Jpn. J. Cancer Res. **93**, 917–924, August 2002

Natural Antigenic Peptides from Squamous Cell Carcinoma Recognized by Autologous HLA-DR8-restricted CD4$^+$ T Cells

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A large number of human tumor antigens recognized by CD8$^+$ cytotoxic T lymphocytes (CTL) have been identified. Some of them have been employed in clinical trials and have achieved some objective responses. However, little is known about those that are recognized by CD4$^+$ T cells, except for a very few that were identified from melanomas. Previously, we reported that an oral squamous cell carcinoma (SCC) cell line, OSC-20, was effectively lysed by HLA-DRB1*08032 (HLA-DR8)-restricted autologous CD4$^+$ T cell line, TcOSC-20. In this study, we performed two steps of chromatographic purification of the tumor cell lysate in combination with mass spectrometry. We found one reverse-phase high-performance liquid chromatography (RP-HPLC) fraction that was effectively recognized by the T cells. We analyzed the fraction by nano-liquid chromatography/electrospray ionization ion trap mass spectrometry (LC/MS/MS) and found six representative ions. We could determine the primary amino acid sequence of each of the six ions. Three of them contained a potential HLA-DR8 binding motif, and TcOSC-20 showed a rather strong cytotoxic response to one of the synthetic peptides, namely, amino acid residues 321–336 of human α-enolase. Thus, several gene products of squamous cancer cells are endogenously processed and may be presented on HLA class II molecules, so that they could constitute target molecules for autologous CD4$^+$ T cells.

Key words: Natural antigenic peptide — CD4$^+$ killer T cell — HLA-DR8 — Squamous cell carcinoma

It is well established that cytotoxic T lymphocytes (CTL) recognize antigenic peptides presented by major histocompatibility complex (MHC) class I molecules on the cell surface and lyse these target cells. Generally, CD8$^+$ CTL are thought to play a major role in cytotoxic action,[1)] since MHC molecules expressed on most of the cell surface are MHC class I. To date, a large number of genes encoding human tumor antigens recognized by CD8$^+$ T cells have been identified, the majority of which were isolated from melanomas.[2,3)] Although it appeared more difficult to establish an autologous tumor-specific CTL line or clone from non-melanoma patients, such reports of isolation of T-cell-defined tumor antigens from epithelial tissues have been increasing.[4–14)]

On the other hand, CD4$^+$ T cells are considered to control both humoral and cellular immune responses; however, it remains unclear whether CD4$^+$ T cells can directly react against autologous tumor cells.[15)] Very recently, several MHC-class-II-restricted, CD4$^+$ T-cell-defined antigens have been reported. However they were only derived from melanoma studies.[16–28)] These reports strongly suggested that CD4$^+$ T cells could also play an important role in tumor immunity.

We previously established a squamous cell carcinoma cell line, OSC-20, and an HLA-DRB1*08032 (HLA-DR8)-restricted autologous CD4$^+$ T cell line, TcOSC-20. It appeared that TcOSC-20 lysed tumor cells.[29)] In the present study, we applied biochemical approaches to isolate natural antigenic peptides from the tumor cell lysate, i.e., two steps of reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. We succeeded in determining the primary amino acid sequences of six peptides. Three of the peptides contained a DR8 binding motif. Furthermore, one of them, human α-enolase-derived peptide, may possibly be immunogenic, since TcOSC-20 lysed L-cell transfectant-expressing human DR8 molecules when pulsed with the synthetic peptide. Our data suggest that endogenous peptides processed in squamous cancer may be presented on class II molecules and recognized by autologous CD4$^+$ T cells.

