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Identification of Natural Antigenic Peptides of a Human Gastric Signet Ring Cell Carcinoma Recognized by HLA-A31-Restricted Cytotoxic T Lymphocytes

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Peptides of human melanomas recognized by CD8⁺ CTLs have been identified, but the nature of those of nonmelanoma tumors remains to be elucidated. Previously, we established a gastric signet ring cell carcinoma HST-2 and HLA-A31 (A*31012)-restricted autologous CTL clone, TcHST-2. In the present study, we determined the natural antigenic peptides of HST-2 cells. The purified preparation of acid-extracted Ags was submitted to the peptide sequencer, and one peptide, designated F4.2 (Tyr-Ser-Trp-Met-Asp-Ile-Ser-Cys-Trp-Ile), appeared to be immunogenic. To confirm the antigenicity of F4.2 further, we constructed an expression minigene vector (pF4.2ss) coding adenovirus E3, a 19-kDa protein signal sequence plus F4.2. An introduction of pF4.2ss minigene into HST-2 and HLA-A31(+)-alloplastic tumor cells clearly enhanced and induced the TcHST-2 reactivity, respectively. Furthermore, when synthetic peptides of F4.2 C-terminal-deleted peptides were pulsed to HST-2 cells, F4.2-2 (nonamers), but not F4.2-8 or F4.2-7 (octamer or heptamer, respectively), enhanced the reactivity of TcHST-2, suggesting that the N-terminal ninth Trp might be a T cell epitope. This was confirmed by lack of antigenicity when using synthetic substituted peptides as well as minigenes coding F4.2 variant peptides with Ala or Arg at the ninth position of F4.2. Meanwhile, it was indicated that the sixth position Ile was critically important for the binding to HLA-A31 molecules. Thus, our data indicate that F4.2 may work as an HLA-A31-restricted natural antigenic peptide recognized by CTLs. The Journal of Immunology, 1999, 163: 2783–2791.

A n increasing amount of evidence has indicated that a patient’s immune system can respond against his or her own neoplastic cells (1–5). This is particularly true for melanoma patients. CD8⁺ CTLs from these patients can recognize MHC class I-bound tumor antigenic peptides (6–14). There is the possibility that the elucidation of human tumor Ags may directly lead to a drastic improvement in tumor immunotherapy and establish a new modality of cancer therapy. Indeed, it is very interesting that therapeutic attempts using the antigenic peptides of these tumor Ags frequently resulted in the regression of primary melanoma tumors as well as metastatic tumor foci (15–18).

Materials and Methods

CTls and human tumor lines

The procedure for establishing CTL against tumor lines was previously described (21). In the current investigation, we used a gastric signet ring cell carcinoma line, HST-2, and CD8⁺ CTL clone, TcHST-2 (HLA haplotype: HLA-A2, A31, B38, B54, C1, C7, DR4,1, DRw53, DQ3, DQ4).
TCCHST-2 is specifically cytotoxic to autologous HST-2 cells in the context of HLA-A31 restriction, because this cytotoxicity was completely blocked by anti-HLA-A31-specific mAb as previously reported (22). iL-2 was kindly provided by Takeda Pharmaceutical (Osaka, Japan) and Shionogi Pharmaceutical (Osaka, Japan). We also employed HLA-A31(-) allogeneic lines, such as M-EB (EB virus-transformed B cell line), CIR (B cell line), MKN28 (gastric tumor line), and HOBC8 (HeLa cells transfected with SV40 large T). HOBC8 was kindly provided by Dr. P. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium).

HLA-A31 DNA typing of HST-2 cells, and introduction of HLA-A31 gene to allogeneic tumor cells

A cDNA library of HST-2 cells was made by a poly(A) Tract mRNA purification kit (Promega, Madison, WI) and cDNA synthesis kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s manuals. PCR was employed for HLA-A typing of HST-2 cells by using HLA-A-specific primer set including SalI site and HindIII site (forward primer, 5' -GGG CCTG CGA CCC CAG ACG CCG AGG ATG GCC-3'; backwarsd primer, 5' -CGG CAA GCT TTT GGG GAC GGA GCA CAG GTG GGA AG-3'). The reagents and condition of the PCR were the same as previously described except that we used Vent DNA polymerase (New England Biolabs, Beverly, MA) (25). The 1.3-kb PCR products were inserted into the T-vector derived from pBluescript SK+ and 20 subclones were sequenced by using M13 primers and other HLA primers (ABC25F, ABC35F, and unpublished sequence data), which were kindly provided by Dr. M. Takiguchi (Kumamoto University School of Medicine, Kumamoto, Japan). The sequence data was identical with that of HLA-A*31012 (26).

For the expression of HLA-A*31012 cDNA in allogeneic tumor lines, the 1.3-kb full-length HLA-A*31012 cDNA was inserted into the Xhol/Xhol site of the pBl mammalian expression vector containing neo resistance (27). Several tumor lines (M-EB, CIR, MKN28, and HOBC8) were transfected with pBJ-A*31012 by the electroporation method (Gene pulser II; Bio-Rad, Richmond, CA) or Lipofectin Reagent (Life Technologies, Gaithersburg, MD), and selected with 100 – 400 μg/ml of G418 (Life Technologies) containing medium. Cells were subjected to a single cell cloning, and MKN28-A31-2 and HOBC8-A31-12 clones were obtained. All of these clones were positive for the expression of HLA-A31 molecules on the cell surface as assessed by FACScan with anti-HLA-A31-specific mAb, 2D12, as described below.

