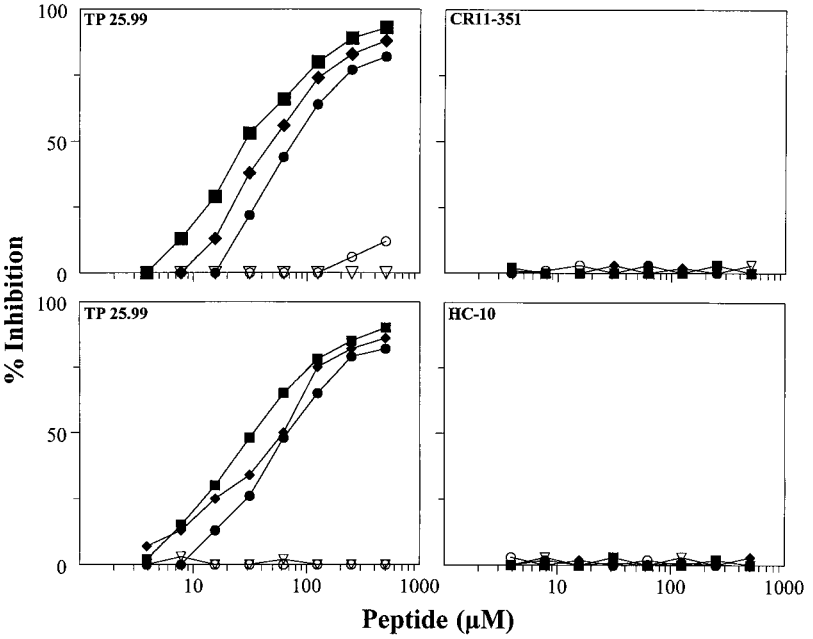

FIGURE 7. Dose-dependent inhibition by the cyclic LX-8 no. 1 peptide of the reactivity of mAb TP25.99 with β_2m-associated and β_2m-free HLA class I heavy chains. Varying amounts of the cyclic LX-8 no. 1 peptide (●), the linear LX-8 no. 1 C→S peptide (○), the linear X_{19} no. 5 peptide (■), the combined linear X_{19} no. 5 and cyclic LX-8 no. 1 peptides (♦) and the control no. 7 peptide (▽) were incubated with biotinylated mAb TP25.99. The mixture was then transferred to wells coated with β_2m-associated HLA class I heavy chains (*upper panels*) or with wells coated with β_2m-free HLA class I heavy chains (*lower panels*). Binding of biotinylated mAb TP25.99 to HLA Ags was detected using SA-HRP conjugate. Inhibition of the reactivity of biotinylated mAb CR11-351 with β_2m-associated HLA-A2 heavy chains (*upper right panel*) and of biotinylated mAb HC-10 with β_2m-free HLA class I heavy chains (*lower right panel*) were used as specificity controls.

identified by mAb TP25.99, respectively, share no sequence homology. Hence inhibition assays were performed to determine whether the two peptides bind independently to two noninteracting binding sites on mAb TP25.99 or act concurrently and bind to the same or overlapping binding sites. As shown in Fig. 7, combination of the cyclic LX-8 no. 1 peptide and the linear X_{19} no. 5 peptide inhibited the binding of mAb TP25.99 to β_2m-associated and β_2m-free HLA class I heavy chains to a lower extent than the linear X_{19} no. 5 peptide and to a greater extent than the cyclic LX-8 no. 1 peptide. The inhibitory activity of the mixture of the two peptides is consistent with their differential ability to inhibit the binding of mAb TP25.99 to HLA class I Ags. Furthermore, this data is compatible with the possibility that the two peptides compete for binding to the same or spatially close binding sites on mAb TP25.99. This interpretation is supported by the dose-dependent inhibition by the linear X_{19} no. 5 peptide of the reactivity of mAb TP25.99 with the cyclic LX-8 no. 1 peptide (Fig. 8).

Discussion

The present study has shown that the anti-HLA class I mAb TP25.99 has the unique characteristic to recognize a conformational and a linear determinant on a single protein molecule. Although the conformational determinant is expressed on all known β_2m-associated HLA class I allospecificities, the linear determinant is expressed on β_2m-free HLA class I heavy chains of a subset of HLA class I allospecificities. The latter includes all those encoded by the HLA-B locus except HLA-B73 and <50% of those encoded by the HLA-A locus.