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MATERIALS AND METHODS

$CD4^+$ CTL and tumor cell lines OSC-20 is a human squamous cell carcinoma (SCC) cell line of the tongue, and TcOSC-20 (HLA type: HLA-A2, A11, B55, B46, Cw1, Cw9, DR8, DR12, DR52, DQ1, and DQ7) is a $CD4^+$ T cell line. The procedures for establishing OSC-20 and TcOSC-20 were previously described.[29] In addition to HLA-class I molecules, OSC-20 cells express HLA-DR8 (DRB1*08032), DQ1 and DQ7. However, HLA-DR12 and DR52 are deleted at the genomic level.[29] Cytotoxic activity of TcOSC-20 against OSC-20 is blocked by anti-HLA-DR (TC-8B1) and anti-HLA-DR8, 12 (HU-39) specific monoclonal antibodies (mAbs). This indicates that TcOSC-20 might recognize peptides in the context of HLA-DRB1*08032.[29] In this study we also used an autologous EBV-transformed B-cell line, M-EB,[29] and an allogeneic erythroleukemia cell line K562. LDR0803, a stable transfectant cell line of mouse L-cells into which human *HLA-DR* α and *HLA-DRB1*08032* genes had been introduced, was kindly provided by Dr. Y. Nishimura (Kumamoto University School of Medicine, Kumamoto). rIL-2 was a kind gift from Takeda Pharmaceutical Co. (Osaka) and Shionogi Pharmaceutical Co. (Osaka).

Cytotoxicity assay The cytotoxicity assay has been described previously.[12] We used TcOSC-20 cells as effector cells and OSC-20, M-EB, K562, and LDR0803 cells as target cells. In the current study, the fractions of reverse-phase HPLC (RP-HPLC) and synthetic peptides were assessed for the ability to stimulate TcOSC-20 in cytotoxicity assays. ^{51}Cr-labeled target cells were pulsed with the fractionated samples for 60 min at 37°C in a CO_2 incubator, washed once with phosphate-buffered saline (PBS), and mixed with effector cells at a certain effector-to-target (E/T) ratio. After 6 h of incubation, supernatants were harvested and radioactivity was measured with a γ-counter.

Acid extraction and separation of the antigenic peptides The isolation procedure for acid-extraction of the HLA-class-I-bound peptides from the cells was previously described.[12] We applied this procedure to isolate HLA-class-II-bound peptides of OSC-20 cells. Briefly, approximately 5×10^9 OSC-20 cells were harvested from *in vitro* culture in RPMI 1640 medium plus 10% fetal calf serum (FCS). Cells were washed with PBS and centrifuged at 2000 rpm for 5 min. The resultant cell pellet was dissolved in lysis buffer (10 m*M* Tris-HCl [pH 7], 150 m*M* NaCl, 0.02% NaN_3, and protease-inhibitor-cocktail tablet ["Complete," Boehringer Mannheim GmbH, Germany]), destroyed by freeze-thawing, and incubated with 0.1% trifluoroacetic acid (TFA) for 30 min at room temperature. After centrifugation at 10 000*g*, the supernatant was ultrafiltered (Centricon-10, Japan Millipore Co., Tokyo) to obtain molecules of less than 10 kDa. Then antigenic peptides were purified by RP-HPLC in two sequential steps. In the first step, the samples were applied to a ZORBAX-ODS Column (4.6×250 mm; Rockland Technologies, Gilbersville, PA) and eluted with a linear gradient of buffers A (0.1% TFA in water) and B (acetonitrile:water 9:1 with 0.095% TFA); the sample was eluted with buffer A for 30 min, and then a linear gradient from 0 to 50% buffer B for 50 min (from 30 to 80 min) was applied. The sample was fractionated every 3 min from 30 to 80 min. In the second step, fractions of the first RP-HPLC that were immunogenic in the cytotoxicity assay were applied to the same column and eluted with a linear gradient of 0 to 40% buffer B for 80 min. Each peak was fractionated. In all steps of RP-HPLC, the flow rate was set at 0.5 ml/min.

The antigenicities of the fractions of the first and second step RP-HPLC were assessed in a ^{51}Cr release cytotoxicity assay by using TcOSC-20 against relevant target cells. In these experiments, each fraction was lyophilized and dissolved in AIM-V, and 10% of each fraction was added to wells containing ^{51}Cr-labeled LDR0803 target cells in 100 μl of medium. After incubation for 1 h in a CO_2 incubator, 100 μl of medium containing TcOSC-20 at a certain E/T ratio was added and the cytotoxicity was measured after 6 h.