Establishment of anti-HLA-A31-specific mAb

BALB/c mice were immunized i.p. with CIR-A*31012 cells at weekly interval for 4 wk, and hybridoma cells were established as previously described (28). A screening of mAb production was done with a positive reaction to CIR-A*31012, but negative to CIR and CIR-A*3303 cells, CIR-A*31012 (27). Several tumor lines (M-EB, CIR, and HOBC8) were transfected with pBJ-A*31012 by the electrotransfection method (Gene pulser II; Bio-Rad, Richmond, CA) or Lipofectin Reagent (Life Technologies, Gaithersburg, MD), and selected with 100 – 400 μg/ml of G418 (Life Technologies) containing medium. Cells were subjected to a single cell cloning, and MKN28-A31-2 and HOBC8-A31-12 clones were obtained. All of these clones were positive for the expression of HLA-A31 molecules on the cell surface as assessed by FACScan with anti-HLA-A31-specific mAb, 2D12, as described above.

Acid extraction and separation of the antigenic peptides, amino acid sequencing, and synthesis of the antigenic peptides

The isolation procedure for the natural antigenic peptides of cells was previously described by Falk et al. (29) and York and Rock (30). Approx. 5 × 10⁶ cells of tumor lines were homogenized in vitro cultivation with RPMI 1640 medium plus 10–15% FCS in the presence of 50 U/ml of IFN-γ (Chugai Pharmaceutical, Tokyo, Japan). Cells were washed with PBS three times, homogenized, 0.1% trifluoroacetic acid (TFA) was added, and the mixture was incubated for 30 min at room temperature. After centrifuging at 10,000 × g, the supernatant was applied to Sephadex G-25 column (20 × 300 mm) chromatography to recover samples of less than 5 kDa in molecular size. Then, antigenic peptides were purified from these samples by reverse-phase HPLC (RP-HPLC) in three steps. In the first and the second step, these samples were loaded to the preparative C18 column (μBondasphere, 5 μ, 150 × 150 mm, Nihon Waters, Tokyo, Japan) and applied with a linear gradient of buffer A (0.1% TFA in H₂O) and B (0.1% TFA in acetonitrile) as described below; a linear gradient from 0 to 80% buffer B for 30 min and from 40 to 50% buffer B for 30 min were employed in the first and second step of RP-HPLC, respectively. In the final (third) step, samples including antigenic peptides were loaded to a ZORBAX-ODS column (4.6 × 250 mm; Rockland Technologies, Gilbertsville, PA) and applied with a linear gradient from 0 to 60% buffer B for 60 min. In all steps of RP-HPLC, the flow rate was 1 ml/min.

We determined the fraction of the first and second step RP-HPLC that contained the cytotoxicity of TCHST-2 clone against autologous HST-2 cells in a ⁵¹Cr release cytotoxicity assay. In these experiments, 3 μl of each fraction was added to a 100 μl medium/well containing 5 μCi labeled target cells, such as HST-2 and HLA-A31(-) M-EB cells, and incubated for 60 min in a CO₂ incubator. A 100-μl volume of medium containing TCHST-2 was then added and cultured for 6–10 h. We applied the antigenic fraction to the third step of RP-HPLC. The amino acid sequencing of each peak of the third step RP-HPLC was then performed by Edman’s degradation method (477A Protein Sequencer, Applied Biosystems, Foster City, CA).

Based upon these sequences, peptides were synthesized (431A Peptide Synthesizer, Applied Biosystems) and purified with a ZORBAX-ODS column. We also utilized synthetic C-terminal-deleted peptides, peptides substituted with Ala or Arg at certain positions, and HLA-A31-binding heptapeptide peptides. These peptides were purified similarly in PBS containing 1% DMSO, and were tested in cytotoxicity and TNF production bioassays by TCHST-2 CTL clone as described below.

Treatment of cells with peptides, and cytotoxicity and TNF assays

The cytotoxicity assay has been described previously (21, 22). In the current investigation, the fractions of RP-HPLC and synthetic peptides were assessed for their ability to stimulate and enhance the TCHST-2 reactivity in the cytotoxicity and/or TNF production assays. For the cytotoxicity assay, ⁵¹Cr-labeled target cells, such as HST-2 cells, were pulsed with the samples for 60 min in a CO₂ incubator, washed with PBS, and then applied with TCHST-2 at certain E:T ratios. Thereafter, we followed the procedure as previously noted (21, 22). The TNF assay used was as described by Espevik and Nissen-Meyer (31). HST-2 cells were treated with certain amounts of the peptides for 60 min in a CO₂ incubator. The culture supernatant was harvested, and its TNF production was assessed using W13 (WEHI-164 clone 13) cells as described previously (31). Briefly, W13 cells were plated in the presence of 2 μg/ml of actinomycin D and 40 mM LiCl, and the experimental supernatant was distributed onto the plate. After incubation for 20 h, viability of the W13 cells was analyzed by MTT assay. W13 was kindly provided by Dr. P. Coulie. The TNF content of the supernatant was determined from the standard TNF solution.

