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Presentation of human minor histocompatibility antigens by HLA-B35 and HLA-B38 molecules

(non-HLA antigens/HLA class I restriction/chimeric gene/epitopes/cytotoxic T lymphocyte clones)

JUNJI YAMAMOTO*, AI KARIYONE*, NOBUO AKIYAMA†, KYOICHI KANO*, AND MASAFUMI TAKIGUCHI*‡

Departments of *Immunology and †Clinical Transplantation, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

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ABSTRACT **Cytotoxic T lymphocyte (CTL) clones specific for human minor histocompatibility antigens (hmHAs) were produced from a patient who had been grafted with the kidneys from his mother and two HLA-identical sisters. Of eight CTL clones generated, four recognized an hmHA (hmHA-1) expressed on cells from the mother and sister 3 (second donor); two recognized another antigen (hmHA-2) on cells from the father, sister 2 (third donor), and sister 3; and the remaining two clones recognized still another antigen (hmHA-3) on cells from the father and sister 3. Panel studies revealed that CTL recognition of hmHA-1 was restricted by HLA-B35 and that of hmHA-2 and hmHA-3 was restricted by HLA-B38. The HLA-B35 restriction of the hmHA-1-specific CTL clones was substantiated by the fact that they killed HLA-A null/HLA-B null Hmy2CIR targets transfected with HLA-B35 but not HLA-B51, -Bw52, or -Bw53 transfected Hmy2CIR targets. These data demonstrated that the five amino acids substitutions on the α_1 domain between HLA-B35 and -Bw53, which are associated with Bw4/Bw6 epitopes, play a critical role in the relationship of hmHA-1 to HLA-B35 molecules. The fact that the hmHA-1-specific CTLs failed to kill Hmy2CIR cells expressing HLA-B35/51 chimeric molecules composed of the α_1 domain of HLA-B35 and other domains of HLA-B51 indicated that eight residues on the α_2 domain also affect the interaction of hmHA-1 and the HLA-B35 molecules.**

Human minor histocompatibility antigens (hmHAs) are believed to induce rejection of the organ graft and graft vs. host reaction in bone marrow transplantation between HLA-identical siblings. However, our knowledge about hmHAs (1) has been very limited. Previous studies (2–8) demonstrated that the hmHAs could be recognized by cytotoxic T lymphocytes (CTLs), whereas antibodies against these antigens were difficult to detect.

Thus far the best characterized hmHAs are those H-Y antigens identified by CTLs (3, 9–12). Other hmHAs were also detected by CTLs (2, 4–8). As in the mouse, responses of these CTLs were restricted by major histocompatibility complex class I antigens. Only a limited number of HLA allospecificities have been reported as restriction molecules in the recognition of hmHAs. Among them, HLA-A2 and -B7 were two major ones, although there are examples of restriction by HLA-A1 and -Bw62 (6, 13). These studies strongly suggested that certain HLA-A/B molecules can present hmHAs to T cells.

In the present study, we attempted to generate CTL clones recognizing hmHAs from a recipient of multiple renal grafts and to characterize in detail CTL clones and corresponding hmHAs. Furthermore, the restriction elements in the CTL recognition of hmHAs were investigated by using Hmy2CIR cells transfected with HLA class I genes.

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MATERIALS AND METHODS

Cells. B cells from the patient and the second donor (sister 3; S3 in Fig. 1) were transformed by Epstein–Barr virus (EBV). These transformed B-cell lines were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% (vol/vol) fetal calf serum. EBV-transformed cell lines HVB-B5, Leo10, FS, CRB-087B, JBUSH, and AMAI were kindly supplied by F. Otani (Kitasato University, Kanagawa, Japan) and grown in the same medium. Peripheral blood lymphocytes (PBL) from six healthy staff members (Sug, YO, KT, YK, ST, and Tm) as well as those from the father, mother, sister 2 (S2), and S3 of the patient were stimulated twice by 0.2% phytohemagglutinin (PHA; Difco). The PHA-induced T cells were used as target cells for the CTL assay.

