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Identification of Cyclophilin B-derived Peptides Capable of Inducing Histocompatibility Leukocyte Antigen-A2-restricted and Tumor-specific Cytotoxic T Lymphocytes

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We recently suggested that cyclophilin B (Cyp-B) is a tumor antigen recognized by histocompatibility leukocyte antigen (HLA)-A24-restricted and tumor-specific cytotoxic T lymphocytes (CTLs). In this study, we tried to identify Cyp-B-derived epitopes, which can induce HLA-A2-restricted and tumor-specific CTLs in cancer patients. The tumor-infiltrating lymphocytes (TILs) from an HLA-A0207 patient with colon cancer were found to respond to COS-7 cells when co-transfected with the *Cyp-B* gene and either *HLA-A0201*, *-A0206*, or *-A0207* cDNA. These TILs contained CTLs capable of recognizing either the Cyp-$B_{129–138}$ or the Cyp-$B_{172–179}$ peptide among 28 different peptides, all of which were prepared based on the HLA-A2 binding motif. Both Cyp-B peptides possessed the ability to induce tumor-specific CTLs in HLA-$A2^+$ cancer patients. Cyp-$B_{172–180\ (V)}$, which is a 9-mer peptide with valine added at the C terminus, showed no clear superiority over the parental Cyp-$B_{172–179}$ peptide in an *in vitro* sensitization experiment. *In vitro*-sensitized T cells with these peptides responded to cancer cells in an HLA-A2-restricted manner. These two Cyp-B peptides could be useful for specific immunotherapy of HLA-$A2^+$ cancer patients.

Key words: Cyclophilin B — Cytotoxic T lymphocytes — Peptide — HLA-A2

Many peptide antigens capable of inducing histocompatibility leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocytes (CTLs) have been identified from melanoma cDNA.[1)] Specific immunotherapy utilizing these peptides has been under investigation for treatment of HLA-$A1^+$ or -$A2^+$ metastatic melanoma patients, and has resulted in partial clinical responses in the initial trials.[2, 3)] However, there are few peptides useful for specific immunotherapy for patients with adenocarcinoma or squamous cell carcinomas, histologically the two major malignancies in the world.[4)] We have found several genes coding antigenic peptides of these cancers capable of inducing HLA class I-restricted CTLs in the peripheral blood mononuclear cells (PBMCs).[4–8)] One such antigen, cyclophilin B (Cyp-B), was identified using HLA-A24-restricted and tumor-specific CTLs established from a patient with lung adenocarcinoma.[5)] Although the HLA-A24 allele is most commonly expressed in Japanese (60%),[9, 10)] HLA-A2 is also expressed in Japanese at a relatively high frequency (40%), and appears in other ethnic populations (e.g., with an estimated frequency of 50% in Caucasians).[11)] In this study, we investigated whether the Cyp-B antigen possesses antigenic peptides available for HLA-$A2^+$ cancer patients as cancer vaccines, and we present evidence that the Cyp-B antigen contains immunogenic epitopes capable of inducing HLA-A2-restricted and tumor-specific response in HLA-$A2^+$ cancer patients.

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MATERIALS AND METHODS

Generation of HLA-A2-restricted CTLs The HLA-A2-restricted and tumor-specific CTL (OK-CTL) lines were established from tumor-infiltrating lymphocytes (TILs) of a patient (HLA-A0207/31, -B46/51, -Cw1) with colon adenocarcinoma by incubation with interleukin-2 (IL-2, 100 U/ml) alone for more than 50 days, by the method reported previously.[12)] The anti-tumor specificity of these CTL lines was previously reported.[13)]

Typing of HLA-class I alleles Genotypes of HLA-class I alleles of the tumor cells were reported previously.[14)] HLA-class I alleles of PBMCs were serotyped by the conventional method, whereas HLA-A2 subtypes were determined by a sequence-specific oligonucleotide probe method and direct DNA sequencing, as previously reported.[4)]

