Polymorphism of Human Minor Histocompatibility Antigens: T Cell Recognition of Human Minor Histocompatibility Peptides Presented by HLA-B35 Subtype Molecules

By Yoshifumi Beck,*‡ Leonardo Satz,§ Yuji Takamiya,* Setsuko Nakayama,‡ Lin Ling,‖ Yoshihide Ishikawa,‖ Takeshi Nagao,‡ Hisanori Uchida,‡ Katsushi Tokunaga,‖ Claudia Müller,¶ Takeo Juji,‖ and Masafumi Takiguchi*

*From the *Department of Tumor Biology and the ‡Department of Organ Transplantation, Institute of Medical Science, University of Tokyo, Tokyo, Japan; the §Laboratorio de Immunogenetica, Hospital de Clinicas and Department of Microbiology, Faculty of Medicine, University of Buenos Aires, Buenos Aires, Argentina; the ‖Japan Red Cross Central Blood Center, Tokyo, Japan; and the ¶Department of Internal Medicine II and III, University Medical Clinic Eberhard-Karls-University, 7400 Tübingen, Federal Republic of Germany*

Summary

To investigate the polymorphism of human minor histocompatibility (mH) antigens, PBLs from 23 Japanese individuals and 25 German individuals with HLA-B35 were studied by using four human mH antigen–specific, HLA-B35–restricted CTL clones. The CTL clones killed PHA-stimulated PBLs from all 23 Japanese individuals. On the other hand, they killed the PHA-stimulated PBLs from 19 of 25 German individuals and partially killed the PHA-stimulated PBLs from three German individuals (CTL weakly sensitive cell line); those from another three individuals (CTL-resistant cell line) were not killed by the CTL clones. All of three CTL weakly sensitive cell lines carry HLA-B*3503 molecules, whereas the three CTL-resistant cell lines carry HLA-B*3502, B*3507, and B*3508 molecules. The cytotoxicity of the CTL clones for three CTL weakly sensitive cell lines was enhanced by stimulation of human mH peptides isolated from HLA-B*3501 molecules purified from C1R-B*3501 cells. Small amounts of human mH peptides were isolated from B*3503 molecules purified from these three CTL weakly sensitive cell lines. Taken together, these results indicate that weak recognition by the CTL clones of three CTL weakly sensitive cell line results from a small amount of the human mH peptides presented by B*3503 molecules. The CTL-resistant cell line carrying B*3507 loaded with the human mH peptides was killed by four CTL clones, whereas the cell lines carrying B*3502 or B*3508 loaded with the peptides were not. The human mH peptides were not isolated from B*3507 molecules purified from the cell lines expressing this subtype, whereas small amounts of the human mH peptides were isolated from B*3502 and B*3508 molecules purified from the cell lines expressing these subtypes. These results indicate that failure of the CTL recognition of the cell line carrying B*3507 is due to a lack of human mH antigens in this cell line. The failure of the CTL recognition of the cell lines carrying B*3502 and B*3508 is not explained by only the amount of the human mH peptides binding to these B35 subtype molecules because the amount of the human mH peptides eluted from B*3502 and B*3508 molecules purified from the cell lines carrying these B35 subtypes is almost the same as that eluted from B*3503 molecules purified from the cell lines carrying B*3503. It is suspected that the conformational structure of the human mH peptides bound to these HLA-B35 subtype molecules changes and initiates a lack of the recognition of the CTL clones. The currrent study showed that the human mH antigens are conserved beyond race and that recognition by human mH antigen–specific T cells is critically influenced by the subtypes of HLA class I molecules.

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Minor histocompatibility (mH)[1] antigens have not been studied in detail even though they are very important in transplantation, since incompatibility of these antigens induces the rejection of grafts in organ transplantation and graft versus host disease in bone marrow transplantation (1). In these diseases, mH-specific cytotoxic T cells but not specific antibodies are induced (2–7). T cells recognize mH antigens in association with MHC antigens (4, 5, 7). Recent studies have demonstrated that mH antigens are presented as peptides by MHC class I molecules in the mouse (8–10) and human (11–13).

It is suspected that mH peptides are derived from self-proteins or endogenously integrated viral protein, and the peptides are transported into the endoplasmic reticulum via transporters after they are generated in the cytoplasm as peptides derived from self-proteins and viral proteins (14–19). Peptides take part in the assembly of MHC class I molecules in the endoplasmic reticulum (20, 21). Assembled MHC class I molecules are transported to the cell surface and are recognized by T cells.

In the mouse ~50 mH antigen genes have been mapped using congenic strains, and a limited polymorphism is known (1). In contrast, genes encoding human mH antigens have not been mapped, and the polymorphism of human mH antigens is not known. A recent study of population genetics using HLA-A2–restricted human mH antigen–specific CTL clones (22) has shown that the phenotype frequencies of three human mH antigens are 69, 16, and 7%.

There are many HLA subtypes that are distinguished by alloreactive CTL clones, biochemical analysis using two-dimensional electrophoresis, or molecular analysis using sequencing of the genes encoding HLA antigens. For example, 12 subtypes of HLA-A2 have been reported. Only three of these subtypes can be distinguished by serology (23). Since amino acid substitutions found among the subtypes affect the presentation of virus antigens to specific T cells (24–28), it is assumed that a given subtype of HLA class I antigen cannot present human mH peptides to specific T cells. Thus, population genetic analysis using mere recognition by CTL clones may not reflect the frequencies of mH antigens in a given population.

To investigate phenotype frequencies of human mH antigens in detail, we analyzed the frequencies of human mH antigens that are recognized by an HLA-B35–restricted CTL clone using an acid elution method for peptides and sequencing of HLA-B35 genes. Moreover, the current study demonstrated a molecular mechanism for the presentation of human mH peptides by HLA class I molecules.

Materials and Methods

Cells. PBLs from 23 Japanese individuals and 25 German individuals with HLA-B35 were collected. HLA haplotypes of these individuals were serologically determined. These PBLs were stimulated with PHA. PHA-induced T cells were cultured in RPMI 1640 medium with 10% FCS and 50 U/ml recombinant human IL-2, and the cells were used for CTL assays. EBV-transformed cell lines from German individuals, EBV-transformed self-B cell line from a recipient of HLA-identical renal grafts (Pt cells) (7), EBV-transformed B cell lines from a donor (Dn-2) (7), and Hmy2C1R (C1R) cells were maintained in RPMI 1640 medium with 10% FCS. T2 cells expressing HLA-B*3501 (T2-B*3501) were previously generated (29), and they were cultured in RPMI 1640 medium with 10% FCS.