Minigenic construction, transfection, and assays

To express peptide Ags in endogenous form, we constructed an expression minigenic vector, pF4.2s, pcDSRα-E3, which contains an adenovirus E3, 19-kDa protein signal sequence (32) under the control of SRα promoter, was kindly gifted by Drs. E. De Plaen and P. Chomez (Ludwig Institute for Cancer Research, Brussels, Belgium) and Dr. J. R. Miller (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). pF4.2s was constructed by insertion of oligonucleotides corresponding to F4.2 peptide into the PsI and XhoI site of pcDSRα-E3 expression vector. pF4.2s and pcDSRα-E3 were transfected to HST-2, MKN28-A31-2 and HOBC8-A31-12 cells by lipofectamid reagent. At first, the reactivity of TCHST-2 against HST-2, HOBC8-A31-12, and K562 cells, which were introduced with pF4.2s and pcDSRα-E3, was assessed in transient assays for 48 h transfaction of genes using TNF production from TCHST-2. Then, the obtained stable transfextant lines of HOBC8-A31-12 and MKN28-A31-2, which were cotransfected with pF4.2s and an SRα-responsive gene, pBabe Puro (33, 34), at a 20:1 molar ratio of DNA. The cells were selected with 1 μg/ml of puromycin, and the cell lines were obtained. The insertion of oligonucleotide sequence coding F4.2 in these lines was confirmed by PCR analysis. These lines were used in the cytotoxicity experiment by TCHST-2. We also constructed an expression minigenic vector, pF4.2sA130, which contains the agonogenic peptides substituted with Ala at the ninth position Trp of F4.2 wild peptide.

Binding assays of peptides to purified HLA-A31 molecules

Purified HLA-A31 molecules were obtained from the lysates of CIR-A*31012 cells by using immunoaffinity chromatography with anti-HLA-A31-specific mAb 2D12. Namely, CIR-A*31012 cells were homogenized

<sup>1</sup> Abbreviations used in this paper: TFA, trifluoroacetic acid; RP-HPLC, reverse phase-HPLC.
with the lysate buffer (10 mM Tris-HCl (pH 7.0) 150 mM NaCl, and 0.02% NaN₃) and protease inhibitor cocktail tablet (complete; Boehringer Mannheim, Mannheim, Germany) in the presence of 0.1% octyl-glycoside (Sigma, St. Louis, MO). centrifuged at 15,000 rpm for 30 min, and supernatant was obtained as the cell lysate. The lysate was incubated with mAb 2D12 immobilized Affi-Gel 10 beads (Bio-Rad) at 4°C for 12 h. After washing with the buffer (0.1 M Tris-HCl (pH 8.0) and 0.2 M NaCl), purified HLA-A31 molecules were eluted with 0.1 M glycine buffer (pH 2.5) and immediately neutralized with 1.0 M Tris. A small sample of eluate was run on 10% SDS-PAGE and, purification was confirmed by silver staining of gel.

The binding of peptides to purified HLA-A31 molecules was determined by the method described by Tsai et al. (35). This assay was based on the competitive inhibition of radiolabeled standard peptide binding to HLA-A31 molecules with test peptides. We used the standard peptide Lys-Ile-Met-Lys-Trp-Asn-Tyr-Glu-Arg (KIMKMWYNEH), which was shown to bind strongly to HLA-A31 by Falk et al. (36) and Rammensee et al. (37). This peptide was radiolabeled with 125I by the Iodo-Beads method (Pierce, Rockford, IL) under the manufacturer’s protocol. Approximately 14 pM HLA-A31 molecules were admixed with 125 pM standard radiolabeled peptide by 50% (IC50) was also calculated.

The concentration of test peptides necessary to inhibit the binding of radiolabeled HLA-A31 molecules was determined by a scintillation gamma counter. The percent inhibition was calculated as "radioactivity (CPM) of bound HLA-A31 molecules was determined by the method described by Tsai et al. (35). This assay was based on the competitive inhibition of radiolabeled standard peptide binding to HLA-A31 molecules with test peptides. We used the standard peptide Lys-Ile-Met-Lys-Trp-Asn-Tyr-Glu-Arg (KIMKMWYNEH), which was shown to bind strongly to HLA-A31 by Falk et al. (36) and Rammensee et al. (37). This peptide was radiolabeled with 125I by the Iodo-Beads method (Pierce, Rockford, IL) under the manufacturer’s protocol. Approximately 14 pM HLA-A31 molecules were admixed with 125 pM standard radiolabeled peptide by 50% (IC50) was also calculated.