***In vitro* transfection** To determine whether the *Cyp-B* gene encodes antigenic epitopes recognized by the OK-CTLs, both the *Cyp-B* gene and one of the HLA-class I cDNAs (*HLA-A0201*, *-A0206*, *-A0207*, *-A2402*, or *-A2601*) were co-transfected into COS-7 cells followed by a test of their activity to stimulate interferon (IFN)-γ production by the OK-CTLs by methods reported previously.[4)]

IFN-γ ELISA To determine the level of IFN-γ, both anti-human IFN-γ coating monoclonal antibody (mAb) and anti-human IFN-γ rabbit polyclonal antibody, which was prepared in our laboratory and used as a detecting antibody, were used. Thereafter, DAKO EnVision^{+TM} Peroxidase-conjugated anti-rabbit antibody (DAKO Corp., Carpinteria, CA) was used. The development was carried

out using the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD). After stopping the enzyme reaction with 1 *M* H_3PO_4, absorbance at 450 nm was determined with an ELISA spectrophotometer.

Peptides The Cyp B-derived peptides capable of binding to the HLA-A2 molecules were determined based on the HLA-A2-binding motifs,[15, 16] and 28 different peptides were synthesized. The peptides (>95% purity) were kindly provided by Dr. Takasu (Research Division of Sumitomo Pharmaceutical Co., Osaka). To determine which Cyp-B peptides could be recognized by the OK-CTLs, prepared peptides were loaded onto T2 cells at a concentration of 10 μM for 2 h. Thereafter, the culture supernatants were harvested and the level of IFN-γ was determined by ELISA.

CTL induction by the peptides For induction of tumor-specific CTLs by the peptides, PBMCs from 5 HLA-$A2^+$ patients with adenocarcinona were incubated with a peptide (10 μM). These PBMCs were re-stimulated at days 7, 14, and 21 with irradiated (30 Gray) autologous PBMCs, as antigen-presenting cells, that had been pulsed with the same peptide at the same dose for 2 h. These cultured cells at day 27 were tested for their tumor-specific IFN-γ production and cytotoxicity by a standard 6 h ^{51}Cr-release assay. The assay of cytotoxicity was performed, as previously reported.[4] The surface phenotypes of the cultured cells were determined by indirect staining with anti-CD8 mAb, followed by staining with FITC-conjugated goat anti-mouse IgG antibody. For inhibition of IFN-γ production by the cultured cells, 10 μg/ml of anti-class I (W6/32, mIgG2a), anti-class II (H-DR1, mIgG2a), anti-CD4 (Nu-Th/I, mIgG1), anti-CD8 (Nu-Ts/c, mIgG2a), anti-A2 (BB7.2, mIgG2b), and anti-A23/24 (DU41HA, mIgG2a) as anti-HLA-A24 mAb antibodies were used as reported previously.[5]

RESULTS

Recognition of the Cyp-B-derived antigen by HLA-A2-restricted OK-CTLs Several HLA-A2-restricted and tumor-specific CTL lines were established from the TILs of a patient (HLA-A0207/31, -B46/51, -Cw1) with colon adenocarcinoma by incubation with 100 U/ml IL-2. These CTL lines showed a response to several types of cancer cells expressing HLA-A2 molecules, as previously reported.[13] One (OK-CTL) of these CTL lines was utilized to determine which Cyp-B peptides could induce HLA-A2-restricted and tumor-specific CTLs in cancer patients. As shown in Fig. 1A, the OK-CTL cells produced a higher level of IFN-γ when the COS-7 cells were co-transfected with both *Cyp-B* and *HLA-A0207* cDNAs than when co-transfected with both control cDNA and *HLA-A0207* cDNA. The OK-CTL cells failed to produce IFN-γ when the COS-7 cells were co-transfected with both *Cyp-B* and *HLA-A2402* cDNAs. These results indicate that the OK-CTL line contains T cells recognizing Cyp-B-derived antigens in an HLA-A0207-restricted manner.