Analysis of antigenic peptides by nano-LC/ESI IT MS/CID MS The primary amino acid sequence of the immunogenic fraction was analyzed by nano-liquid chromatography/electrospray ionization ion trap mass spectrometry (nano-LC/ESI IT MS), as described.[30] The fractions were loaded onto a nano-liquid chromatography column (0.075 mm i.d.×150 mm) and separated peptides were passed to an electrospray ion source in an LCQ ion trap MS (Finnigan, CA). The peptides were eluted at 300 nl/min with a gradient of 90% to 95% buffer A (0–10 min), 30% to 90% buffer A (10–50 min), 2% to 30% buffer A (50–60 min), and 20 min with 2% buffer A. ESI IT MS was performed on an LCQ equipped with a stainless electrospray ion source that was operated at a potential of 2.9–3.2 kV. To achieve a stable electrospray over the course of the gradient elution, 50% methanol containing 1% acetic acid was used as a sheath liquid at a flow rate of 300 nl/min. Mass spectra were obtained by scanning the range of m/z between 300 and 2000. The most abundant ions were further analyzed by collision-induced dissociation mass spectrometry (CID MS). LC/MS and LC/MS/MS were run in an automated LC/MS-LC/MS/MS mode that monitored for the signal threshold and MS/MS was performed on the base peak when the threshold criterion was exceeded. In the CID mode, the threshold to trigger ion selection was 3.0×10^4, and the default collision energy was set at 35%. MS/MS spectra were analyzed using the SEQUEST software against the OWL database to identify the amino acid sequence and original protein of the antigenic peptides.[31]

Peptide synthesis Six peptides determined by mass spectrometric analysis and a control peptide (FTFTISRLEPED-

FAVYYC) derived from the immunoglobulin κ chain (63–80 amino acid residues)[32] with the HLA-DR8-binding motif were used in the cytotoxicity assay. Peptides were synthesized according to the standard solid-phase synthesis method and purified by RP-HPLC. The purity and identity of peptides were determined by mass spectrometric analysis. Peptides were dissolved in 10% dimethyl sufoxide (DMSO) at 10 m*M* as a stock solution and stored at −80°C.

RESULTS

Cytotoxic profiles of TcOSC-20 First, we tested the cytotoxic activity of $CD4^+$ TcOSC-20 against autologous OSC-20 squamous cancer cells, autologous EBV-transformed B-cells, M-EB, allogeneic K562 cells and an L-cell transfectant, LDR0803. As shown in Fig. 1, TcOSC-20 lysed autologous OSC-20 and showed approximately 60% and 40% cytotoxicities at E/T ratios of 30:1 and 10:1, respectively. TcOSC-20 did not significantly lyse an autologous EBV-B cell line, M-EB, or K562 cells as compared with OSC-20. It was previously demonstrated that TcOSC-20 was an HLA-DR8-restricted $CD4^+$ T cell line.[29] Next, we tested whether LDR0803, which expressed human HLA-DR α and HLA-DRB1*08032, was recognized by TsOSC-20. TcOSC-20 did not lyse LDR0803 (Fig. 1). Since our previous study suggested that TcOSC-20 lysed tumor cells via a Fas-mediated pathway,[29] we also investigated whether Fas antigen was expressed on LDR0803 cells. Fas antigens were expressed on the surface of LDR0803 (data not shown), suggesting that LDR0803 could be used as antigen-presenting cells in a cytotoxicity assay.

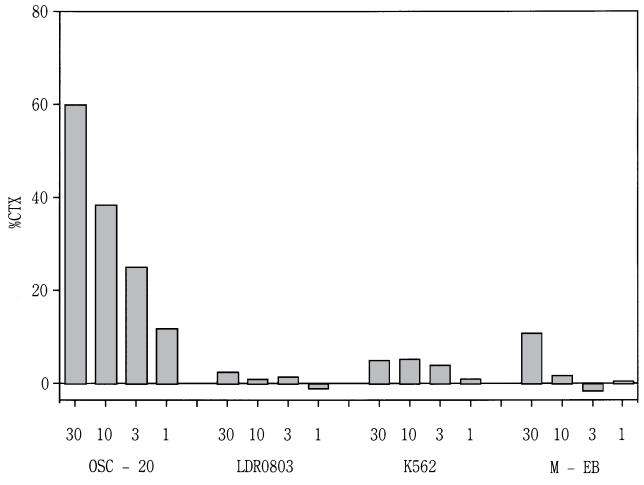

Fig. 1. Percent cytotoxicity of TcOSC-20 against ^{51}Cr-labeled OSC-20, M-EB, LDR0803, and K562 cells at E/T ratios of 30, 10, 3, and 1.