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Results
Separation of TFA-extracted antigenic peptides of HST-2 tumor cells recognized by TcHST-2
To determine the natural antigenic peptides that are recognized by TcHST-2, 4 × 10⁷ HST-2 cells were harvested and treated with 0.1% TFA solution. The supernatant was applied to a Sephadex G-25 column, and molecules less than 5 kDa in molecular size were obtained. They were then applied to the first (Fig. 1A) and second (Fig. 2A) step of preparative RP-HPLC. We examined whether the samples of each fraction could enhance the cytotoxicity by TcHST-2 CTL clone against 3H-Cr-labeled autologous HST-2. As a negative control, we used HLA-A31(-) target cells such as M-EB cells. In this experiment, we employed an E:T ratio of 10:1. Because this condition confers a base line of specific cytotoxicity of only <10% against HST-2 target cells in the absence of exogenously added peptide treatment, it allowed determination of whether peptide fractions have antigenic activity.

As shown in Fig. 1B, it was suggested that fraction no. 12 (arrow) in the first step of RP-HPLC, which corresponded to ~43–44% of acetonitrile concentration, contained immunogenic molecules that enhanced the cytotoxicity of TcHST-2 against HST-2 cells but not against HLA-A31(-) M-EB B cells. We also performed the second step of preparative RP-HPLC of the no. 12 fraction by using the same preparative RP-HPLC column with 40–50% acetonitrile (thick arrows in Fig. 2A) linear gradient and determined the immunogenic fraction. As shown in Fig. 2B, it appeared that fraction no. 17, corresponding to 43–44% of acetonitrile in elution on RP-HPLC (thin arrow in Fig. 2A), enhanced the cytotoxicity of TcHST-2 against HST-2 cells, although its extent of enhancement was not drastic (thick arrow in Fig. 2B). However, the enhancing effect of the TcHST-2 reactivity with fraction no. 17 was detected, and this effect was reproducible. Furthermore, such effect was not seen against M-EB cells. Therefore, we concluded that the antigenic peptide was contained in fraction no. 17.

Determination of amino acid sequence
Fraction no. 17 of the second step of preparative RP-HPLC (Fig. 2A) was further purified by a ZORBAX-ODS RP-HPLC column. As shown in Fig. 3, several peaks were demonstrated, and we characterized each of these as to whether they contained peptides. It was shown by using Edman’s degradation method for protein sequencing that only fractions 4, 5, and 6 contained amino acids, and the other fractions did not. Both fraction 4 (fr. 4) and fraction 6 (fr. 6) (arrows in Fig. 3) were shown to be composed of 10 aa, as demonstrated in Table 1; fraction 4 demonstrated the primary amino acid sequence of Tyr-Ser-Trp-Met-Asp-Tyr-Ser-Cys-Trp-Ile (designated F4.1) or Tyr-Ser-Trp-Met-Asp-Ile-Ser-Cys-Trp-Ile (F4.2). They appeared to differ from one another only in N-terminal sixth amino acid, because two clear peaks corresponding to Tyr and Ile were detected with almost the same quantity in cycle 6 of the HPLC chart of Edman’s degradation method. Meanwhile, fraction 6 showed Ile-Ala-Pro-Cys-Pro-Leu-Arg-Arg-Pro-Ala (designated F6). It was shown that the third amino acid Trp of F4.1 and F4.2, and the sixth Ile of F4.2, corresponded to the anchor motif of HLA-A31-bound peptides (36, 37). This was also true for the sixth amino acid Leu of F6. Fraction 5 was shown to be composed of only 4 aa. Because peptides that bind to HLA-class I molecules are usually 8–13 aa (29, 30), we omitted fraction 5 as a candidate of antigenic peptides.

Three peptides (F4.1, F4.2, and F6), whose primary sequences were obtained from analyses of fraction 4 and fraction 6 in the third (final) step of RP-HPLC, were synthesized and purified with RP-HPLC. To determine the antigenicity of these peptides, we used the cytokine production assay by CTLs, because this method seems to be highly sensitive in determining the antigenicity of peptides (11, 38). Namely, we determined if TcHST-2 could enhance the TNF production when HST-2 cells were pulsed with exogeneously added synthetic peptides. In the experiments, certain amounts of each peptide were pulsed to HST-2 cells. TcHST-2 was added at an E:T ratio of 10:1 following in vitro cultivation for 6 h, and the TNF production was assessed in each culture supernatant of wells with the MTT assay using W13 cells (31). As shown in Table 2, at an E:T ratio of 10:1, base line TNF production was indicated at 200–300 pg/ml in response to HST-2 cells without peptide treatment. Meanwhile, when peptides were pulsed to HST-2 cells, it appeared that TcHST-2 enhanced the production of TNF most preferentially with F4.2 peptides. Other peptides seemed to fail to enhance the TNF production by TcHST-2. Furthermore, none of these synthetic peptides at any concentration stimulated TcHST-2 when they were pulsed to M-EB cells. We also determined the specificity of TcHST-2 response against peptides. As shown in Fig. 4, F4.2 peptide could enhance the TNF production in a dose-dependent manner, whereas HBA31 (STL PETTVVRR), which is the antigenic peptide of CTL specific for the HLA-A31-restricted hepatitis B Ag (39), could not affect TcHST-2 reactivity.