HLA-A2 subtypes share similar binding motifs.[17, 18] For example, HLA-A0201 and -A0207 have a dominant anchor leucine at position 2, and HLA-A0201 shares dominant and strong anchors leucine and valine at position 9 with -A0206 and -A0207, respectively. In addition, these OK-CTL lines possess HLA-A2-restricted and tumor-specific CTL activity toward antigenic epitope(s) expressed on HLA-A0201, -A0206, -A0207 molecules of epithelial cancer cells.[13] Therefore, we next determined whether the OK-CTL line could recognize Cyp-B-derived antigens in association with HLA-A0201, -A0206, or -A0207 molecules. Fig. 1B shows that the OK-CTL line responded to the COS-7 cells when transfected with *Cyp-B* cDNA in

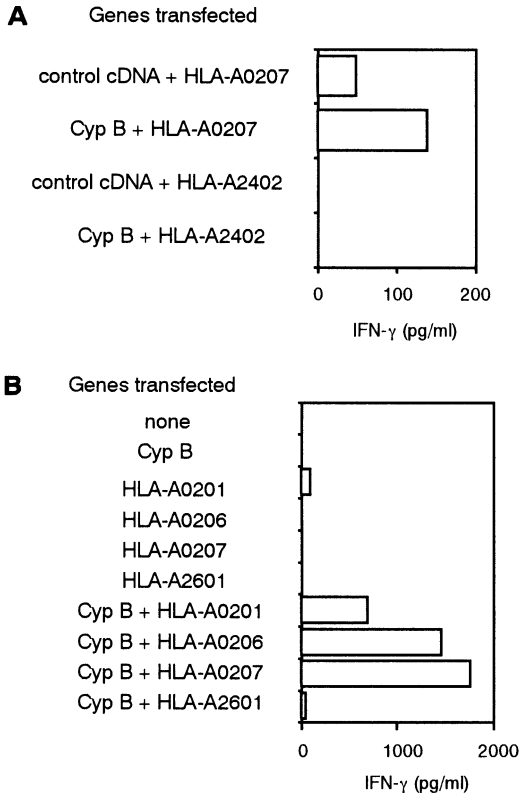

Fig. 1. Recognition of the Cyp-B-derived antigens by the OK-CTLs. (A) COS-7 cells were co-transfected with either the *Cyp-B* gene or the control cDNA, and with either *HLA-A0207* or *HLA-A2402* cDNA. (B) COS-7 cells were co-transfected with either or both the *Cyp-B* gene, *HLA-A0201*, *-A0206*, *-A0207*, or *-A2601* cDNA. These transfected COS-7 cells were cultured with the OK-CTL cells for 24 h. Thereafter, the supernatant was harvested and the level of IFN-γ was determined by ELISA. Values represent the means of the triplicate determinations.