Chromatographic fractionation of OSC-20 cell extracts and isolation of a positive fraction by TcOSC-20 To determine the natural antigenic peptides recognized by TcOSC-20, 5×10^9 OSC-20 cells were harvested and treated with 0.1% TFA solution. The supernatant was ultrafiltered to obtain molecules of less than 10 kDa. The sample was loaded on an octadecyl silica (ODS) column, and fractionated every 3 min up to sixteen fractions between 30 and 80 min in the first step of RP-HPLC (Fig. 2A). The samples that were eluted with water for the first 30 min were excluded from analysis since the molecules in these samples could not bind to the ODS column in the water phase. We performed a cytotoxity assay to deter-

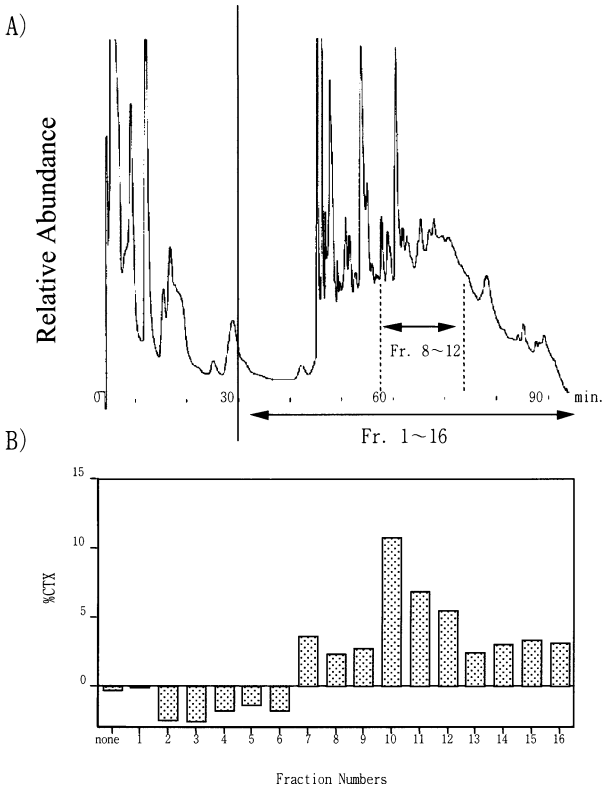

Fig. 2. Elution profile (A) of the first RP-HPLC of TFA-extracted OSC-20 and determination of antigenicity (B) of fractions. A) 5×10^9 OSC-20 cells were treated with 0.1% TFA and prepared as a sample for RP-HPLC as indicated in "Materials and Methods." After harvesting molecules <10 kDa in molecular size, a sample was applied to an ODS column (4.6×250 mm) and loaded with only buffer A (0.1% TFA in water) for 30 min and then a linear gradient from 0% to 50% buffer B (0.1% TFA in acetonitrile) for 50 min. The sample was fractionated every 3 min between 30 and 80 min. *Y*-axis, relative abundance at UV 210 nm; *X*-axis, time (min). B) The 10% volume of each fraction was pulsed onto ^{51}Cr-labeled LDR0803 cells followed by co-incubation with TcOSC-20 at an E/T ratio of 20. Cytotoxic activity was measured after 6 h.