Table I. The primary sequence of F4 and F6 peptides in fractions no. 4 and no. 6, respectively, of RP-HPLC.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>From fraction no. 4</td>
<td>Tyr-Ser-Trp&lt;sup&gt;b&lt;/sup&gt;-Met-Asp-Tyr&lt;sup&gt;c&lt;/sup&gt;-Ser-Cys-Trp-Ile</td>
</tr>
<tr>
<td>F4.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Tyr-Ser-Trp&lt;sup&gt;b&lt;/sup&gt;-Met-Asp-Ile&lt;sup&gt;c&lt;/sup&gt;-Ser-Cys-Trp-Ile</td>
</tr>
<tr>
<td>F4.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Tyr-Ser-Trp&lt;sup&gt;b&lt;/sup&gt;-Met-Asp-Ile&lt;sup&gt;c&lt;/sup&gt;-Ser-Cys-Trp-Ile</td>
</tr>
<tr>
<td>From fraction no. 6</td>
<td>Ile-Ala-Pro-Cys-Pro-Leu-Arg-Arg-Pro-Ala</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples from fractions no. 4 and no. 6 in the final (third) step of RP-HPLC were subjected to the Edman’s degradation method for protein sequencing.

<sup>b</sup> Amino acids in underline are compatible with the peptide binding motif to HLA-A31 (36, 37).

<sup>c</sup> Two clear peaks, which correspond with Tyr and Ile, were detected in cycle 6 HPLC chart of Edman’s degradation method. Because these peaks were almost the same in quantity, both of these two amino acids appeared to be candidates for N-terminal sixth amino acid of the antigenic peptides. The peptides containing Tyr and Ile were designated as F4.1 and F4.2, respectively.

Table II. The antigenicity of synthetic peptides F4.1, F4.2, and F6.

<table>
<thead>
<tr>
<th>Synthetic Peptides</th>
<th>TNF Production (pg/ml ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HST-2</td>
</tr>
<tr>
<td>no</td>
<td>292 ± 25.8</td>
</tr>
<tr>
<td>F4.1</td>
<td>360 ± 24.3</td>
</tr>
<tr>
<td>F4.2</td>
<td>623 ± 30.4</td>
</tr>
<tr>
<td>F6</td>
<td>336 ± 24.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> The antigenicity of synthetic peptides F4.1, F4.2, and F6, to stimulate the reactivity of TcHST-2, was determined. HST-2 and HLA-A31 (−) M-EB cells were pulsed with 50 μM synthetic peptides or without peptides, and mixed for 10 h with TcHST-2 at an E:T ratio of 10:1. The TNF production by TcHST-2 was assessed for each well using TNF-sensitive W13 cells and MTT assays as described in Materials and Methods.
Antigenicity of minigene encoding F4.2 peptide

Although it appeared that TcHST-2 could respond to pulsed F4.2 peptide in the highly sensitive TNF production assay, we attempted to express F4.2 peptide in an endogenous form (32) by constructing an expression vector pF4.2ss. Because it is uncertain whether F4.2 peptide could enter into the endoplasmic reticulum via TAP, we used the expression vector encoding the signal sequence plus F4.2 peptide. pF4.2ss minigene encodes adenovirus E3, 19-kDa protein signal sequence plus F4.2 peptide, as shown in Fig. 5.

HST-2 cells were transfected for 48 h with pF4.2ss or a control pcDSRe-E3 that encodes the adenovirus E3, 19-kDa protein signal sequence alone, and TcHST-2 was added. As shown in Fig. 6A, the transient TNF production assay indicated that TcHST-2 enormously enhanced its responsiveness to HST-2 cells transfected with pF4.2ss, whereas it only responded on a background level with a control pcDSRe-E3. It was also true that HOBC8-A31-12 cells transfected only with pF4.2ss could stimulate TcHST-2 in a dose-dependent manner (Fig. 6B). However, when MHC class I (HLA-A31)-deficient K562 cells were used as the target, pF4.2ss introduction did not result in the stimulation of TcHST-2 at all (Fig. 6C).

We confirmed further the antigenicity of endogenously expressed F4.2 peptide in the cytotoxicity assays by TcHST-2. To this end we used HOBC8-A31-12 and MKN28-A31-2 cells. In the TNF production assays, HOBC8-A31-12 cells did not stimulate TcHST-2, whereas our preliminary data indicated that MKN28-A31-2 cross-reacted with TcHST-2, suggesting that the MKN28-A31-2 gastric tumor line, but not HOBC8-A31-12, may have endogenous F4.2 peptide on the cell surface. Both HOBC8-A31-12 and MKN28-A31-2 cells were cotransfected with pF4.2ss pluspBabe Puro at a 20:1 molar DNA ratio, selected with puromycin, and stable transfectant lines were obtained. These cells...
were then assessed in a cytotoxicity experiment by TcHST-2. The data showed that pF4.2ss minigene introduction to HOBCC8-A31-12 cells clearly conferred cytotoxic susceptibility on HOBCC8-A31-12 cells to TcHST-2 (Fig. 7A). Furthermore, the cytotoxic susceptibility of MKN28-A31-2 cells transfected with pF4.2ss minigene was clearly enhanced to TcHST-2 as compared to that with pBabe Puro alone (Fig. 7B). Taken together, these data suggest that F4.2 is the peptide antigen to TcHST-2 in the context of HLA-A31 molecules.