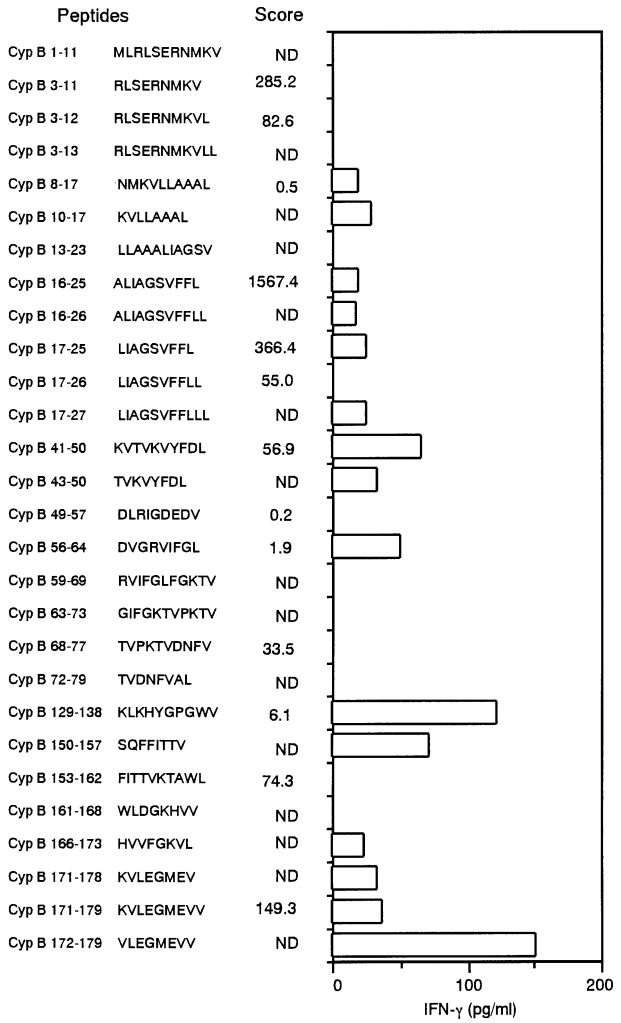

Fig. 2. Recognition of Cyp-B peptides by the OK-CTLs. Twenty-eight different Cyp-B-derived peptides were loaded onto T2 cells at a concentration of 10 μM for 2 h. After having been washed with the culture medium, the OK-CTLs were then added at an E/T ratio of 10/1, and incubation was continued for 18 h. Thereafter, the culture supernatants were harvested and the level of IFN-γ was determined by ELISA. Values indicate the means of the triplicate determinations. The background of IFN-γ production (192 pg/ml) by the OK-CTLs in the presence of unpulsed T2 cells was subtracted from the values in the figure. Score represents an estimated half-time of disassociation of the Cyp-B peptide binding to HLA-A2 molecules (Ref. 15). ND: not determined.

combination with either *HLA-A0201*, *-A0206*, or *-A0207* cDNA. On the other hand, co-transfection with *HLA-A2601* cDNA resulted in no production of IFN-γ. These results indicate that the OK-CTLs contain T cells capable of recognizing Cyp-B-derived antigens in the context of three HLA-A2 subtypes.

Next, the 28 Cyp-B peptide candidates were prepared, based on the HLA-A2-binding motif,[15, 16] and we investigated whether these peptides could be recognized by the OK-CTLs (Fig. 2). Although 8-mer and 11-mer peptides were not common as peptides binding to class I molecules and it was impossible to determine the score (an estimated half-time of disassociation of the peptide binding to HLA-A2 molecules) by computer search, these were also included. Among them, two Cyp-B peptides at positions 129–138 and 172–179 were found to be recognized by the OK-CTLs. The score of the Cyp-$B_{129-138}$ peptide was 6.1 (low), but that of the Cyp-$B_{172-179}$ peptide was undetermined (it was an 8-mer peptide). Because the Cyp-$B_{172-179}$ peptide was an 8-mer and because the optimal C-terminal anchor residue for HLA-A2 binding peptides has been shown to be valine,[15, 16] a modified Cyp-B peptide, which had valine added at position 180 and was designated as Cyp-$B_{172-180\ (V)}$, was prepared. Although the score of the modified peptide was 3.1 (low), this peptide was also tested in the following experiments.

Induction of tumor-specific CTLs by peptides These Cyp-B peptides were examined for the ability to induce tumor-specific CTLs from 5 cancer patients (Table I). The PBMCs of patients #1 and #2 were repeatedly stimulated with the Cyp-$B_{129-138}$, Cyp-$B_{172-179}$, and Cyp-$B_{172-180\ (V)}$ peptides, respectively, and the *in vitro*-sensitized PBMCs were examined for IFN-γ production in response to HLA-$A2^+$ or HLA-$A2^-$ tumor cell lines. Sensitized cells stimulated with any of the three peptides produced a higher level of IFN-γ in response to the HLA-$A2^+$ tumor cell lines (Panc-1, SW620 and CA9-22) than to the HLA-$A2^-$ tumor cell lines (QG-56, RERF-LC-MS and colo-320). PBMCs of patients #3, from whom the OK-CTLs were established, and patients #4 and #5 were repeatedly stimulated with either the Cyp-$B_{129-138}$ or Cyp-$B_{172-179}$ peptide, respectively, and these sensitized PBMCs were also examined for IFN-γ production. In these cases, although the difference was not as apparent as that in the cases of patients #1 and #2, the sensitized cells also produced a higher level of IFN-γ in response to the HLA-$A2^+$ tumor cell lines than to the HLA-$A2^-$ tumor cell lines. These results suggest that these three Cyp-B peptides could be useful for specific immunotherapy.