*Transfection of the HLA-B*3502 Gene into C1R Cells.* The HLA-B*3502 genomic gene was previously cloned (30). This gene was cotransfected with a hygromycin-resistance gene into HLA-A, B null C1R cells as previously described (31). C1R cells expressing HLA-B*3502 (C1R-B*3502) were selected by flow cytometry using SFR8-B6 anti–HLA-Bw6 mAb (32). C1R cells expressing B*3501 (C1R-B*3501) were previously generated (33). C1R-B*3502 and C1R-B*3501 cells were cultured in RPMI 1640 medium with 10% FCS and 0.15 mg/ml hygromycin B.

Flow Cytometry Analysis. Approximately 5×10^5 cells, suspended in 50 ml of PBS with 2% FCS, were incubated with 50 ml of appropriately diluted SFR8-B6 anti–HLA-Bw6 or 4D12 anti–HLA-B5, B35 cross-reacting antigen mAb (34) for 30 min on ice. After two washes with PBS containing 2% FCS, the cells were incubated with 50 ml of FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (Silenus Laboratories, Hawthorne, Australia) at a dilution of 1:40 for 30 min on ice. The cells were washed three times with PBS containing 2% FCS, and the mean intensity of fluorescence was measured by a FACStar (Becton Dickinson, Mountain View, CA).

CTL Clones. Four human mH antigen–specific, HLA-B35–restricted CTL clones, NH-5.2, NH-5.3, NH-5.5, and NH-5.9, were previously generated and characterized (7). These CTL clones were maintained by repeated stimulations with the EBV-transformed B cell line Dn-2 at 1-wk intervals and feeding with RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FCS, and 50 U/ml human recombinant IL-2 every third day.

CTL Assay. Target cells (5×10^5) were incubated for 60 or 90 min with 100 μCi of $Na_2{}^{51}CrO_4$ in PBS and washed three times with RPMI 1640 medium containing 10% FCS. Target cells (5×10^3 per well) were added to serial dilutions of effector cells at E/T ratios of 4:1–0.5:1, and the mixtures were incubated for 4 or 6 h at 37°C in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark). The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the counts per minute in the supernatant in wells containing only target cells. The maximum release was determined by measuring the release of ^{51}Cr from target cells in the presence of 2.5% Triton X-100. The specific lysis was calculated using the following formula: percent specific lysis = $[(cpm_{exp} - cpm_{spn})/(cpm_{max} - cpm_{spn})] \times 100$, where cpm_{exp} is the counts per minute in the supernatant in wells containing target and effector cells, cpm_{spn} is the spontaneous release, and cpm_{max} is the maximum release.

Isolation and Characterization of HLA-B35 cDNA Clones. Total cellular RNA was extracted from EBV-transformed cell lines by standard methods (35). mRNA was reverse transcribed using random hexamers (first strand DNA synthesis kit; Pharmacia LKB Biotechnology Inc., Piscataway, NJ,). cDNA was then PCR amplified with HLA-B locus–specific primers, as described (36). Amplified DNA was subcloned in the SalI/HindIII sites of pUC18. Recombinant clones were identified by colony hybridization to an HLA class I cDNA probe, pHLA-2 (37). Double-stranded DNA plasmids were sequenced by the chain termination method using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH) and exon-

[1] *Abbreviation used in this paper:* mH, minor histocompatibility.

specific primers. The sequence was obtained for both DNA strands and was confirmed in four independent clones derived from each individual.

Acid Extraction of Naturally Occurring Human mH Peptides from EBV-transformed Cell Lines. Cultured EBV-transformed cell lines and C1R-B*3501 cells (10^9) were homogenized in 0.1% (vol/vol) TFA solution and then sonicated for 30 s. The homogenates were ultracentrifuged for 30 min (10,000 *g*). These procedures were repeated for the pellets. Supernatants were loaded on a Sephadex G-25 column (Pharmacia) equilibrated with 0.1% TFA solution. Material of low molecular weight eluted in the column volume was collected, lyophilized, and separated on a Superpac Pep S reverse-phase HPLC column (Pharmacia) by forming a linear gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile (60% per 35 min). Each 1-ml fraction was collected and lyophilized for CTL assay.

Acid Extraction of Naturally Occurring Human mH Peptides from Purified HLA-B35 Subtype Molecules. 2×10^9 C1R-B*3501 and EBV-transformed B cells expressing four HLA-B35 subtypes were lysed in 2 ml of 1.5% NP-40 detergent in 10 mM PBS containing 1 mM PMSF, 10 mg/ml trypsin inhibitor A, 10^{-5} M pepstatin A, 10^{-5} M leupeptin, and 10^{-4} M iodoacetamide. The resulting suspension was stirred for 30 min at 4°C and centrifuged for 30 min (1,800 *g*). The resulting supernatant was incubated with CNBr-activated Sepharose CL4B beads (Pharmacia), to which 4D12 or W6/32 mAb had been covalently coupled. The sample was again rotated six times at 4°C to precipitate HLA class I molecules. After washing in 10 mM PBS containing 0.1% NP-40, loaded beads were subjected to 50 mM diethylamine (pH 11.5). Eluted sample were lyophilized, resolved in 2 ml of 0.1% TFA, and separated in a Superpac Pep S reverse-phase HPLC column. Each fraction (1 ml) was collected and lyophilized for CTL assay.

CTL Assay for B Cell Lines Stimulated with Isolated Human mH Peptides. Dried HPLC fractions were dissolved in 200 μl of PBS. 50 μl of this solution was added to 50 μl of the medium containing 5×10^3 ^{51}Cr-labeled B cell lines in round-bottom wells of a 96-well microplate. After incubation for 60 min at 37°C, 100 μl of the medium containing 2×10^4 CTL clones was added to yield a final volume of 200 μl (4:1 E/T ratio). Plates were then incubated for 6 h at 37°C. The supernatants were collected and analyzed with a gamma counter.

Results

Recognition of Human mH Antigens on Cell Lines Derived from Japanese and German Individuals with HLA-B35 by HLA-

Table 1. *Expression of Human mH Antigens Presented by HLA-B35 on PHA-induced T Cell Lines from the Japanese Population*