**T cell epitope analysis of F4.2 peptide**

We further studied the characteristics of F4.2 peptide. As shown in an upper panel of Fig. 8A, we synthesized and purified peptides such as F4.2-9, F4.2-8, and F4.2-7, which were deleted with C-terminal 1, 2, and 3 aa residues, respectively, from F4.2 decamer wild peptide. Then these peptides were assessed for antigenicity to TcHST-2 in the TNF production assay. HST-2 cells were pulsed with 0.1, 1.0, and 10.0 μM peptides, and mixed with TcHST-2 at an E:T ratio of 3:1. As shown in a lower panel of Fig. 8A, F4.2 and F4.2-9 peptides could enhance TcHST-2 reactivity in a dose-dependent manner, whereas F4.2-8 and F4.2-7 lost almost completely the antigenicity to TcHST-2. Because these data suggested that the N-terminal ninth Trp might act as the T cell epitope for TcHST-2, we studied the interaction by using synthetic peptides which are substituted with Ala (F4.2-Ala9) or Arg (F4.2-Arg9) at this ninth position (upper panel of Fig. 8B). Although F4.2-Ala9 may show very weak reactivity with higher concentrations of peptides, it may be concluded that neither of these peptides could enhance TcHST-2 reactivity (lower panel of Fig. 8B). It appears that F4.2-Arg9 has a strong anchor motif for the binding to HLA-A31 at the ninth position (36, 37), but it almost completely failed to stimulate TcHST-2. Although the content of TNF production by TcHST-2 was different in Fig. 8A and Fig. 8B, this seemed due to the different level of activation state of TcHST-2 by recombinant IL-2.

We further confirmed that the ninth Trp of F4.2 peptide is critical to the enhancement of TcHST-2 reactivity by using HOBCC8-A31-12 and a minigene (pF4.2ss-Ala9) coding F4.2-Ala9 peptide. In an upper panel of Fig. 9A, the construction and nucleotide sequence of minigenes is illustrated. HOBCC8-A31-12 cells were transfected for 48 h with pF4.2ss and pF4.2ss-Ala9, and TcHST-2 was added. As shown in a lower panel of Fig. 9B, TcHST-2 could react with HOBCC8-A31-12 cells transfected with pF4.2ss, but not with pF4.2ss-Ala9. Thus, these data strongly suggest that the N-terminal ninth Trp might act as the T cell epitope for TcHST-2 clone.

**Binding of F4.2 peptide to HLA-A31 molecule**

We determined amino acid residue(s) of F4.2 peptide which acts as the aggretope in the binding to HLA-A31 molecules. To this end, we developed anti-HLA-A31 mAb 2D12. This mAb reacts with C1R-A*31012, but not with C1R or C1R-A*3303. 2D12 also does not react with HLA-A1, A2, A3, A11, A24, A26, B7, B18, B35, B44, B51, B55, B60, B61, C1, C7, and C9-positive cells, indicating that 2D12 is highly specific for HLA-A31. Therefore, HLA-A31 molecules were purified with mAb 2D12 from the lysates of C1R-A*31012 cells. As shown in Fig. 10, it is indicated that mAb 2D12 could precipitate molecules corresponding to HLA class I heavy and light chains. Then, the binding of synthetic peptides to HLA-A31 molecules was assessed in a competitive inhibition with HLA-A31-restricted radiolabeled standard peptide, Lys-Ile-Met-Lys-Trp-Asn-Tyr-Glu-Arg (KIMKWNYER) (36, 37). The radioactivity of peptide-bound HLA-A31 molecules was assessed in a competitive inhibition with HLA-A31-restricted radiolabeled standard peptide, Lys-Ile-Met-Lys-Trp-Asn-Tyr-Glu-Arg (KIMKWNYER) (36, 37). The radioactivity of peptide-bound HLA-A31 molecules was determined, and the concentration of test peptides that could inhibit the radioactivity of peptide-bound HLA-A31 molecules was calculated. As shown in Table 3, F4.2 wild peptide indicated the strongest binding affinity to HLA-A31 molecules among all the peptides. Although TcHST-2 CTL responded to F4.2-9, but not to F4.2-8, both of these C-terminal-deleted peptides showed almost similar binding capability. As compared with F4.2, F4.2-Arg9 showed almost the same binding affinity, suggesting that the ninth Trp of F4.2 peptide is critical for binding to HLA-A31.

![FIGURE 7](http://www.jimmunol.org/)

Cytotoxicity by TcHST-2 against stable transfectants of HOBCC8-A31-12 and MKN28-A31-2 cells with pF4.2ss minigene. HOBCC8-A31-12 (A) and MKN28-A31-2 (B) cells were transfected with pF4.2ss and pBabe Puro at 20:1 of DNA molar ratio, or with pBabe Puro alone. The cells were selected in the presence of 1.0 μg/ml of puromycin, and the cell lines were obtained. Then the cells were labeled with 51Cr and mixed with TcHST-2 at various E:T ratios. Bars represent mean ± SE.