Hmy2CIR cells expressing HLA-B35, -B51, or -Bw52 antigens or HLA-B51/35 chimeric antigens were previously generated (14–16). The HLA-Bw53 genomic gene has been cloned and transfected into Hmy2CIR cells (unpublished data). These cells were grown in the same medium supplemented with hygromycin B (0.2 mg/ml). Untransfected Hmy2CIR cells were grown in the medium without hygromycin B.

Antibodies. W6/32 (17) and ME40.5 (18) HLA class I monomorphic, L243 HLA-DR monomorphic (19), anti-Leu-10 HLA-DQ monomorphic (20), B7/21 HLA-DP monomorphic (21), OKT3 anti-human CD3 (22), OKT8 anti-human CD8 (22), OKT4 anti-human CD4 (22), and WT31 anti-human α,β T-cell receptor monoclonal antibodies (mAbs) were used. The MB40.5 mAb was kindly supplied by Peter Parham (Stanford University).

Generation and Maintenance of hmHA-Specific CTL Clones. PBLs were obtained from the patient carrying the third renal graft (from S2, Fig. 1): 10^7 PBLs were stimulated three times at 1-week intervals by 10^7 irradiated PBLs of the second donor (S3). After specific killing of S3 target cells by the bulk culture cells was confirmed, the CTLs were cloned in a 96-well flat-bottom microtiter plate (Nunc) with irradiated PBLs from S3 and recombinant human interleukin 2 (25 units/ml) (Shionogi, Tokyo). The T-cell clones grown in each well were examined for their specific killing against S3 cells. The CTL clones were maintained by repeated stimulation of S3 cells at 1-week intervals and were fed with the medium containing recombinant human interleukin 2 every 3 days.

Measurement of CTL Reactivity by ^{51}Cr Release. Target cells (10^6) were incubated for 60 min with 50 μCi (1 Ci = 37 GBq) of $Na_2\,^{51}CrO_4$ in RPMI 1640 medium with 20% fetal calf serum and washed three times in the medium with 5% fetal calf serum. The target cells (5×10^3 cells per well) were added to serial dilutions of the effector cell suspension in 96-well

Abbreviations: hmHA, human minor histocompatibility antigen; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; mAb, monoclonal antibody; S2, sister 2; S3, sister 3.

‡To whom reprint requests should be addressed.

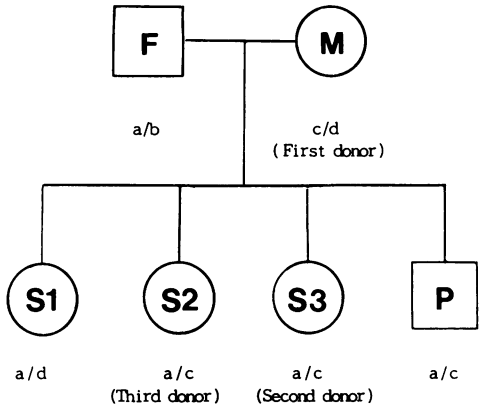

FIG. 1. Haplotype of the patient's family. F, father; M, mother; S1, sister 1; S2, sister 2; S3, sister 3; P, patient.

round-bottom microtiter plates (Nunc). After 4 hr of incubation at 37°C, the supernatants were harvested from each well by using a Titertek supernatant collection system (Skatron, Sterling, VA) and analyzed in a γ counter. Spontaneous ^{51}Cr release was determined by measuring cpm of the supernatant of wells containing only target cells (cpm_{spn}). The maximum release (cpm_{max}) was determined by the release of ^{51}Cr from target cells in the presence of 2.5% Triton X-100. Specific lysis was calculated according to the formula: % specific lysis = $[(cpm_{exp} - cpm_{spn})/(cpm_{max} - cpm_{spn})] \times 100$, where cpm_{exp} is the cpm in the supernatant of wells containing target and effector cells.