Source of target cells	HLA type			CTL clones			
				NH-5.2	NH-5.3	NH-5.5	NH-5.9
KaS	A24/A –	B35/B54	Cw1/Cw10	**54.8***	**54.7**	**57.4**	**56.0**
KoE	A26.1/A11	B35/B46	Cw9/Cw11	**56.3**	**57.1**	**60.5**	**65.7**
SuM	A2/A31	B35/B –	Cw9/Cw –	**60.2**	**64.6**	**68.7**	**73.4**
SiR	A2/A24	B35/B46	Cw11/Cw –	**49.3**	**67.4**	**60.6**	**65.6**
MiN	A26.3/A26.4	B35/B52	Cw9/Cw –	**26.9**	**40.8**	**31.3**	**38.7**
MuM	A11/A –	B35/B67	Cw7/Cw9	**61.6**	**70.7**	**69.2**	**73.1**
OoT	A2/A24	B35/B61	Cw – /Cw –	**50.9**	**58.1**	**56.1**	**54.6**
KaO	A24/A26.3	B35/B61	Cw9/Cw –	**53.1**	**73.4**	**60.5**	**56.3**
WaN	A24/A26.3	B35/B52	Cw9/Cw –	**40.4**	**68.1**	**52.6**	**42.1**
SuR	A2/A26.1	B35/B54	Cw1/Cw9	**46.6**	**70.2**	**58.2**	**58.1**
MaU	A24/A26.3	B35/B62	Cw9/Cw –	**42.8**	**76.4**	**53.9**	**47.4**
NoH	A2/A –	B35/B62	Cw9/Cw –	**45.9**	**67.9**	**56.7**	**52.9**
UsT	A11/A26.3	B35/B –	Cw9/Cw –	**69.7**	**68.0**	**72.0**	**69.6**
InT	A24/A33	B35/B48	Cw9/Cw –	**61.1**	**65.2**	**59.1**	**56.1**
OnN	A2/A –	B35/B54	Cw9/Cw –	**66.8**	**64.3**	**68.3**	**62.5**
TsH	A2/A26.4	B35/B39	Cw7/Cw –	**68.4**	**63.9**	**74.3**	**64.2**
MuT	A2/A24	B35/B52	Cw9/Cw –	**56.7**	**69.9**	**63.0**	**55.7**
MaS	A2/A33	B35/B44	Cw9/C4451	**39.2**	**43.2**	**37.1**	**33.4**
MuU	A2/A26.3	B35/B –	Cw9/Cw –	**57.9**	**61.6**	**61.5**	**56.6**
IsS	A24/A26.3	B35/B7	Cw7/Cw9	**60.1**	**62.4**	**58.8**	**54.1**
MiT	A2/A31	B35/B51	Cw – /Cw –	**50.8**	**64.9**	**65.8**	**58.3**
Sak	A2/A24	B35/B51	Cw9/Cw –	**47.1**	**58.7**	**62.3**	**53.3**
MiM	A11/A26	B35/B51	Cw1/Cw9	**64.6**	**73.3**	**67.0**	**65.6**

* Percent specific lysis at a 1:1 E/T ratio. More than 20% of percent specific lysis was evaluated as positive killing (*boldface*).

*B*3501-restricted Human mH Antigen-specific CTL Clones.* Our previous study (7) showed that four mH antigen-specific, HLA-B*3501-restricted CTL clones (NH-5.2, NH-5.3, NH-5.5, and NH-5.9) killed B cell lines derived from all of five individuals with HLA-B35. These findings suggest that the human mH antigens recognized by these CTL clones are very well conserved among Japanese or other populations and that only members of this family lack these antigens. To identify the distribution of human mH antigens in populations, we examined the recognition by the CTL clones of a panel of cells derived from Japanese and German individuals. As shown in Table 1, four CTL clones killed all of 23 cell lines from Japanese individuals with HLA-B35. On the other hand, the CTL clones killed 19 of 25 cell lines from German Caucasian individuals; they partially killed three cell lines (12405, 13159, and 21254), but did not kill another three cell lines (18514, 20073, and 22338) (Table 2). Taken together, these data suggest that the human mH antigens recognized by these four CTL clones may be less conserved within the Caucasian population than the Japanese population, though they are very well conserved in the Japanese population.

It is speculated that poor recognition of the CTL clones for three cell lines (the CTL weakly sensitive cell lines) or lack of recognition of three cell lines (the CTL-resistant cell lines) may be due to low or even absent expression of HLA-B35 antigens on the cell lines, respectively. To exclude this possibility, we examined the surface expression of HLA-B35 antigens on these cell lines by flow cytometry with 4D12 anti-B5, B35 mAb. As shown in Table 3, all of the cell lines expressed HLA-B35. Mean intensities of fluorescence of cell lines 13159 and 19635 were higher than those of other cell lines expressing HLA-B35. This is explained by the fact that mAb 4D12 cross-reacts with HLA-B15 (34). The mean intensities of fluorescence of the other five cell lines (13.9 to 29.0) were the same as those of six CTL-sensitive cell lines (10.5 to 28.4). These results indicate that weak or lacking

Table 2. *Expression of Human mH Antigens Presented by HLA-B35 on PHA-induced T Cell Lines from the German Population*

Source of target cells	HLA type			CTL clones			
				NH-5.2	NH-5.3	NH-5.5	NH-5.9
83A2	A1/A11	B35/B7	Cw4/Cw7	**71.2***	**77.1**	**67.3**	**68.2**
M1202	A2/A3	B35/B39	Cw4/Cw –	**59.6**	**74.5**	**61.2**	**58.6**
M1228	A3/A29	B35/B44	Cw4/Cw –	**57.1**	**58.4**	**50.8**	**57.2**
22337	A2/A26	B35/B55	Cw3/Cw4	**58.6**	**82.2**	**56.9**	**46.0**
22322	A1/A3	B8/B35	Cw4/Cw7	**65.9**	**86.7**	**77.4**	**72.0**
12717	A2/A11	B7/B35	Cw4/Cw7	**56.2**	**69.5**	**51.1**	**63.6**
20058	A24/A29	B35/B44	Cw – /Cw –	**74.4**	**100.0**	**71.6**	**77.2**
19635	A3/A68	B35/B35	Cw4/Cw4	**74.7**	**91.9**	**89.2**	**83.5**
22358	A2/A –	B35/B39	Cw4/Cw7	**83.9**	**100.0**	**81.1**	**80.5**
17772	A2/A3	B27/B35	Cw2/Cw4	**48.9**	**79.0**	**58.4**	**58.0**
22780	A3/A –	B27/B35	Cw2/Cw4	**66.4**	**78.3**	**72.5**	**70.6**
21142	A2/A24	B27/B35	Cw1/Cw4	**52.0**	**100.0**	**51.1**	**48.2**
22008	A3/A –	B35/B44	Cw6/Cw –	**65.3**	**61.1**	**73.3**	**60.1**
16213	A3/A –	B7/B35	Cw4/Cw7	**82.8**	**57.3**	**51.7**	**67.7**
22339	A2/A24	B35/B44	Cw4/Cw5	**67.0**	**41.7**	**43.6**	**47.4**
11985	A3/A24	B35/B44	Cw4/Cw5	**70.3**	**82.2**	**61.3**	**84.4**
10476	A1/A3	B8/B35	Cw4/Cw7	**72.5**	**58.7**	**56.4**	**61.5**
20072	A30/A68	B27/B35	Cw2/Cw4	**82.9**	**70.4**	**71.7**	**66.3**
12893	A2/A24	B35/B49	Cw4/Cw –	**80.5**	**100.0**	**100.0**	**81.1**
12405	A3/A28	B18/B35	Cw4/Cw –	18.5	**29.5**	15.1	17.9
13159	A2/A24	B35/B62	Cw3/Cw –	16.5	18.8	11.9	15.1
21254	A2/A3	B7/B35	Cw4/Cw7	9.8	14.6	8.6	6.9
18514	A1/A3	B7/B35	Cw4/Cw –	– 1.3	0.7	– 1.0	0.0
20073	A3/A32	B35/B64	Cw4/Cw8	– 1.0	– 0.9	– 1.7	– 0.6
22338	A1/A24	B35/B41	Cw4/Cw –	– 0.2	0.1	– 1.7	– 1.3

* Percent specific lysis at a 1:1 E/T ratio. More than 20% of percent specific lysis was evaluated as positive killing (*boldface*).