Next, the cytotoxicity of these *in vitro*-sensitized PBMCs was examined (Fig. 3). In patient #1, the *in vitro*-sensitized PBMCs with the Cyp-$B_{172-179}$ peptide showed a higher cytotoxicity to HLA-$A2^+$ tumor cell lines (Panc-1 and 1-87) than to HLA-$A2^-$ tumor cell lines (QG-56 and RERF-LC-MS). However, no difference in cytotoxicity was observed when PBMCs were *in vitro*-sensitized with either the Cyp-$B_{129-138}$ or the Cyp-$B_{172-180\ (V)}$ peptide (data not shown). On the other hand, in patient #3, the *in vitro*-sensitized PBMCs with the Cyp-$B_{129-138}$ peptide showed a higher cytotoxicity to HLA-$A2^+$ SW620 than to other

tumor cell lines. In patient #4, the *in vitro*-sensitized PBMCs with the Cyp-$B_{129-138}$ peptide showed a higher cytotoxicity to HLA-$A2^+$ tumor cell lines (Panc-1 and SW620) than to HLA-$A2^-$ tumor cell lines (QG-56 and RERF-LC-MS). However, no difference in cytotoxicity was observed when PBMCs from patients #3 and #4 were *in vitro*-sensitized with either the Cyp-$B_{172-179}$ or the Cyp-$B_{172-180\ (V)}$ peptide, respectively (data not shown). Overall, although peptides to induce tumor-reactive CTLs with cytotoxicity varied among cancer patients, these results suggest that both Cyp-$B_{129-138}$ and Cyp-$B_{172-179}$ peptides appear to be effective for specific immunotherapy of HLA-$A2^+$ cancer patients, and that the Cyp-$B_{172-180\ (V)}$ peptide was not necessarily superior to the parental Cyp-$B_{172-179}$ peptide.

HLA-A2 restriction of the peptide-induced CTLs Finally, we tried to confirm HLA-A2 restriction of the CTLs that had been *in vitro*-sensitized by the Cyp-$B_{129-138}$ and the Cyp-$B_{172-179}$ peptides. PBMCs from patients #5 and #3 were repeatedly stimulated with these peptides. As

Table I. Induction of HLA-A2-restricted CTLs by *in vitro* Sensitization with Cyp-B Peptides

Donors	Disease/stage	Peptides	CD8 (%)	Stimulator cells					
				Panc-1 (A0201/1101)	SW-620 (A0201/2402)	CA9-22 (A0207/2402)	QG-56 (A2601/)	RERF-LC-MS (A1101/)	colo-320 (A2402/)
Patient #1	Lung cancer/$T_4N_2M_0$	Cyp-$B_{129-138}$	45.8	122	257	99	0	0	0
(A*0206/−)	(adenocarcinoma)	Cyp-$B_{172-179}$	64.5	128	1000	173	0	0	0
		Cyp-$B_{172-180\ (V)}$	70.5	80	3584	288	21	34	0
Patient #2	Lung cancer/$T_2N_0M_0$	Cyp-$B_{129-138}$	33.8	1711	576	371	286	113	128
(A*0206/−)	(adenocarcinoma)	Cyp-$B_{172-179}$	62.6	775	512	326	199	149	14
		Cyp-$B_{172-180\ (V)}$	48.9	1636	2000	932	737	176	0
Patient #3	Colon cancer/$T_2N_1M_0$	Cyp-$B_{129-138}$	ND	286	152	ND	0	0	ND
(A*0207/31)	(adenocarcinoma)	Cyp-$B_{172-179}$	ND	573	504	ND	313	153	ND
Patient #4	Gastric cancer/$T_1N_0M_0$	Cyp-$B_{129-138}$	ND	167	458	ND	150	0	ND
($A2^+$)	(adenocarcinoma)	Cyp-$B_{172-179}$	ND	724	521	ND	292	32	ND
Patient #5	Gastric cancer/$T_2N_1M_0$	Cyp-$B_{129-138}$	ND	272	386	ND	133	61	ND
($A2^+$)	(adenocarcinoma)	Cyp-$B_{172-179}$	ND	139	145	ND	94	67	ND