![FIGURE 8](http://www.jimmunol.org/)  
**A** The antigenicity of F4.2 C-terminal-deleted synthetic peptides (A) and variant F4.2 peptides substituted at the ninth position (B). Identical amino acid as F4.2 amino acid sequence. HST-2 cells were pulsed with 0.1, 1.0, and 10.0 μM of peptides or without peptides, and mixed for 6 h with TcHST-2 at an E:T ratio of 3:1. The TNF production by TcHST-2 was determined as in Fig. 4.

![FIGURE 9](http://www.jimmunol.org/)  
**A** We further confirmed that the ninth Trp of F4.2 peptide is critical to the enhancement of TcHST-2 reactivity by using HOBC8-A31-12 and a minigene (pF4.2ss-Ala9) coding F4.2-Ala9 peptide. In an upper panel of Fig. 9A, the construction and nucleotide sequence of minigenes is illustrated. HOBCC8-A31-12 cells were transfected for 48 h with pF4.2ss and pF4.2ss-Ala9, and TcHST-2 was added. As shown in a lower panel of Fig. 9B, TcHST-2 could react with HOBC8-A31-12 cells transfected with pF4.2ss, but not with pF4.2ss-Ala9. Thus, these data strongly suggest that the N-terminal ninth Trp might act as the T cell epitope for TcHST-2 clone.

![FIGURE 10](http://www.jimmunol.org/)  
**B** We determined amino acid residue(s) of F4.2 peptide which acts as the aggretope in the binding to HLA-A31 molecules. To this end, we developed anti-HLA-A31 mAb 2D12. This mAb reacts with C1R-A*31012, but not with C1R or C1R-A*3303. 2D12 also does not react with HLA-A1, A2, A3, A11, A24, A26, B7, B18, B35, B44, B51, B55, B60, B61, C1, C7, and C9-positive cells, indicating that 2D12 is highly specific for HLA-A31. Therefore, HLA-A31 molecules were purified with mAb 2D12 from the lysates of C1R-A*31012 cells. As shown in Fig. 10, it is indicated that mAb 2D12 could precipitate molecules corresponding to HLA class I heavy and light chains. Then, the binding of synthetic peptides to HLA-A31 molecules was assessed in a competitive inhibition with HLA-A31-restricted radiolabeled standard peptide, Lys-Ile-Met-Lys-Trp-Asn-Tyr-Glu-Arg (KIMKWNYER) (36, 37). The radioactivity of peptide-bound HLA-A31 molecules was assessed in a competitive inhibition with HLA-A31-restricted radiolabeled standard peptide, Lys-Ile-Met-Lys-Trp-Asn-Tyr-Glu-Arg (KIMKWNYER) (36, 37). The radioactivity of peptide-bound HLA-A31 molecules was determined, and the concentration of test peptides that could inhibit the radioactivity of peptide-bound HLA-A31 molecules was calculated. As shown in Table 3, F4.2 wild peptide indicated the strongest binding affinity to HLA-A31 molecules among all the peptides. Although TcHST-2 CTL responded to F4.2-9, but not to F4.2-8, both of these C-terminal-deleted peptides showed almost similar binding capability. As compared with F4.2, F4.2-Arg9 showed almost the same binding affinity, suggesting that the ninth Trp of F4.2 peptide is critical for binding to HLA-A31.
same binding capability, and F4.2-Ala\textsuperscript{9} showed a minimally reduced binding capability. However, these peptides could not stimulate TcHST-2 reactivity, confirming our notion that the N-terminal ninth Trp may act as the T cell epitope residue. F4.2-7 showed much lower binding capability, but it seemed that this peptide could still bind to HLA-A31 molecules, since the N-terminal sixth Ile appeared to be the most important residue as the agretope. Indeed, when we substituted F4.2 with Ala (F4.2-Ala\textsuperscript{6}) at the N-terminal sixth Ile, the binding capability was drastically impaired. Meanwhile, F4.2-Ala\textsuperscript{3} minimally lost its binding affinity to HLA-A31 molecules. Thus, in F4.2 peptide it appeared that the most critical residue to the binding to HLA-A31 was the sixth Ile.

**Discussion**

Recent investigations have clarified that patients’ immune systems can recognize tumor cells derived from their own cells and tissues (1, 2, 40 – 45). This is particularly true for the melanomas (6 –14, 46 – 48), and these promising attempts lead directly to a new modality for cancer treatment (49, 50). Meanwhile, it becomes increasingly important to study the tumor Ags recognized by CTLs in human nonmelanoma tumors of epithelial tissue origins such as the stomach, pancreas, liver, colon, lung, and breast. The clinical incidence of these epithelial tumors is considerably higher than that of melanomas. Therefore, we have attempted to determine the structure of the tumor antigenic peptides in such tumors.