Blocking of CTL Reactivity by mAbs. CTL clones were preincubated with serial dilutions of mAbs (OKT8 or OKT4) in a 96-well microtiter plate at 37°C for 60 min, and then 5×10^3 labeled target cells were added to each well. Specific lysis of the CTL clones was determined as described above. In the reverse experiments, the labeled target cells were preincubated with serial dilutions of mAbs against HLA class I or class II (HLA-DR, -DQ, and -DP) molecules at 37°C for 60 min, and then 10^4 CTLs were added. After 4 hr of incubation, the supernatants were analyzed in a γ counter. Percent inhibition of specific lysis was calculated according to the following formula: % inhibition = (1 − % specific lysis of CTL clones with mAb/% specific lysis of CTL clones without mAb) × 100.

RESULTS

Three Different hmHAs Are Recognized by the CTL Clones. hmHA-specific CTLs were generated from PBL of the patient by using irradiated PBL from the HLA-identical second donor (S3) as the stimulator. Eight CTL clones were isolated by a limiting dilution method. These CTL clones showed ≈46–72% specific killing at an effector-to-target ratio of 0.5:1 against EBV-transformed B cells and ≈20–66% against PHA-induced T cells from S3 but not B and T target cells from the patient. These results indicated that hmHAs recognized by these eight CTL clones were expressed on both T and B cells of S3.

Flow-cytometric analyses of these CTL clones revealed that all of them express CD3, CD8, α,β T-cell receptor, HLA class I molecules, and HLA-DR (class II) molecules but not CD4 molecules. The killing activity of four CTL clones was inhibited by anti-CD8 mAb but not by anti-CD4 mAb,

Table 1. Three different hmHAs are recognized by CTL clones

Source of target cells	E:T ratio	% specific lysis NH-5.2	NH-5.4	NH-5.17
Father	2:1	−1	53	68
	1:1	0	50	63
Mother	2:1	40	−1	2
	1:1	36	−3	3
S2 (third donor)	2:1	−3	50	0
	1:1	−2	48	0
S3 (second donor)	2:1	41	50	78
	1:1	34	46	78
Patient	2:1	−3	−1	0
	1:1	−1	1	1

E:T, effector:target. Clones NH-5.3, NH-5.5, and NH-5.9 showed the same pattern of killing as NH-5.2; clone NH-5.7, the same as NH-5.4; and NH-5.13, the same as NH-5.17.

whereas that of the other four was not inhibited by either mAb (data not shown).

The expression of the hmHAs on PBLs from the family of the patient (Fig. 1) was investigated. PHA-induced T cells from the father, mother, S2, and S3 were used as target cells. As shown in Table 1, these CTL clones could be divided into three groups based on their recognition patterns. Four CTL clones (NH-5.2, NH-5.3, NH-5.5, and NH-5.9) killed T cells from the mother and S3 but not those from the father and S2. Two CTL clones (NH-5.4 and NH-5.7) killed T cells from the father, S2, and S3 but not those from the mother. The remaining clones (NH-5.13 and NH-5.17) killed T cells from the father and S3 but not those from the mother and S2. These data demonstrate that the first group of four CTL clones recognizes hmHAs (hmHA-1) derived from the mother, whereas the second and third groups recognize hmHAs (hmHA-2 and hmHA-3, respectively) derived from the father.

The Recognition of hmHAs Is Restricted by HLA-B35 or HLA-B38 Molecules. To investigate whether the recognition of hmHAs by the CTL clones is restricted by HLA class I antigens, inhibition of their killing activity was examined by pretreatment of the target cells with anti-HLA class I mAbs. As shown in Fig. 2, killing activities of all CTL clones were blocked by W6/32 anti-HLA class I mAb but not by anti-HLA class II mAbs. The inhibition of NH-5.4, NH-5.7, NH-5.13, and NH-5.17 CTL clones by W6/32 mAb seemed to be weaker than that of other CTL clones. The inhibition of these CTL clones by MH40.5 anti-HLA class I mAb also showed the same pattern as W6/32 (data not shown). These