PBMCs from five HLA-$A2^+$ patients with adenocarcinoma were stimulated 4 times with the indicated peptides, as described in "Materials and Methods." On day 27, the cultured cells were harvested and cultured with the indicated tumor cell lines at an E/T ratio of 5/1 for 20 h. The level of IFN-γ in the culture supernatant was determined by ELISA. Values represent the means of triplicate determinants. The background production of IFN-γ by the effector cell alone was subtracted from the experimental values. Percentage of $CD8^+$ T cells in the cultured cells was examined by flow cytometry. These 5 cases were classified according to the TNM classification of UICC. ND: not determined.

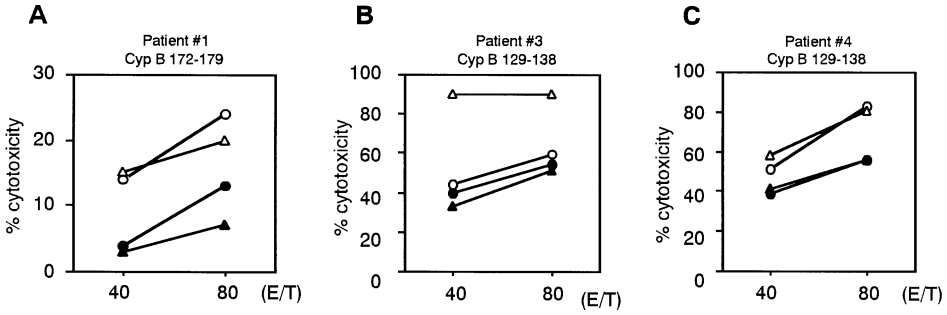

Fig. 3. Induction of CTLs by the peptides. PBMCs from 3 cancer patients were repeatedly stimulated *in vitro* with the Cyp-$B_{129-138}$, Cyp$B_{172-179}$, and Cyp-$B_{172-180\ (V)}$ peptides, respectively, as described in "Materials and Methods." Thereafter, their respective cytotoxic activity against HLA-$A2^+$ and HLA-$A2^-$ tumor cell lines was determined by a 6 h ^{51}Cr-release assay. Values represent the means of triplicate determinations. The following target cell lines were used; (A) ○ Panc-1 (A0201/1101), △ 1-87 (A0207/1101), ● QG-56 (A2601/), and ▲ RERF-LC-MS (A1101/). (B and C) ○ Panc-1 (A0201/1101), △ SW620 (A0201/2402), ● QG-56 (A2601/), and ▲ RERF-LC-MS (A1101/).

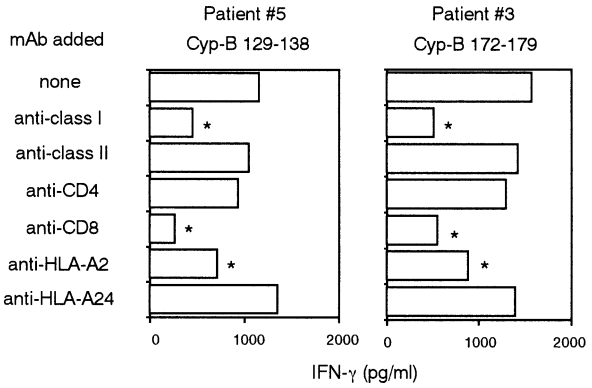

Fig. 4. HLA-A2 restriction of the CTLs induced by the Cyp-B peptides. PBMCs from two HLA-A2$^+$ cancer patients were stimulated weekly *in vitro* with the Cyp-$B_{129-138}$ and the Cyp-$B_{172-179}$ peptide (10 μM), respectively, 4 times, followed by a test for the ability to produce IFN-γ in response to Panc-1 cells (A0201/1101) at an E/T ratio of 5/1. The mAbs were added at the initiation of the culture. Values represent the means of triplicate determinations. The background IFN-γ production by the effector cells alone has been subtracted from the experimental values.