Table 3. *Surface Expression of HLA-B35 Antigen on EBV transformed Cell Lines*

Cell line	CTL response	HLA-B haplotype	mAb 4D12	mAb – *
12405	W‡	18,35	14.0§	1.7
13159	W	35,62	78.8	2.0
21254	W	7,35	24.7	2.0
18514	–	7,35	29.0	2.1
20073	–	35,64	13.9	1.9
22338	–	35,41	16.3	2.2
11985	+	35,44	19.9	4.0
12717	+	7,35	16.2	2.7
19635	+	35,35	31.6	3.3
20072	+	27,35	16.3	2.6
21142	+	27,35	14.0	3.1
22780	+	27,35	10.5	2.5
Dn-2	+	35,38	28.4	2.0
Pt	–	35,38	27.2	2.3

* No primary antibody.
‡ Weak response of the CTL clones.
§ Mean intensities of fluorescence.

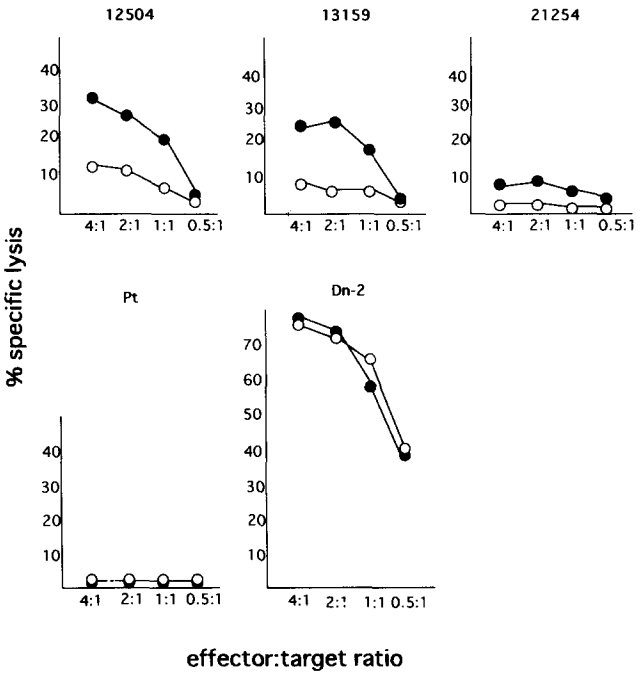

Figure 1. Recognition by CTL clone NH-5.5 of the CTL weakly sensitive cell lines with different incubation times. Killing activity of CTL clone NH-5.5 for the CTL weakly sensitive cell lines (*12504*, *13159*, and *21254*), a self-B cell line (*Pt*), and a donor derived cell line (*Dn-2*) was tested with incubation times of 4 (○) and 6 (●) h.

recognition by the CTL clones of these CTL weakly sensitive and CTL-resistant cell lines does not result from low or absent expression of HLA-B35 molecules.

Three cell lines, 12405, 13159, and 21254, were partially killed by four CTL clones with a 4-h incubation and an E/T ratio of 1:1. The killing of these cell lines was enhanced with a 6-h incubation (Fig. 1), suggesting that HLA-B35 molecules on these cell lines may present a small amount of the human mH peptide to CTL clones.

Sequence of HLA-B35 Genes in Three CTL-resistant Cell Lines and Three CTL Weakly Sensitive Cell Lines. It is speculated that the CTL-resistant or weakly sensitive cell lines express HLA-B35 subtypes that are different from B*3501, since the recognition of four human mH CTL clones is restricted with B*3501 (11). To clarify this possibility, we attempted to clone cDNA encoding HLA-B35 from these cell lines, and the exons 2–4 of the genes were sequenced. The sequence of the HLA-B35 genes derived from cell line 18514 was identical to that of HLA-B*3502 (30). The sequences of HLA-B35 genes derived from cell lines 20073 and 22338 differed from HLA-B*3501 by a single nucleotide substitution at codons 16 and 156, respectively. The glycine codon, GGC, in B*3501 is replaced by the valine codon, GTC, in the gene from cell line 20073, whereas the leucine codon, CTG, in B*3501 is substituted by the arginine codon, CGG, in the gene from cell line 22338. The novel HLA-B35 subtypes on cell lines 20073 and 22338 have been designated as B*3507 and B*3508, respectively (38). The sequences of HLA-B35 genes derived from three cell lines, 12405, 13159, and 21254, were identical to that of HLA-B*3503. Polymorphic residues in these HLA-B35 subtypes are shown in Table 4.

B*3502 differs from B*3501 by three amino acids at positions 109, 114, and 116. Residues 114 and 116 are located on the floor of the β sheet, and the residue 109 is in the outer loop on the β sheet (Fig. 2). B*3503 (39), B*3507, and B*3508 differ from B*3501 only by a single amino acid substitution at residues 116, 16, and 156, respectively. Residue 156 is a peptide-binding site in the α2 helix, and residue 16 is in the outer loop of the β sheet (Fig. 2).

Table 4. *Polymorphic Residues in HLA-B35 Subtypes of Six Cell Lines*

Cell line	Substituted amino acid residue 16	109	114	116	156	Allele
	Gly	Leu	Asp	Ser	Leu	B*3501
18514	–*	Phe	Asn	Tyr	–	B*3502
20073	Val	–	–	–	–	B*3507
22338	–	–	–	–	Arg	B*3508
12405, 13159, 21254	–	–	–	Phe	–	B*3503

* Dashes indicate identity with HLA-B*3501.

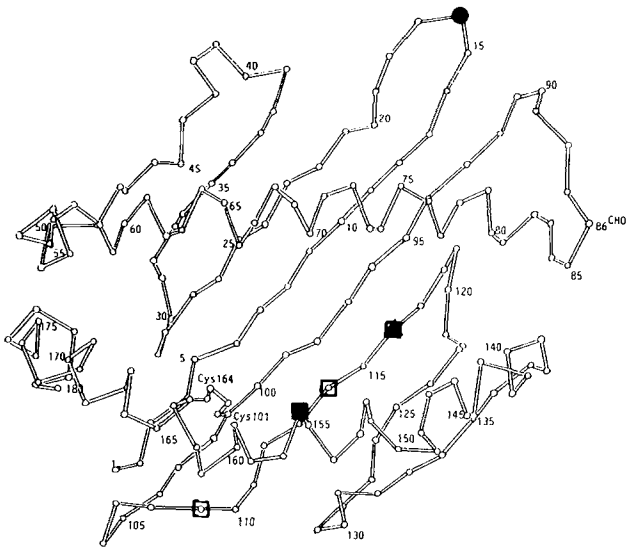

Figure 2. Location of the amino acid substitutions in HLA-B*3501 and other HLA-B35 subtypes. HLA-B*3501 differs from HLA-B*3502 by substitutions at residues 109, 114, and 116 (□), from HLA-B*3503 by substitution at residue 116 (▲), from HLA-B*3507 by substitution at residue 16 (●), and from HLA-B*3508 by substitution at residue 156 (■).