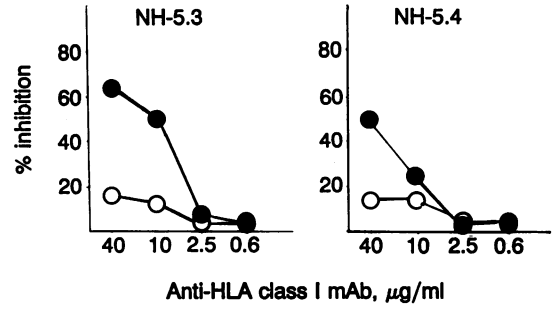

FIG. 2. Blocking of hmHA-specific CTL clones by anti-HLA class I mAbs. The specific lysis of CTL clones was inhibited by W6/32 anti-HLA class I mAb (●) or L243 anti-HLA-DR mAb (○). The percent inhibition of these CTL clones by anti-HLA-DQ mAb and anti-HLA-DP mAb was <15%. Three other hmHA-1-specific HLA-B35-restricted CTL clones showed the same blocking pattern as NH-5.3. Three other HLA-B38-restricted CTL clones showed the same blocking pattern as NH-5.4.

Table 2. Killing patterns of cells by CTL clones that recognize three hmHAs

Source of target cells	HLA type	E:T ratio	% specific lysis							
			NH-5.2	NH-5.3	NH-5.5	NH-5.9	NH-5.4	NH-5.7	NH-5.13	NH-5.17
S3	A2/A2, B35/B38, Cw3/Cw7	2:1	85	90	74	67	58	61	44	74
		1:1	79	66	55	49	38	63	29	70
Patient	A2/A2, B35/B38, Cw3/Cw7	2:1	0	−1	0	1	3	1	1	1
		1:1	1	−2	2	0	1	0	−1	0
Sug	A24/A26, B35/Bw52, Cw⁻/Cw⁻	2:1	69	67	57	63	−5	−2	−5	−5
		1:1	57	51	56	43	−6	−5	−4	−7
HVB-B5	A1/A32, B35/Bw41, Cw4/Cw⁻	2:1	9	19	12	12	−2	1	−2	0
		1:1	8	13	8	4	−1	2	1	−2
YO	A2/A24, B35/Bw61, Cw3/Cw⁻	2:1	73	77	73	75	0	1	1	−1
		1:1	77	82	72	72	0	0	1	−1
KT	A2/A11, B7/B35, Cw3/Cw7	2:1	77	80	77	77	3	3	3	−1
		1:1	77	82	76	67	4	3	4	−1
Leo 10	A26/A19, B35/B38, Cw⁻/Cw⁻	2:1	84	91	75	77	21	30	32	53
		1:1	70	76	68	67	16	19	18	44
FS	A26/A26, B38/B44, Cw⁻/Cw⁻	2:1	3	1	5	0	40	41	38	53
		1:1	2	2	1	2	41	33	24	49
CRB-087B	A2/A26, B38/B44, Cw⁻/Cw⁻	2:1	−2	3	5	1	7	8	56	81
		1:1	2	3	6	−2	5	11	41	68
JBUSH	A32/A32, B38/B38, Cw⁻Cw⁻	2:1	−3	−6	−4	4	29	47	2	1
		1:1	−5	−3	2	−1	31	48	0	−3
YK	A2/A-, B40/Bw52, Cw1/Cw3	2:1	−2	−2	−1	−1	−2	−1	−1	0
		1:1	−3	−1	−2	−2	−2	0	−1	0
ST	A2/A24, B7/Bw55, Cw3/Cw7	2:1	−1	−2	−2	−3	−2	3	−1	2
		1:1	0	−4	−3	−3	−3	1	−2	2
Tm	A11/A24, Bw52/Bw52, Cw7/Cw⁻	2:1	−1	0	0	2	1	2	1	1
		1:1	1	−1	0	0	1	3	1	2
AMAI	Aw68/Aw68, Bw53/Bw53, Cw4/Cw4	2:1	0	−2	12	−2	−1	−2	−2	6
		1:1	0	−2	−1	−3	−1	0	−2	6

E:T, effector:target.

results strongly suggest that the recognition of hmHAs by these CTL clones is restricted by HLA class I molecules.