mine which of the sixteen fraction samples contained antigenic peptides. Each fraction was lyophilized and dissolved in AIM-V, and 10% of each fraction volume was pulsed onto ^{51}Cr-labeled LDR0803 cells. After pulsing for 1 h, TcOSC-20 cells were added at an E/T ratio of 20:1, and cytotoxic activities were measured after 6 h. As shown in Fig. 2B, fractions 8 through 12 were recognized by TcOSC-20. This result was reproduced in three independent experiments. Fractions 8–12 were pooled and subsequently applied to the second step of RP-HPLC. Approximately twelve peaks (F1–12) were detected and fractionated, as shown in Table I. Each fraction sample was studied in the same manner as in the case of the first-step RP-HPLC. The data indicated that TcOSC-20 showed the strongest cytotoxicity when LDR0803 cells were pulsed with fraction 6 (F6) of the second RP-HPLC. It showed approximately 52% specific lysis at an E/T ratio of 20:1 (Table I). Therefore, it is highly likely that F6 included antigenic peptides recognized by TcOSC-20. Although the cytotoxicity of TcOSC-20 to the samples from the first RP-HPLC was only about 10% (Fig. 2B), the second chromatography remarkably enhanced the cytotoxicity (Table I), suggesting that the antigenic peptide in F6 should be further analyzed.

Determination of the amino acid sequence of fraction 6 by nano-LC/ESI IT MS/CID MS Since it was suggested that F6 in the second RP-HPLC might contain antigenic peptides, F6 was characterized by nano-LC/ESI IT MS. Fig. 3 shows the elution pattern of F6 with a base peak mass chromatogram. Twelve fractions around representative peaks were collected and further characterized by nano-LC/MS. Apparent non-peptide products were excluded from the analysis. From one peak, 2 to 10 ions were protonated and were further analyzed in the next CID MS analysis. We finally detected six ions (ions 1–6) from the 12 peaks and they are depicted in Fig. 3. The retention times of ions 1–6 approximately corresponded to peaks of 22.6, 28.3, 28.7, 30.3, 30.4, and 31.0 min, respectively. Ions that had similar retention times could not be detected in F5 (data not shown). The MS spectrum of a representative ion, ion 5, was automatically analyzed, and m/z 878 was indicated to be the most prominent ion at 30.4 min (Fig. 4A). The protonated molecular ions were analyzed by CID MS (Fig. 4B). The pattern of the fragment ions that were dissociated from the m/z 878 peak in CID MS was analyzed using the SEQUEST search program against the OWL database. It turned out to be identical to that of N-terminal amino acid residues 321–336 of human α-eno-

Table I. Antigenic Activities of the 12 Fractions from the Second RP-HPLC

Fraction No.	1	2	3	4	5	6	7	8	9	10	11	12		
Time (min)	42.6	43.8	48.1	49.3	50.9	53.4	54.7	60.0	62.3	64.5	66.9	68.5	Medium	OSC-20
% Cytotoxicity	6.8	−9.3	5.7	−13.0	1.9	51.8	−12.9	−11.1	−11.1	5.9	−10.6	−11.8	6.8	75.9

Fractions 8–12 of the first RP-HPLC were collected and subjected to the second RP-HPLC. Twelve peaks were collected (F1–12) at indicated elapsed time points. Then 10% of the volume of each fraction was pulsed onto ^{51}Cr-labeled LDR0803 cells and cytotoxic activities of TcOSC-20 were determined. Medium and OSC-20 represent the cytotoxic activity of TcOSC-20 in response to LDR0803 cells alone and autologous tumor cell line OSC-20 at an E/T ratio of 20, respectively. Boxes show the cytotoxic response of TcOSC-20 to fraction 6 and positive control OSC-20.