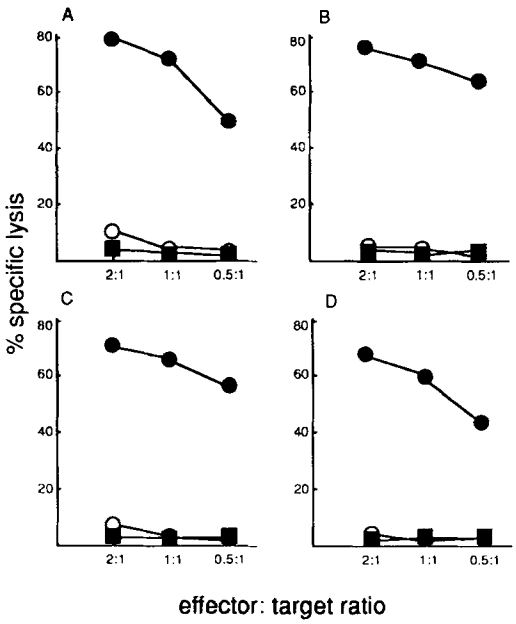

Figure 3. Recognition by HLA-B*3501-restricted, human mH antigen-specific CTL clones of C1R-B*3502 cells. Killing activity of NH-5.2 (*A*), NH-5.3 (*B*), NH-5.5 (*C*), and NH-5.9 (*D*) for C1R-B*3501 (●), C1R-B*3502 (■), or untransfected C1R (□) cells was tested. Surface expression of HLA-B35 antigens on C1R-B*3501, C1R-B*3502, and C1R cells was examined by flow cytometry using mAb SFR8-B6. Mean intensities of fluorescence of C1R-B*3501, C1R-B*3502, and C1R cells were 188.3, 243.8, and 6.7, respectively.

*Recognition of Human mH Antigens on C1R Cells Transfected with the HLA-B*3502 Gene.* Our previous study (7) showed that four human mH antigen-specific, HLA-B*3501-restricted CTL clones can kill C1R cells transfected with the HLA-B*3501 gene (C1R-B*3501), but not untransfected C1R cells. These findings indicated that C1R cells possess the human mH antigens presented by HLA-B*3501 molecules. To investigate the presentation of human mH antigens by HLA-B*3502 molecules, the HLA-B*3502 gene was transfected into C1R cells, and the transfectant highly expressing HLA-B*3502 (C1R-B*3502) was selected by flow cytometry using SFR8-B6 anti-Bw6 mAb. As shown in Fig. 3, four human mH antigen-specific CTL clones killed C1R-B*3501 cells but not C1R-B*3502 cells. These results indicated that HLA-B*3502 molecules cannot present human mH antigens to the T cell clones.

Binding of the Human mH Peptide to HLA-B35 Antigens on Three CTL-resistant Cell Lines and Three CTL Weakly Sensitive Cell Lines. Our recent studies showed that four human mH antigen-specific, HLA-B*3501-restricted CTL clones recognize the human mH peptides presented by HLA-B*3501 molecules (11, 12, 29). Therefore, the results of experiments using C1R-B*3502 and the sequence data of the HLA-B35 gene in CTL-resistant cell lines and CTL weakly sensitive cell lines suggest that B*3502 molecules in the 18514 cell line and B*3508 in the 22338 cell line do not bind the human mH peptides, whereas B*3503 molecules in three CTL weakly sensitive cell lines bind a relatively small amount of the human mH peptides. To clarify this possibility, we investigated direct binding of the human mH peptides to HLA-B35 subtype molecules on these cell lines. Naturally occurring human mH peptides were isolated by reverse-phase HPLC from HLA-B*3501 molecules that were purified from C1R-B*3501 cells. The HPLC fraction containing human mH peptides was detected using four CTL clones as previously described (11) and was used as the source of human mH peptides. The cells expressing HLA-B35 subtypes were cultured at 26°C overnight and then used as target cells for four CTL clones after stimulation with human mH peptides. As shown in Fig. 4, the 18514 cell line expressing B*3502 molecules and the 22338 cell line expressing B*3508 molecules stimulated with human mH peptides were not killed by the CTL clones, whereas 20073 cells expressing B*3507 molecules loaded with human mH peptides were killed by the CTL clones. Three CTL weakly sensitive cell lines (12405, 13159, and 21254) expressing B*3503 molecules stimulated with the human mH peptides were more effectively killed by the CTL clones than were unstimulated cell lines. These results indicate that B*3502 and B*3508 molecules cannot bind the human mH peptides, whereas B*3503 and B*3507 molecules can bind the same amount of the human mH peptides as B*3501. Moreover, since 20073 cells were not killed by the CTL clones, these results together suggest that the specific human mH peptides are not produced in the 20073 cell line expressing B*3507.

Isolation of Naturally Occurring Human mH Peptides from CTL-resistant Cell Lines and CTL Weakly Sensitive Cell Lines. We attempted to isolate the naturally occurring human mH peptides from HLA-B35 subtype molecules on CTL-resistant cell lines and CTL weakly sensitive cell lines by acid elution. Relatively low molecular mass material extracted by acid elution