To identify the restriction molecules, a panel of cells with different HLA phenotypes was examined (Table 2). Because the patient, S2, and S3 shared HLA-A2/A2, -B35/B38, and -Cw3/Cw7, these five HLA class I antigens are candidates for the restriction molecules. Therefore, the EBV-transformed B cells or PHA-induced T cells from individuals with these HLA class I antigens were selected as target cells. Four CTL clones (NH-5.2, NH-5.3, NH-5.5, and NH-5.9) recognizing hmHA-1 killed five groups of target cells expressing HLA-B35. On the other hand, these CTL clones did not kill the target cells expressing HLA-A2, -B38, -Cw3 or -Cw7 without HLA-B35. These data indicate that the recognition of these CTL clones is restricted by HLA-B35 molecules. Weak killing of HVB-B5 targets expressing HLA-B35 by these CTL clones was noted, suggesting that this subject might carry a variant B35 molecule.

Four other CTL clones (NH-5.4, NH-5.7, NH-5.13, and NH-5.17) killed three groups of target cells expressing HLA-B38, but they did not kill the cells without HLA-B38 (Table 2). These data indicate that HLA-B38 is the restriction molecule in the recognition of hmHA-2 and hmHA-3 by these CTL clones; rather weak blocking of the CTL activity by anti-HLA class I mAbs was observed on these CTL clones. Although both hmHA-2 and the hmHA-3 are derived from the father (Table 1), they exhibit distinct specificities; hmHA-2 is expressed by both sisters (S2 and S3), whereas hmHA-3 is expressed only by S3. As seen in Table 2, the NH-5.4 and NH-5.7 clones killed JBUSH cells but not CRB-087B cells, whereas NH-5.13 and NH-5.17 killed CRB-087B cells but not JBUSH cells. These results imply that the target specificities (hmHA-2 and hmHA-3) recognized by these CTL clones using the same B38 restriction molecule are independently distributed in the general population.

hmHA-1 Specificity Is Presented to the CTL Clones by HLA-B35 Molecules Expressed on Hmy2CIR Cells After Gene Transfer. Previous studies (14) have demonstrated that HLA-B35 molecules on the HLA-A/B null Hmy2CIR cells transfected with the B35 gene were fully recognized by corresponding alloantisera. Therefore, we expected that the

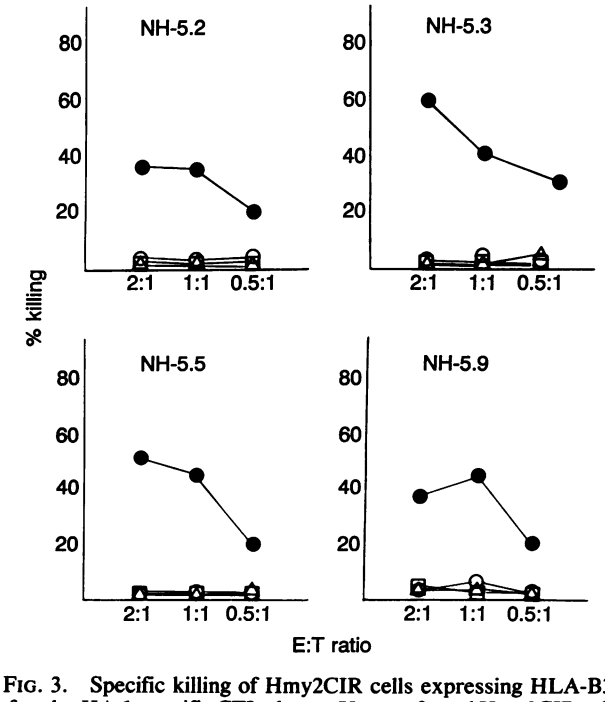

FIG. 3. Specific killing of Hmy2CIR cells expressing HLA-B35 by four hmHA-1-specific CTL clones. Untransfected Hmy2CIR cells (○) and Hmy2CIR cells expressing HLA-B35 (●), HLA-51 (△), or HLA-Bw52 (□) were used as target cells. E:T, effector:target.

hmHA-1-specific CTL clones would kill Hmy2CIR cells expressing HLA-B35 if hmHA-1 antigens were on Hmy2CIR cells. As shown in Fig. 3, all four CTL clones recognizing hmHA-1 killed HLA-B35-transfected Hmy2CIR cells but not HLA-B51 or -Bw52 transfected and untransfected Hmy2CIR cells. These data showed clearly that the hmHA-1 was presented to these CTL clones by HLA-B35 molecules on the transfectants.