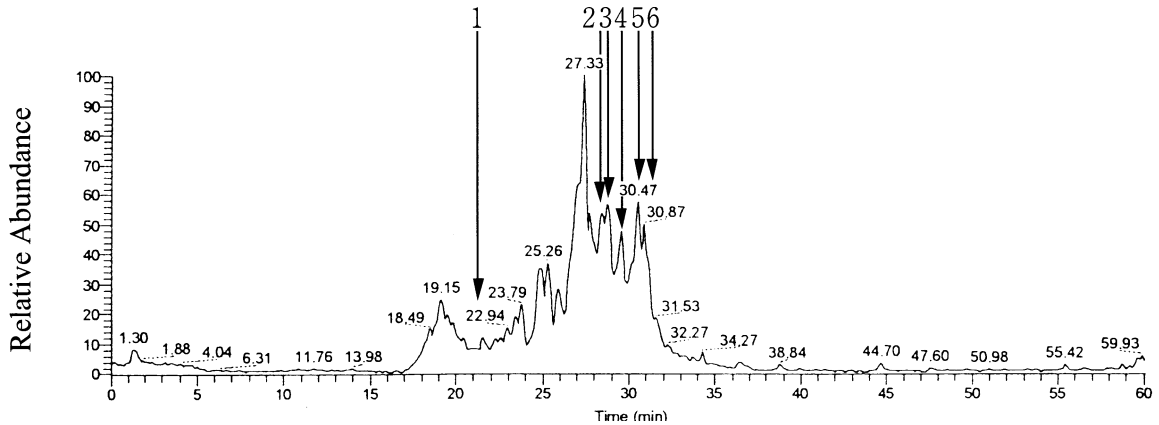

Fig. 3. Base peak mass chromatograms of fraction 6 in the second RP-HPLC. Twelve fractions around representative peaks were collected and further analyzed by nano-LC/MS/MS. *Y*-axis, relative abundance; *X*-axis, time (min). Numbers represent the ions 1 through 6 that finally matched the database.