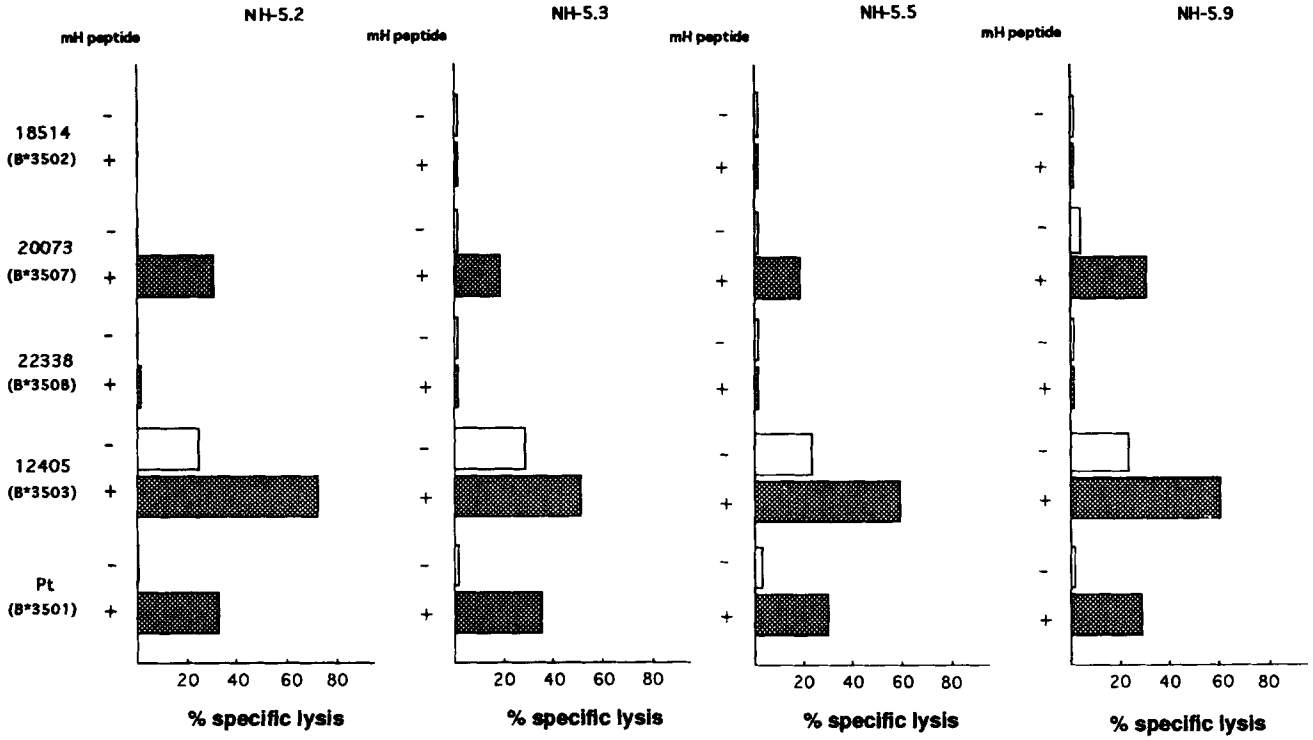

Figure 4. Enhancement of T cell recognition for the cell lines expressing HLA-B35 subtypes by loading with human mH peptides isolated from HLA-B*3501 molecules purified form C1R-B*3501 cells. ^{51}Cr-labeled B cell lines were incubated with fraction 31, which contains the human mH peptides isolated from HLA-B*3501 molecules derived from 2.5 × 10^8 C1R-B*3501 cells. Killing activity by four CTL clones was tested.

from the cell line lysates was separated by reverse-phase HPLC. Fractions 29–32 were tested for recognition of the NH-5.5 clone using Pt cells as the target, since the human mH peptides that the CTL clones recognized were isolated in fraction 30 or 31 (11). A small amount of the human mH peptides was detected in fraction 30 from extracts of the CTL-resistant cell line 22338, whereas no human mH peptides were isolated from extracts of other cell lines (Fig. 5). Since three CTL weakly sensitive cell lines were partially killed by the CTL clones, it is suspected that the amounts of peptides isolated from these cell lines are too small to be detected by the CTL assay using Pt cells as target cells. Therefore, we used HLA-B*3501–transfected T2 cells (T2-B*3501) as target cells in the assay in order to detect a small amount of the human mH peptides because the T2 transfectants express many empty HLA-B35 molecules. Pt and T2-B*3501 cells were stimulated with titrated fraction 30 containing the human mH peptides from C1R-B*3501 cells and then tested by using the NH-5.5 clone. As shown in Fig. 6, the capacity of the CTL clone to detect the human mH peptides was enhanced 10-fold when T2-B*3501 cells were used as target cells.

Fractions 29–32 were again tested for recognition by the NH-5.5 clone using T2-B*3501 cells (Fig. 7). The human mH peptides were detected in fraction 31 from three CTL weakly sensitive cell lines as well as the 18514 and 22338 cell lines. In contrast, no human mH peptides were isolated from the 20073 cell line. These results demonstrated that only the 20073 cell line does not possess human mH antigens.

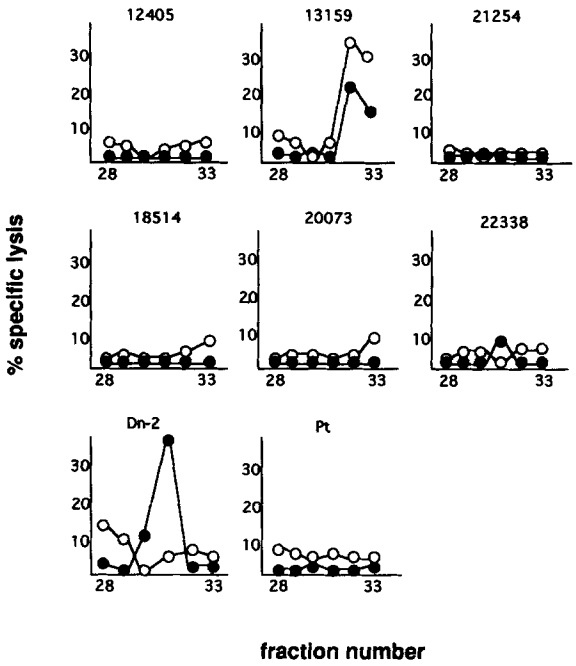

Figure 5. Isolation of naturally occurring human mH peptides from cell lines expressing HLA-B35 subtypes. Fraction 28–33 derived from each cell line expressing the HLA-B35 subtypes were tested for recognition by CTL clone NH-5.5 (●) and for cytotoxicity without CTL clone NH-5.5 (○) by using Pt cells as target cells.

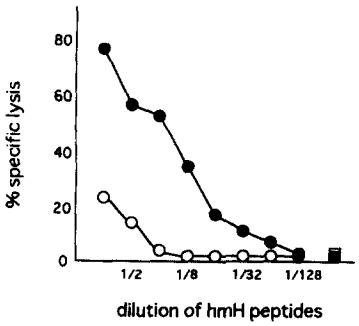

Figure 6. Enhancement of sensitivity to detect naturally occurring human mH peptides by using T2-B*3501 cells as target cells. Fraction 31 derived from 10^9 C1R-B*3501 cells was dissolved in 200 μl of PBS and diluted. 50 μl of each diluted sample was tested for the recognition by CTL clone NH-5.5 using T2-B*3501 (●) and Pt (○) cells. Percent specific lysis for T2-B*3501 cells loaded without peptide (■) and that for Pt cells loaded without peptide (□) are ~0%.