As shown in Fig. 4, Hmy2CIR cells expressing HLA-Bw53 were not killed by hmHA-1-specific CTL clones. Since only five amino acid substitutions (positions 77, 80, 81, 82, and 83) associated with HLA-Bw4/Bw6 epitopes were observed between HLA-B35 and -Bw53 (unpublished data), these five substitutions are definitely involved in the binding between HLA-B35 and hmHA-1 antigens.

The HLA-B35/51 chimeric gene containing the α_1 domain of B35 and the other domains of B51 was previously generated and transfected into Hmy2CIR cells (16). Hmy2CIR cells expressing this chimeric antigen were not killed by the hmHA-1-specific CTL clones (Fig. 4). HLA-B35 and B35/51 chimeric antigens differ by eight amino acid substitutions (positions 94, 95, 97, 103, 114, 116, 152, and 171) on the α_2 domain (14, 16). Therefore, these data indicate that amino acid substitutions at the eight positions of the α_2 domain also affect the binding of hmHA-1 to HLA-B35 antigens.

DISCUSSION

It has been suspected for many years that rejection of some HLA-identical grafts and graft vs. host reaction following bone marrow grafts from HLA-identical siblings are due to disparity of hmHAs. Thus the biological importance of hmHAs in the field of clinical transplantation is well established. Avoidance of incompatibility at such hmHAs would improve survival and function of long-term organ grafts and prevent the catastrophic acute graft vs. host disease seen in some recipients of HLA-identical bone marrow grafts. However, previous studies on hmHAs have been hampered by the lack of alloantibodies and have been limited to those recognized by CTLs in the unfractionated PBLs (3, 6), so-called CTL cell lines (2, 3, 7, 13), or putative "CTL clones" with low lytic activities (8). Therefore, establishment of hmHA-specific CTL clones and delineation of their specificities as well as restriction elements by using such clones have been needed for the advancement of clinical transplantation.

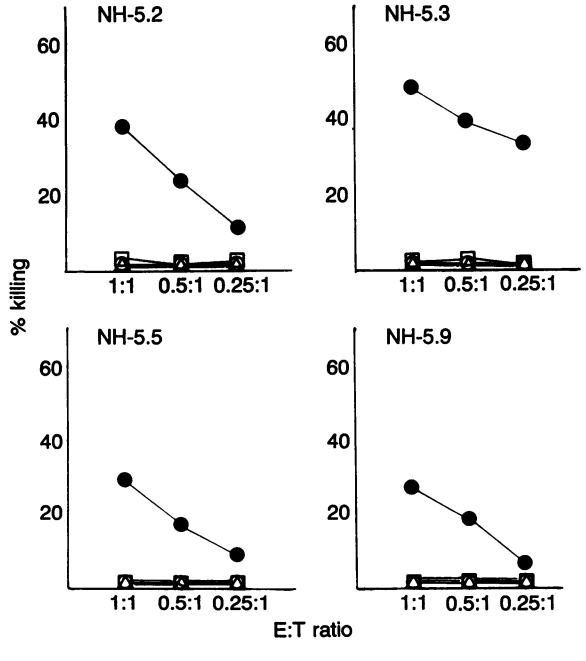

FIG. 4. Failure of killing the Hmy2CIR cells expressing HLA-B35/51 or HLA-Bw53 by the hmHA-1-specific CTL clones. Untransfected Hmy2CIR cells (○) and Hmy2CIR cells expressing HLA-B35 (●), HLA-B35/51 (△), or HLA-Bw53 (□) were used as target cells. E:T, effector:target.

In the present study, we have succeeded in generating hmHA-specific CTL clones with respectable and reproducible lytic activities (see Tables 1 and 2). Studies of the lytic activities of eight CTL clones against target cells from family members demonstrated that they recognize three distinct hmHA specificities. This was also supported by the three distinct killing patterns against a panel of cells (Table 2).