shown in Fig. 4, both types of *in vitro*-sensitized cells produced a significant level of IFN-γ in response to Panc-1, an HLA-A2-expressing adenocarcinoma cell line. IFN-γ production was significantly diminished by the addition of anti-class I, anti-CD8, or anti-HLA-A2 antibodies. On the other hand, none of the anti-class II, anti-CD4, or anti-HLA-A24 antibodies showed any inhibitory effect on IFN-γ production. These results indicate that the Cyp-B peptide-induced CTLs can show a response to HLA-A2-expressing tumor cells in an HLA-A2-restricted manner.

DISCUSSION

Cyp-B is a self antigen that plays an important role in protein folding through its peptidyl-prolyl isomerase activity.[19, 20] Cyp-B transmits a signal from T-cell receptors to calnecium in conjunction with calcium-modulating Cyp-B binding protein, which in turn is involved in regulation of intracellular calcium for lymphocyte activation.[21] The region of Cyp-B from positions 85 to 100 is important in terms of biological activity of signal transmission, and is highly conserved in other members of the cyclophilins.[20] In addition, this region contains the sequence critical for binding to cyclosporin A.[22] Interestingly, both the Cyp-B_{84-92} and the Cyp-B_{91-99} peptides, which can be recognized by HLA-A24-restricted and tumor-specific CTLs, are located in this region,[5] whereas the Cyp-$B_{129-138}$ and Cyp-$B_{172-179}$ peptides, which were revealed to be capable of inducing HLA-A2-restricted and tumor-specific CTLs, are not. It is presently unclear whether this region is an important site relative to the composition of antigenic epitopes. Identification of additional Cyp-B epitopes in the context of other HLA-class I alleles is needed to determine this.

In this study, we identified two Cyp-B-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients. In screening by IFN-γ, both Cyp-B peptides possessed the ability to induce HLA-A2-restricted and tumor-reactive CTLs in all 5 HLA-A2$^+$ cancer patients (Table I), although not all peptide-induced CTLs showed cytotoxicity against HLA-A2-expressing tumor cells, as shown in Fig. 3. These results suggest that tumor-specific CTLs, which could recognize Cyp-B peptides in association with HLA-A2 molecules and subsequently produce IFN-γ, could not necessarily lyse the tumor cells. Each tumor cell line might have a unique machinery by which to show resistance to CTL-mediated cytolysis. Alternatively, cytokine production by CTLs might not necessarily be associated with their cytolytic activity. We can not explain the discrepancy. In addition, in the cytotoxicity assay, the ability to generate T cells with cytotoxicity against HLA-A2-expressing tumor cells varied among the peptides. These findings may imply that CTL precursor frequency varies among cancer patients, and that confirmation of the presence of CTL precursors prior to immunotherapy would greatly improve the efficacy of a subsequent vaccination with the relevant peptides.

There are several major subtypes of HLA-A2 alleles.[17] The frequencies of HLA-A0201, -A0206, and -A0207 among HLA-A2$^+$ Japanese are about 45%, 36%, and 17%, respectively, whereas HLA-A0201 is the predominant subtype among HLA-A2$^+$ Western Caucasians (96%), African Blacks (62%), and Sardinian Caucasians (56%). Interestingly, the OK-CTLs (HLA-A0207) not only recognized the two Cyp-B peptides presented on T2 cells (HLA-A0201), but also responded to HLA-A0201-expressing tumor cell lines.[13] In addition, as shown in this study, the *in vitro*-sensitized PBMCs from HLA-A0206 or -A0207 cancer patients showed a response to HLA-A0201-expressing tumor cell lines. These results suggest that the Cyp-$B_{129-138}$ and Cyp-$B_{172-179}$ peptides would be appropriate for use in specific immunotherapy of a vast majority of cancer patients with different HLA-A2 subtypes.

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