The fine specificity of the mAb TP25.99 is different from that of mAb Q1/28 (5), A1.4 (6), and 5H7 (30), which also recognize determinants expressed both on β_2m-free and β_2m-associated HLA class I heavy chain α3 domains. mAb Q1/28 and 5H7 do not react with the peptides identified with mAb TP25.99 (unpublished results). Furthermore, the different effects of mAb A1.4 and mAb TP25.99 on staphylococcal enterotoxin B-induced T cell proliferation suggest that the two mAb recognize distinct, although spatially close, determinants on the HLA class I α3 domain (31).

Screening of the LX-8 and X_{15} peptide libraries with mAb TP25.99 has resulted in the isolation of sequentially different peptides that are homologous with distinct regions of HLA class I heavy chain α_3 domains. The cyclic LX-8 no. 1 peptide, which is constrained by a disulfide bond, contains residues identical with aa 194–198 of HLA-B heavy chains. Replacement of I with V at position 194, as it occurs in HLA-A allospecificities, does not cause detectable changes in the reactivity of mAb TP25.99 with β_2m-associated HLA-A heavy chains. In contrast, replacement of S and H with F and Y, respectively, at positions 195 and 197 causes loss of reactivity with mAb TP25.99, because the corresponding determinant is not detectable on β_2m-associated HLA-G heavy chains (32). The linear X_{19} peptide is homologous to residues 239–242, 245, and 246 of HLA-B heavy chains. Comparison of the amino acid sequences of the HLA-A and HLA-B heavy chains which react with mAb TP25.99 with the sequence of the HLA-A allospecificities which do not react with mAb TP25.99, indicates that the two alanine residues at positions 245 and 246 and the glutamic acid residue at position 253 play a crucial role in the expression of the antigenic determinant recognized by mAb TP25.99. Replacement of at least one of these residues accounts for the lack of reactivity of mAb TP25.99 with β_2m-free HLA-A2, A10, A29, A31, A32, A33, A34, A66, A68, A69, A74, and B73 heavy chains. The charge change associated with the replacement of E by Q at position 253 suggests that changes in the conformation of the HLA class I heavy chains may account for the loss of reactivity of mAb TP25.99 with β_2m-free HLA class I heavy chains.

Comparison of the three peptide sequences isolated from the LX-8 library revealed no homology among themselves. The LX-8 peptide QCVETWNRIECK and the linear X_{19} no. 5 peptide share several amino acids. Whether they play a crucial role in the interaction of mAb TP25.99 with HLA class I Ags remains to be determined. Lastly, the cyclic LX-8 no. 1 peptide and the linear X_{19} no. 5 peptide corresponding to the conformational and linear determinants recognized by mAb TP25.99 on β_2m-associated and β_2m-free HLA class I heavy chains, respectively, have no sequence homology. The structural homology, shown by nuclear magnetic resonance analysis of the two peptides in the presence of mAb TP25.99 (analysis by NMR spectroscopy of the structural homology between the linear and the cyclic peptide recognized by

Table III. *Sequence of the synthetic peptides corresponding to peptide inserts present in phage clones identified by panning the LX-8 and X_{15} phage display peptide libraries with mAb TP25.99*

Peptide	Sequence
	644448
Cyclic LX-8 no. 1	QCTNFISDHECH
Linear LX-8 no. 1	QCTNFISDHECH
Linear LX-8 no. 1, C→S	QSTNFISDHESH
Linear X_{15} no. 4	IDPVGWGNERTFQVP
Linear X_{19} no. 5 (X_{15} + AAEG)	IDPVGWGNERTFQVPAAEG
Linear X_{17} no. 6 (X_{19} − EG)	IDPVGWGNERTFQVPAA
Control no. 7	KDKEKATNEEKKKNRENEK(spacer)C

anti-HLA class I mAb TP25.99 (33)) indicates that this mAb recognizes a structural motif rather than a sequence-dependent determinant. An analysis of the x-ray structure of HLA class I Ags indicates that the linear determinant is not available for binding of mAb TP25.99 to β_2m-associated HLA class I Ags, because it is masked by the β_2m. This phenomenon may account for the immunogenicity of mAb TP25.99 defined determinant in BALB/c mice, because the amino acid sequence of the linear determinant is conserved through phylogenetic evolution (34). Furthermore, the homology of the linear and cyclic peptide sequences with two distinct areas of HLA class I α3 domain, strengthens the hypothesis that mAb TP25.99 recognizes two determinants independently and argues against the possibility that mAb TP25.99 recognizes a split epitope. The latter phenomenon has been reported for the CD39 B cell marker by Maliszewski et al. (35).