Isolation of Naturally Occurring Human mH Peptides from HLA-B35 Subtype Molecules Purified from CTL-resistant Cell Lines and CTL Weakly Sensitive Cell Lines. The human mH peptides recognized by the NH-5.5 clone were isolated from whole-cell extract from five cell lines expressing HLA-B*3502, B*3503, or B*3508 (Fig. 7). This does not demonstrate direct isolation of the specific human mH peptides from these HLA-B35 subtype molecules. Therefore, we attempted to isolate the specific human mH peptides from purified HLA-B35 subtype molecules. HLA-B35 molecules were isolated from six cell lines expressing HLA-B35 sybtypes as well as from Pt and Dn-2 cells using Sepharose beads coupled with 4D12 anti-B5, B35 mAb. Peptides bound to these HLA-B35 molecules were isolated by reverse-phase HPLC after purified HLA-B35 molecules were resolved in 0.1% TFA solution. Fractions 29–32 were tested for recognition by the NH-5.5 clone using T2-B*3501 cells as target cells (Fig. 8). The specific human mH peptides were again isolated from HLA-B*3502, B*3503, and B*3508 molecules purified from the cell lines expressing these HLA-B35 subtypes. The amount of the specific peptides isolated from these subtype molecules was significantly smaller than that that from HLA-B*3501 mole-

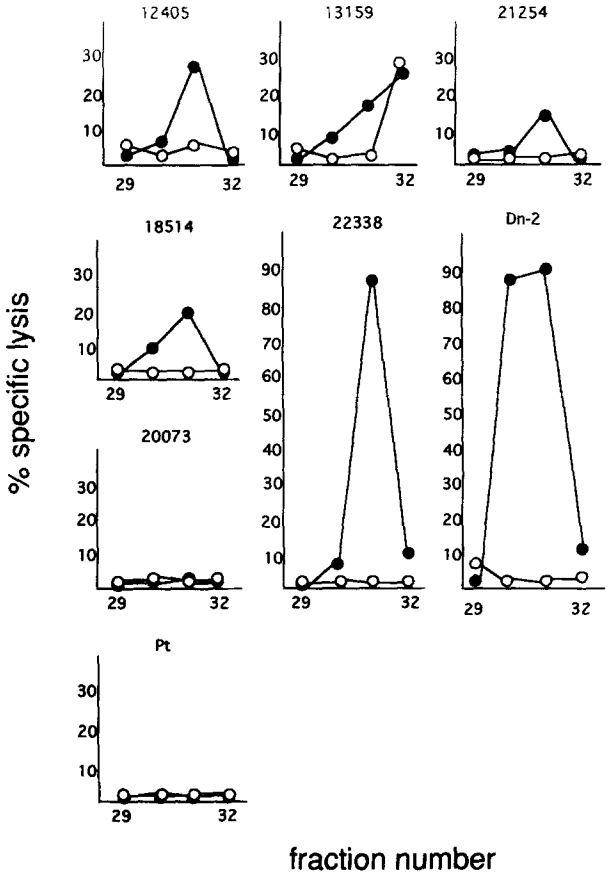

Figure 7. Detection of naturally occurring human mH peptides derived from whole-cell lysates from cell lines expressing HLA-B35 subtypes by using T2-B*3501 cells as target cells. The same fractions shown in Fig. 5 were tested for the recognition by CTL clone NH-5.5 (●) and cytotoxicity without the CTL clone NH-5.5 (○) using T2-B*3501 cells as target cells.

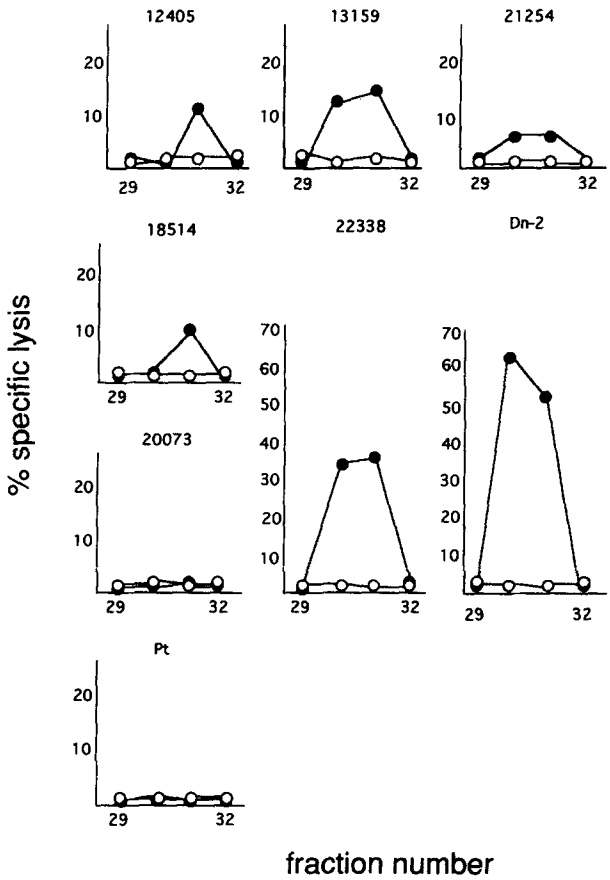

Figure 8. Detection of naturally occurring human mH peptides isolated from HLA-B35 subtype molecules purified from cell lines expressing HLA-B35 subtypes. The same fractions shown in Fig. 5 were tested for recognition by CTL clone NH-5.5 (●) and cytotoxicity without CTL clone NH-5.5 (○) using T2-B*3501 cells as target cells.

cules. These results indicate that three HLA-B35 subtype, B*3502, B*3503, and B*3508 molecules, are able to bind a small amount of the peptides recognized by the NH-5.5 clone. Similarly, the peptides recognized by the NH-5.2, NH-5.3, and NH-5.9 clones were isolated in the same HPLC fraction from the same cell lines that yielded the peptides recognized by clone NH-5.5 (Table 5). Taken together, these findings indicate that the human mH peptides recognized by the CTL clones bind to HLA-B*3502, B*3503, and B*3508 molecules in the cell lines expressing these HLA-B35 subtype molecules.

Discussion

Three CTL weakly sensitive cell lines carry HLA-B*3503 molecules that differ from HLA-B*3501 by a single amino acid substitution at position 116 on the floor of the peptide-binding groove. Since residue 116 is located on the bottom of the F pocket (40, 41), it is suspected that the substitution of phenylalanine for serine at this position reduces the binding of human mH peptides. The reduction in the binding of human mH peptides may cause weak recognition of the cells expressing HLA-B*3503 by the specific CTL clones. In fact, the current study demonstrated that a small amount of the specific peptides is eluted from the cells carrying HLA-B*3503. In contrast, the HLA-B*3503 molecules appear to have the ability to bind effectively the human mH peptides on the cell surface because the killing of cells expressing HLA-B*3503 by the CTL clone is enhanced by the stimulation of human mH peptides from C1R-B*3501 cells. These results imply that HLA-B*3503 molecules bind other self-peptides more effectively than the specific human mH peptides.