Our previous report indicated that a gastric signet ring cell carcinoma, HST-2 cells, was lysed by autologous PBL-derived CD8\textsuperscript{+} CTL clone, TcHST-2, in the context of HLA-A31 (22). The TCR gene structure analysis of CTLs in this system has suggested that HST-2 cells may express the tumor Ag on the cell surface, because MLTC of patient’s PBL frequently resulted in the clonal expansion of CTLs which have the same DNA sequence in the complementarity-determining region-3 (CDR-3) as TcHST-2 (23, 24). Therefore, using an autologous pair of HST-2 tumor cells and TcHST-2 CTLs, we have attempted to determine the primary structure of the antigenic peptide of HST-2 cells. In the current investigation, we used TFA elution of Ags and purified the antigenic peptide with several steps of biochemical procedures, including RP-HPLC. This is the first report demonstrating the primary amino acid sequence of human gastric signet ring cell tumor Ag recognized by CD8\textsuperscript{+} CTLs. Our data suggested that TcHST-2 could preferentially recognize 10 aa composed of Tyr-Ser-Trp-Met-Asp-Ile-Ser-Cys-Trp-Ile. We designated this peptide as F4.2.

The immunogenic activity of fractions no. 12 and no. 17 from the first and second step, respectively, of preparative RP-HPLC could be detected in the cytotoxicity assays. However, although that of synthetic peptides was detected in TNF production assays, it was rather obscure in the conventional cytotoxicity assays. Perhaps these observations were due to the sensitivity of the assay system. TNF assays using W13 cells are more sensitive than the cytotoxicity assays, and can detect even <10 pg/ml of TNF. These

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC\textsubscript{50} (nM)\textsuperscript{a}</th>
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<tbody>
<tr>
<td>F4.2</td>
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<td>836</td>
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<td>F4.2-Ala\textsuperscript{6}</td>
<td>YSWMDASCW</td>
<td>35,111</td>
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\textsuperscript{a} A HLA-A31-binding standard synthetic peptide, LIMKWNYER, was radiolabeled with \textsuperscript{125}I. Purified HLA-A31 molecules by mAb 2D12 were admixed with standard radiolabeled peptide in 0.1 ml PBS containing 1 nM \textbeta\textsubscript{2}-microglobulin and protease inhibitors. Nonlabeled competitor peptides were added, and IC\textsubscript{50} were calculated as described in Materials and Methods.
facts may imply that F4.2 peptide has a relatively weak immunogenicity. Meanwhile, one more possible explanation is that there may exist a conformational difference between natural antigenic peptides and synthetic ones. It is also possible that the peptide exchange was relatively ineffective between exogenously added F4.2 peptides and already-bound endogenous, i.e. resident, peptides on the cell surface of HLA-A31 molecules.

These possibilities led us to investigate whether the antigenicity of F4.2 could be enhanced when it was expressed in an endogenous form within the cells by using the minigene expression vectors (32). Because we do not know yet whether F4.2 peptide enters into the endoplasmic reticulum via TAP molecules, we constructed a minigene, pF4.2ss, encoding signal sequence plus F4.2 peptide by which means F4.2 peptide could enter directly into the endoplasmic reticulum via signal recognition particles. Our data showed that the introduction of pF4.2ss into autologous HST-2 cells resulted in drastically enhanced TcHST-2 reactivity. Furthermore, the introduction of pF4.2ss to HLA-A31(+) allogeneic tumor lines was also able to stimulate TcHST-2; TcHST-2 could produce TNF in a very efficient amount as well as lysing with these targets. These facts may suggest that the antigenicity of F4.2 peptide itself was not weak, and the relatively low immunogenicity observed in the experiment using F4.2 synthetic peptides may have been due to inefficient peptide exchange between already-bound endogenous, i.e. resident, peptides and exogenously added synthetic ones on the cell surface of HLA-A31 molecules. More recent experiments suggest that F4.2 peptide may be transported via TAP, because the minigene coding F4.2 without signal sequence was similarly effective to pF4.2ss in enhancing TcHST-2 response.

So far there is no homologous protein between F4.2 peptide primary sequence and known proteins when investigated with the computer database, and it appears that the parental molecule of this peptide is an as yet unknown protein. The molecular cloning of the parental protein of F4.2 is very important for understanding the whole characteristic of this Ag, and this is now being undertaken by using degenerated oligonucleotides deduced from F4.2 peptide sequence.

We are also studying the effectiveness of in vitro CTL induction from HLA-A31(+) patients’ PBL with gastric tumors. Our preliminary data indicated that ~30% of patients could clearly induce peptide-specific CTL in vitro. Our data also showed that F4.2 is immunogenic in at least some gastric tumors when they express HLA-A31 molecules; TcHST-2 was cytotoxic against gastric tumor lines MKN-28, but not to MKN-74 and HGC-25, when these lines were transfected with an expression vector of HLA-A31 gene, pBJ-A*31012. Furthermore, F4.2 peptides expressed in endogenous form within the cells could obviously enhance the reactivity of TcHST-2 in HLA-A31(+) allogeneic tumor cells. If this is true for more large panels of HLA-A31(+) gastric tumor cells, it may be recommended for immediate clinical use, because gastric signet ring cell carcinomas belong to the highly malignant cancers with very poor prognosis.

Acknowledgments

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NATURAL ANTIGENIC PEPTIDES OF HUMAN GASTRIC CARCINOMA