HLA-A2 and -B7 have been claimed to be the most frequent restriction molecules, and therefore it was proposed that only a very limited number of HLA class I molecules are used in the hmHA recognition (1). The CTL clones under investigation selected HLA-B35 and -B38 rather than other possible candidates, notably HLA-A2. This would indicate that restriction molecules for hmHA recognition are not necessarily limited to particular HLA specificities such as HLA-A2 and -B7. It may well be that an HLA allospecificity is preferentially selected in the recognition of each hmHA. Further studies are needed to delineate the relationship between each hmHA and its restricting allospecificity of HLA class I molecules.

Linkage to HLA of the hmHAs under investigation is very unlikely. First of all, the hmHA-specific CTLs were generated in response to the genotypically HLA-identical cells, and all these antigens segregated independently from HLA in the family. Furthermore, the haplotype A2-B35-Cw3 of the stimulator cells was found in only two of five hmHA-1-positive cells and the other haplotype, A2-B8-Cw7, was not seen at all in any of hmHA-2- or hmHA-3-positive cells.

There has been little information available on the polymorphism of mHAs in both man and mouse. Several studies (23, 24) indicated that the polymorphism of murine minor histocompatibility antigens is very limited. In the present study, the killing patterns of a panel of cells by the eight CTL clones revealed that hmHA-1 was expressed on five out of five cells expressing HLA-B35, and hmHA-2 and hmHA-3 were expressed on three out of four cells with HLA-B38. These results indicate that the polymorphism of the hmHAs under study is also limited.

Recent studies (25) demonstrated that viral antigens are directly bound to HLA class I molecules and are presented to specific CTLs. Moreover, other studies (26–28) using mutant HLA class I genes and virus-specific CTLs demonstrated the interaction between virus peptides and the allodeterminants of HLA class I molecules. However, the interaction between HLA class I molecules and hmHAs remains unknown, although the recognition of hmHAs was shown to be restricted by HLA class I molecules (2, 4, 7, 8). In this study, we demonstrated directly that four hmHA-1-specific CTL clones were restricted by HLA-B35 molecules on Hmy2CIR cells transfected with HLA-B35. This provided an approach to investigate the interaction between hmHAs and HLA class I molecules.

Interestingly, none of four hmHA-1-specific CTL clones killed Hmy2CIR cells expressing HLA-Bw53. Since only five amino acid substitutions (positions 77, 80, 81, 82, and 83) associated with HLA-Bw4/Bw6 epitopes were observed between HLA-B35 and -Bw53 (unpublished results), it may be concluded that these substitutions are involved in the recognition of hmHA-1 by the CTL clones. On the other hand, two of three HLA-B35 allospecific CTL clones can recognize HLA-Bw53 (unpublished results), suggesting that the differences of HLA-Bw4/Bw6 epitopes may be more critical for the restriction of the hmHA recognition by the CTL clones than recognition of HLA-B35 by allospecific CTLs.

Eight amino acid substitutions (positions 94, 95, 97, 103, 114, 116, 152, and 171) on the α_2 domain can also affect the recognition of hmHA-1 by the HLA-B35-restricted CTL clones, since these CTL clones cannot kill hmHA-1-positive Hmy2CIR cells expressing the HLA-B35/51 chimeric antigens. Six substitutions (positions 94, 95, 97, 103, 114, and 116) are on the β-sheet, while two substitutions (positions 152 and 171) are on the α-helix. Previous studies (28) demonstrated that certain amino acid substitutions on the α-helix of class I molecules can affect presentation of the influenza virus matrix peptides. On the other hand, recent studies (29) demonstrated that recognition by A2.1-restricted influenza peptide-specific CTL was totally eliminated by a single substitution at position 9 on the β-sheet. Thus, as the residues on both the α-helix and the β-sheet can affect presentation of the influenza peptides, any of the eight substitutions may affect the recognition by the HLA-B35-restricted hmHA-1-specific CTL.

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