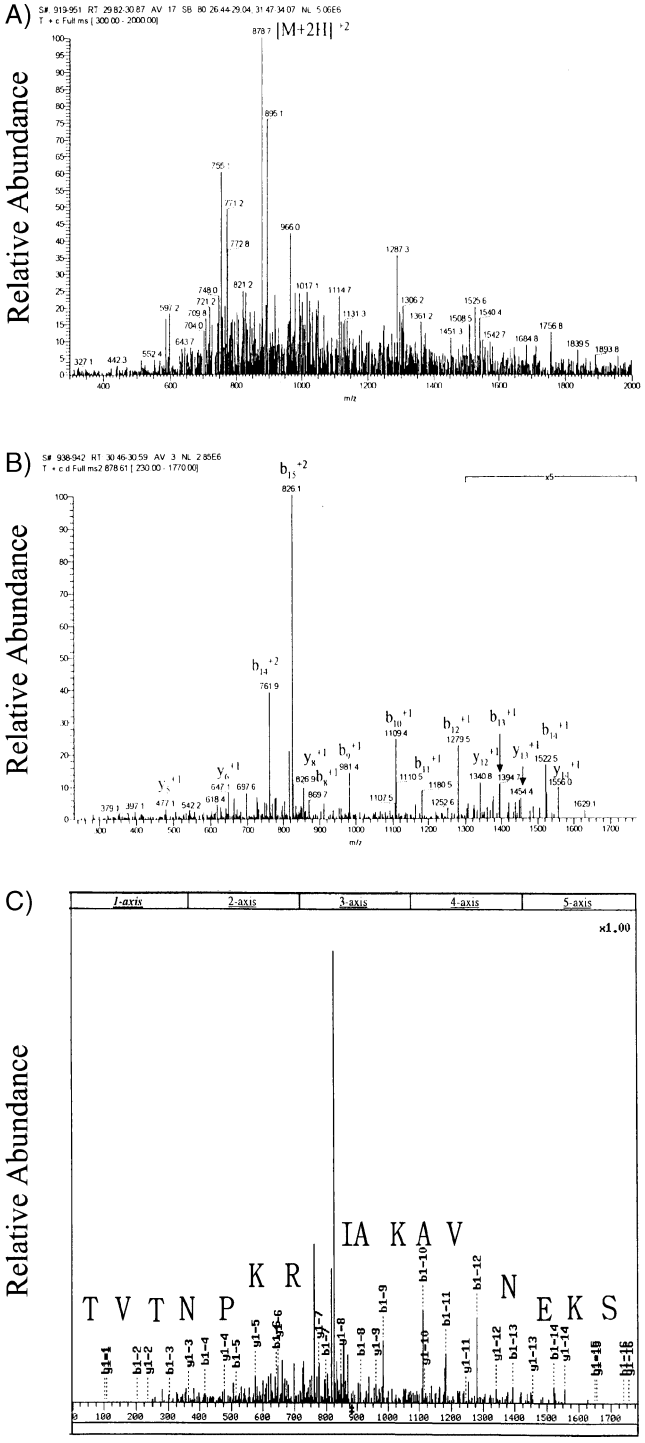

Fig. 4. Nano-LC/MS/MS analysis to identify α-enolase. A) Mass spectra of ionized peak 5 at 30.4 min in Fig. 3. B) Product ion spectrum of the $(M+2H)^+$ ion (m/z 878) by CID MS. C) The search result by the SEQUEST software against the OWL database. *Y*-axis, relative abundance; *X*-axis, m/z. The identified amino acid sequence is depicted. The search results of the six ions are summarized in Table II.

lase (Fig. 4C). Including this, the search results from the six ions that matched the database are summarized in Table II. Three peptides that were obtained from ions 1, 4, and 5 contained the peptide-binding motif to HLA-DR8 molecules.[32]

The antigenic activity of synthetic peptides To determine the antigenicities of the six peptides, all of them were synthesized. They were incubated with LDR0803, and the cytotoxic activity was assessed using TcOSC-20. We could not use F6 in the second RP-HPLC as a control in the cytotoxicity assay, since the total amount of the fraction was limited. ^{51}Cr-labeled LDR0803 cells were pulsed with the six peptides for 1 h. TcOSC-20 were then added at an E/T ratio of 10, and the cytotoxicity was measured after 6 h. TcOSC-20 showed a relatively strong cytotoxic response to the peptide derived from ion 5 (data not shown). We could repeat the experiment only with the four peptides corresponding to the ions 1, 4, 5 and a control peptide that contained a potential HLA-DR8 binding motif, since the availability of TcOSC-20 had dramatically diminished. TcOSC-20 only lysed LDR0803 cells pulsed with the peptide, namely, amino acid residues 321–336 of human α-enolase, corresponding to ion 5, in a dose-dependent manner (Table III). Similar results were obtained in repeated experiments although the cytotoxic responses were less marked. TcOSC-20 did not lyse LDR0803 cells when pulsed with the peptides derived from ion 1, ion 4, or the control peptide that bound to HLA-DR8.

DISCUSSION

In this study, MHC-class-II-restricted natural antigenic peptides isolated from a human oral squamous cell carcinoma cell line were analyzed. This report is the first description of $CD4^+$ T-cell epitopes of human squamous cancer cells. We found six peptides putatively presented by HLA-DR8 molecules. One of the peptides, derived from human α-enolase, provided strong cytotoxic reactivity to TcOSC-20; however, the response was not entirely convincing. It is not clear whether we missed some unidentified peptides in the screening process, since the total amount of the fractionated sample was quite limited. It is likely that we simply found abundant peptides that could be characterized in LC/MS/MS analysis. Thus, we are setting up cDNA transfection experiments to confirm whether α-enolase or another unknown gene product is responsible for the recognition by TcOSC-20. However, experiments using cDNA transfection followed by screening by $CD4^+$ T cells are difficult, since analysis of class II-restricted endogenous pathways is much more complicated than that of class I pathways,[18, 21] especially when the possibility of post translational modification is taken into account.[33] Moreover, the expression level of HLA class II molecules on OSC-20 significantly decreased,

Table II. Peptide Sequence of the Six Ions Determined by Nano-LC/MS/MS

Ion	Time (min)[a)]	MH^+	Sequence[b)]	Homologous protein
1	22.6	1616.5	VLLPKKTESHHKAK	histone H2A.1
2	28.3	1789.3	RVVSSIEQKTEGAEKK	protein kinase C inhibitor-1
3	28.7	2013.0	MRDQYEKMAEKNRKDA	cytokeratin 14
4	30.3	1508.5	KVLKQVHPDTGISS	histone H2B.1
5	30.4	1756.8	TVTNPKRIAKAVNEKS	human α-enolase
6	31.0	1916.8	RVVSSIEQKTEGAEKKQ	protein kinase C inhibitor 1

a) Retention time.
b) Underlined amino acids correspond to the peptide binding motif of HLA-DR8.