One might argue that the reactivity of mAb TP25.99 with two distinct determinants of HLA class I Ags reflects the presence in the Ab preparation of two Ab populations which have the same heavy chain, but different light chains. One of them is derived from the fusing B cell and the other one from the fusing partner. This possibility is highly unlikely, because the myeloma cell line P3-X63-Ag8.653 used to generate the hybridoma TP25.99 does not express IgG heavy and light chains (36). Furthermore, in inhibition assays the cyclic and the linear peptides isolated with mAb TP25.99 inhibit completely its reactivity with β_2m-associated and β_2m-free HLA class I heavy chains. The similar inhibitory activity of the two peptides is not compatible with the possibility that one of the two hypothesized Ab populations is present in the mAb TP25.99 preparations tested in a small amount and, therefore, has no detectable effect on the outcome of the inhibition assay. Lastly, similar results were obtained using different preparations of mAb TP25.99 prepared from batches of the hybridoma kept in culture for at least 2 mo.

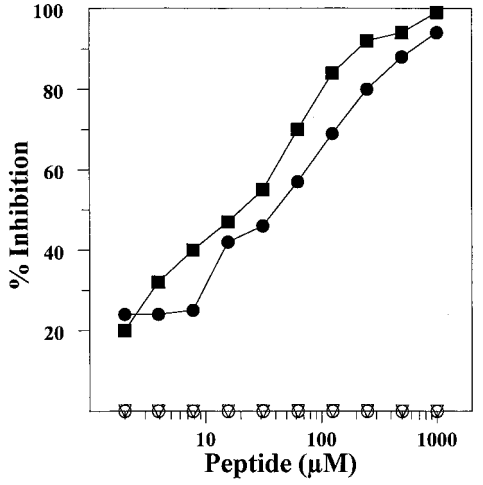

FIGURE 8. Dose-dependent inhibition by the cyclic LX-8 no. 1 peptide and the linear X_{19} no. 5 peptide of the reactivity of mAb TP25.99 with cyclic LX-8 no. 1 peptide coated plates. Varying amounts of the cyclic LX-8 no. 1 peptide (●), the linear LX-8 no. 1 C→S peptide (○), the linear X_{19} no. 5 peptide (■), and the control no. 7 peptide (▽) were preincubated with mAb TP25.99. The mixture was then transferred to wells coated with glutaraldehyde cross-linked cyclic LX-8 no. 1 peptide. Reactivity of mAb TP25.99 with the synthetic peptide was determined by addition of GAM-HRP conjugate.

To the best of our knowledge, the results of this study are unique and identify for the first time a mAb that recognizes two structurally similar cross-reactive epitopes present on the same antigenic structure. In contrast, cross-reactivity among epitopes which are similar in their amino acid sequence or in their structure, but are expressed on disparate Ags has been reported by several investigators (37–39). The latter cross-reactivity provides a molecular basis for the pathogenesis of autoimmune diseases (40).

Panning phage display peptide libraries with mAb has previously isolated peptides that have distinct sequences from the original epitope. Despite the lack of homology with the original epitope (41–44), some of these peptides have been effective in inhibiting the corresponding Ag-Ab interaction and in eliciting an immune response. In contrast, recognition by a mAb of multiple peptides dissimilar in sequence and conformation is an infrequent finding, because, to the best of our knowledge, this has been described so far only in case of an anti-p24 HIV glycoprotein mAb. X-ray crystallographic analysis of the polyspecificity of this mAb indicates interaction of the peptides with different contact residues in the Ag-combining site of the mAb (45, 46). Whether a similar mechanism underlies the reactivity of mAb TP25.99 with multiple peptide conformations remains to be determined.

In recent years, B and TCRs have been described to display a similar or identical specificity in a few antigenic systems (47–49). This finding is unexpected, given the differences in the molecular basis of the recognition of an Ag by B and T cells. The latter recognize processed linear peptides presented by MHC class I Ags, while B cells recognize native folded protein conformations. Recognition by mAb TP25.99 of a similar, if not identical conformational and linear determinant on distinct amino acid stretches of the HLA class I Ag, provides an alternative molecular mechanism for the sharing of specificity by an Ab and a TCR. This mechanism may also account for the unexpected development of not only a humoral, but also a T cell immune response (50) following immunizations with multichain Ags which require the association of distinct subunits for the expression of their immunogenic moiety, such as anti-idiotypic Abs.

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