HLA-B*3502 differs from HLA-B*3501 by 3 amino acid substitutions at positions 109, 114, and 116 on the β sheet. Since the CTL clones cannot kill C1R cells expressing B*3502, it is speculated that HLA-B*3502 molecules cannot bind the human mH peptides. The substitution of asparagine for asparatic acid at residue 114 and tyrosine for serine at residue 116 may affect the binding of the human mH peptides because residues 114 and 116 are located on the floor of the peptide-binding groove and are facing the E and F pockets, respectively (40, 41). The current study demonstrated that a small amount of human mH peptides is eluted from HLA-B*3502 molecules purified from cell line 18514 expressing this subtype. On the other hand, recognition by the CTL clone of cell line 18514 was not induced by stimulation with human mH peptides eluted from B*3501 molecules. These results raise two possibilities: (*a*) HLA-B*3502 molecules can bind a small amount of human mH peptide that is not enough for the recognition by the CTL clones, or (*b*) the substitutions at positions 114 and 116 induce a conformational change of the human mH peptides bound to the HLA class I molecules, resulting in a lack of recognition by the CTL clones. However, it is difficult to explain the lack of recognition of CTL clones for the cell line 18514 expressing HLA-B*3502 by the former hypothesis alone, since the amount of human mH peptides eluted from B*3502 molecules purified from cell line 18514 is almost the same as that from B*3503 molecules purified from the cell lines expressing HLA-B*3503 (12405, 13159, and 21254).

HLA-B*3508 differs from HLA-B*3501 by a single amino acid substitution at position 156. Since this residue is one of the peptide-binding sites facing the E pocket, it is assumed that the substitution of arginine for leucine affects the binding of human mH peptides. The current study shows that the amount of the human mH peptides eluted from B*3508 molecules purified from the 22338 cell line is less than that from B*3501 molecules purified from Dn-2 cells. However, the amount of human mH peptides bound to HLA-B*3508 molecules seems to be sufficient for the recognition by the CTL clones, since it is significantly more than that from B*3503 molecules purified from the cell lines expressing this B35 subtype that were partially killed by the CTL clones. A previous

Table 5. *Isolation of Human mH Peptides Recognized by Four Human mH-specific CTL Clones from Purified HLA-B35 Subtype Molecules*

		HPLC fraction							
		NH5.2		NH5.3		NH5.5		NH5.9	
Cell line	HLA-B35	30	31	30	31	30	31	30	31
18514	B*3502	0*	11.2	0	15.3	0	9.9	0	13.2
12405	B*3503	0	9.5	0	10.1	0.1	11.2	0	9.5
13159	B*3503	14.8	15.2	15.6	16.8	11.7	13.8	20.2	22.4
21254	B*3503	6.5	8.2	9.2	9.4	7.2	7.2	7.4	7.6
20073	B*3507	0	0	0	0	0.3	0	0	0
22338	B*3508	38.4	36.1	37.7	32.4	32.5	31.5	35.5	31.3
DN-2	B*3501	50.3	41.1	69.2	60.2	64.9	51.7	65.4	58.3
Pt	B*3501	0	0	0	0	0	0	0	0

* Percent specific lysis.

study using an HLA-A2 mutant at position 156 (42) demonstrated that 24 of 25 HLA-A2–restricted, influenza virus matrix peptide–specific CTL lines could not recognize HLA-A2 mutant at residue 156 loaded with the peptide, whereas one CTL line recognized it. This study suggested that the amino acid substitution at residue 156 of the HLA-A2 molecule affects the recognition by the specific CTL clones but does not inhibit binding of the peptide to the HLA class I molecules. The current study together with a previous study using HLA-A2 mutant suggests that the substitution at residue 156 changes the conformational structure of the peptides bound to the HLA class I molecules, resulting in a lack of recognition of the CTL clones.

We recently demonstrated that the substitution of glutamic acid for valine at residue 152 of HLA-B*3501 does not affect the binding of the human mH peptides recognized by CTL clones NH-5.2, NH-5.3, NH-5.5, and NH-5.9, but it does abrogate recognition by these CTL clones (29). Since residue 152 is also one of the residues forming the E pocket, it supports the idea that the substitution at the position of the α helix forming the E pocket changes the conformational structure of the human mH peptides bound to the HLA class I molecules. Moreover, the substitution of asparagine for asparaginic acid at residue 114 facing the E pocket and that of tyrosine for serine at residue 116 facing the F pocket seem to change the conformational structure of the human mH peptides bound to HLA-B35 molecules. The amount of human mH peptide bound to HLA-B*3502 molecules purified from the 18514 cell line is more than that bound to HLA-B*3503 molecules purified from three CTLs weakly sensitive cell lines, whereas the CTL clone partially killed PBLs expressing B*3503 but not those expressing B*3502. Taken together, these observations of HLA-B*3502 and B*3508 suggest that T cells recognize a conformational structure of the human mH peptides bound to HLA-B35 molecules.

HLA-B*3507 differs from B*3501 by a single amino acid substitution at position 16. It is thought that this residue hardly affects peptide binding because residue 16 is on the outer loop of the β sheet. Therefore, it is assumed that cell line 20073 expressing HLA-B*3507 molecules does not possess the human mH antigens containing the specific epitope recognized by the CTL clones. Indeed, the current study showed that the specific human mH peptides are not isolated from this cell line, whereas HLA-B*3507 molecules can bind the specific peptides.

In this study, we demonstrated that the human mH peptides recognized by four CTL clones are presented by HLA-B35 molecules in all 23 Japanese individuals and in 24 of 25 German individuals. Since there is no difference in recognition by these CTL clones for a panel of cells expressing HLA-B35 and Pt cells loaded with the peptides eluted from the cells expressing HLA-B35 subtypes, it is suspected that these CTL clones recognize a single peptide. However, it will be confirmed only by identification of the peptides recognized by four CTL clones. Further identification of the peptides recognized by four CTL clones is necessary to clarify the number of human mH peptides recognized by these CTL clones. The current study demonstrated that at least one human mH antigen is conserved in the Japanese and German populations.

Since human mH antigens are recognized by T cells but not by antibodies, their polymorphism was studied only in the specific T cells. This study showed that five of six cell lines that are not killed by the CTL clones possess the human mH peptides. Thus, a simple analysis using killing activity by CTL clones cannot reveal the frequency of a human mH antigen in the population. Analyses of HLA subtypes and human mH peptides are also required to estimate the frequency of human mH antigens in detail. We showed here that only 1 of 25 German people and 23 Japanese people lacks the human mH antigen containing the specific T cell epitopes. This indicates that the human mH antigens are conserved beyond race. The current study also demonstrated that HLA-B*3501–restricted, human mH antigen–specific CTL clones failed to recognize human mH peptides presented by other HLA-B35 subtype molecules. This indicates that precise typing of HLA subtype is particularly required in bone marrow transplantation in which incompatibility of human mH antigens induces graft rejection and graft versus host disease.

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Address correspondence to Masafumi Takiguchi, Department of Tumor Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.

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