Table III. Antigenic Activities of the Synthetic Peptides Induced by TcOSC-20

Ion No.	1		4		5		Control peptide		Medium	OSC-20
Peptide (n*M*)	1	100	1	100	1	100	1	100	(−)	(−)
% Cytotoxicity	1.3	−3.0	3.8	4.2	8.1	12.0	3.7	1.3	−3.7	19.2

^{51}Cr-labeled LDR0803 cells were treated with either 100 n*M* or 1 n*M* of the four peptides that contained the HLA-DR8 binding motif, corresponding to ions, 1, 4, and 5, or control peptide, respectively. Medium and OSC-20 represent the cytotoxic activity of TcOSC-20 in response to LDR0803 cells alone and autologous tumor cell line, OSC-20 at an E/T ratio of 10, respectively. Boxes show the cytotoxic response of TcOSC-20 to a synthetic peptide corresponding to ion 5 and positive control OSC-20. Representative results from repeated experiments are given.

even after treatment with interferon (IFN) γ, with the result that we found it difficult to stimulate and maintain TcOSC-20 by using OSC-20. Unfortunately, the patient died. Therefore we were unable to perform further experiments.

It is well documented that α-enolase is constitutively expressed in the mitochondria of all cells. It is also expressed on the cell membrane of several cell types.[34, 35)] Although recent observations have clarified that endogenously-derived peptides are presented to MHC class II molecules, it is not clear whether the α-enolase-derived antigenic peptide can bind to MHC class II molecules. Recent studies have indicated that the peptide binding motif and requirements for substantial peptide affinity to MHC class II molecules are less rigorous as compared to those of MHC class I molecules. Indeed, Chicz *et al.* reported an extensive biochemical study of the relationship between MHC class II and endogenously derived peptide binding profiles that indicated substantial promiscuity among naturally processed peptides binding to HLA-DR molecules.[32)] Interestingly, they suggested that the naturally processed α-enolase-derived peptide (AEVYHDVAASEFF) presumably complexed with HLA-DR8, although it was not clear whether this peptide could act as an antigenic peptide for T cells. Obviously, this peptide is different from that which we isolated in the current study (TVTNPKRIAKAVNEKS). TcOSC-20 recognized autologous EBV-B cells, although to a much lesser extent as compared with OSC-20 (ref. 29) and Fig. 1 in this report). This may support the processing of α-enolase in professional antigen presenting cells as well as tumor cells in a class-II-restricted manner, though the cytotoxic responses by the CTL were different according to the cell types. In numerous autoimmune diseases, such as primary biliary cirrhosis, SLE and RA, autoantibodies directed against α-enolase exist.[34–37)] These data suggested that α-enolase-derived peptides could be presented on MHC class II molecules and that they could stimulate $CD4^+$ T cells.

We do not yet know how the enolase peptide might work as a tumor antigen. In c-jun-transformed rat fibroblasts, the expression of α-enolase is increased.[38)] It was also reported that α-enolase expression was enhanced in human lung adenocarcinomas and squamous cell carcinomas.[39)] Furthermore, when a similar biochemical approach was applied to isolate tumor-associated protein, α-enolase was identified from a melanoma cell line.[40)] Thus, although this antigen may induce some autoimmune responses, these observations also support the notion that α-enolase may be immunogenic in the context of tumor immunity.

The natural peptide corresponding to α-enolase residues 321–336 contained the binding motif to HLA-DR8 at N-terminal positions 2 and 6. This suggests that the *human α-enolase* gene product might be processed and could be presented on DR8 molecules. Thus, $CD4^+$ T cells could lyse squamous cancer cells in which they recognize the peptide presented on HLA-DR8 molecules. Therefore, our present results may indicate the possibility of a new application of tumor immunotherapy, especially for non-melanomas.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Drs. S. Ichimiya and Y. Kokai of the Department of Pathology for critical reading of the manuscript.

(Received April 26, 2002/Revised May 30, 2002/Accepted June 3, 2002)

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