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Cytotoxicity of Histocompatibility Leukocyte Antigen-DR8-restricted CD4$^+$ Killer T Cells against Human Autologous Squamous Cell Carcinoma

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Although CD8$^+$ killer T cells reacting against human autologous tumor cells have recently been studied in detail, little is known about the cytotoxic mechanism of CD4$^+$ T cells against such tumor cells. In order to investigate this, we have established CD4$^+$ cytotoxic T lymphocyte TcOSC-20 lines. TcOSC-20 showed selective cytotoxic activity against autologous OSC-20 cells, derived from a cancer of the tongue, in an HLA-DR-restricted fashion. HLA-DR8 (DRB1*08032) is the only DR molecule expressed on OSC-20 cells, and anti-DR8 monoclonal antibody could inhibit the cytotoxicity, suggesting that HLA-DRB1*08032 is the tumor rejection antigen-presenting molecule to TcOSC-20. The Fas ligand was expressed on TcOSC-20 lines, and its expression was induced upon mixed lymphocyte-tumor cell culture of autologous peripheral blood lymphocytes. Furthermore, the cytotoxicity of TcOSC-20 was inhibited by anti-Fas ligand antibody. These data imply that TcOSC-20 lines recognize the tumor antigenic peptide presented by HLA-DR8, and exert cytotoxicity against autologous tumor cells via a Fas-mediated cytotoxic pathway.

Key words: CD4$^+$ killer T cell — HLA-DR8 — Squamous cell carcinoma — Autologous tumor — Fas antigen

Cytotoxic T lymphocytes (CTLs) that specifically recognize and lyse human autologous tumor cells have been isolated from tumor-bearing patients, and recently the cytotoxic mechanism has been extensively studied.[1–10] Most of the work has been done by using CD8$^+$, T cell receptor (TCR) α/β^+ type-T cell clones or lines that specifically recognize autologous tumor cells in an HLA class I-restricted fashion. Recently, MHC class I-bound tumor rejection antigens have been defined by molecular cloning[9,11–13] and by reverse-phase high-performance liquid chromatography with trifluoroacetic acid elution.[14,15] These findings may make it possible to evolve more sophisticated cancer vaccination strategies.

It has been suggested that the cellular antigenic peptide derived from tyrosinase protein is recognized by CD4$^+$ T cells from melanoma patients in an HLA class II-restricted fashion.[16] This strongly implies that CD4$^+$ T cells also have cytotoxic potential in tumor immunity.[16–19] However, the mechanism by which CD4$^+$ CTL react against human autologous tumors is not known. We therefore established CD4$^+$ CTL TcOSC-20 that can lyze autologous squamous cell carcinoma OSC-20, and analyzed the cytotoxic mechanism.

Our data demonstrate that TcOSC-20 exerts cytotoxicity against autologous OSC-20 in an HLA-DR-restricted fashion, presumably involving HLA-DRB1*08032. We also demonstrated the involvement of Fas-Fas ligand interaction in the cytotoxicity in this autologous tumor system. These studies may have identified one of the effector mechanisms of host anti-tumor immunity, and should contribute to the improvement of specific cancer immunotherapies.

MATERIALS AND METHODS

Establishment of human autologous tumor cell line and CD4$^+$ CTL line Primary cultures and CTL induction were performed by the methods described previously.[1,2,20] In this study, CTLs reacting against tumor cell lines of the tongue were generated from the patient's peripheral blood lymphocytes (PBLs) by autologous mixed lymphocyte-tumor cell cultures (MLTC). Briefly, a human tumor cell line, OSC-20, was obtained from the metastatic lesion of a 58-year-old female with a moderately differentiated squamous cell carcinoma (SCC) of the tongue.[21] The HLA phenotype of the patient's PBL was HLA-A2, A11, B55, B46, Cw1, Cw9, DR8, DR12, DR52, DQ1, and DQ7. OSC-20 cells were maintained as adherent monolayers in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Prior to MLTC, freshly isolated PBL were cultured in AIM-V (Gibco, Grand Island, NY) medium with rIL-2 (300 u/ml, generously provided by Takeda Chemical Industry, Ltd., Osaka) for one week. Then, T cells were cultured in the absence of

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rIL-2 with irradiated autologous tumor cells (30 Gy) at the effector/target (E/T) ratio of 10:1. After MLTC for 24 h, T lymphocytes were harvested and replaced in AIM-V medium containing rIL-2. The CTL lines were generated by repeating MLTC three times in the same manner, and designated as TcOSC-20.

Monoclonal antibodies (mAbs) and fluorescence-activated cell sorter (FACS) analysis Various mAbs were used in the analysis of cell surface phenotypes and the cytotoxic mechanism between T cells and target tumor cells. MAbs reacting to CD4 (clone TD-4C5), CD8 (TD-3A2) and HLA-DR nonpolymorphic determinant (TC-8B1) were developed in our laboratory.[2,22] TC-8B1 reacts with all the DRB gene products (i.e., DRB1, B3, B4 and B5). MAb W6/32 to HLA-class I and mAb 38.1 to CD3 were purchased from American Type Culture Collection (Rockville, MD). Anti-TCR-α/β (TCR pan-α/β) and anti-TCR-γ/δ (TCRδ1) were purchased from T Cell Diagnostics, Inc. (Cambridge, MA). Anti-HLA DP (BRAFB6) and anti-HLA DQ (SPVL3) nonpolymorphic determinant mAbs were purchased from Serotec Ltd., England, and Immunotech, Marseille, France, respectively. MAbs reacting against HLA-DR52 (0635HA) and DR8/12 (HU-39) were kind gifts from Dr. Y. Iwaki at the University of Southern California and Dr. A. Wakisaka at the Hokkaido University School of Medicine, respectively. MAb NOK-1 to Fas ligand was kindly provided by Dr. H. Yagita, Department of Immunology, Juntendo University School of Medicine.[23] MAb 2D1 reacting to Fas molecules and control mAb 12A5 with the IgM isotype were established in our laboratory.[24] LC4 mAb reacts with TCRα/β expressed on SUP-T13 T lymphoma cells.[25] For FACS analysis, cells were incubated with a saturating amount of mAbs for 30 min on ice, then washed twice with PBS and stained with FITC-conjugated goat anti-mouse IgG/IgM for another 30 min on ice. These cells were analyzed by FACScan (Becton Dickinson, Mountain View, CA).

Target cells, cytotoxicity assays, and Fas-ligand involvement analysis In the cytotoxicity assay, several tumor cell lines were used in addition to autologous OSC-20 cells. OSC-19 (allogeneic tongue SCC line),[26] Daudi (Burkitt's lymphoma cell line), and K562 (erythroleukemia cell line) were prepared as target cells. Epstein-Barr virus-transformed autologous B cells (M-EB) was kindly established by Dr. S. Imai, Department of Virology, Cancer Institute, Hokkaido University School of Medicine. The cytotoxicity assay using ^{51}Cr was previously described.[1,3] Briefly, 1×10^4 target cells labeled with ^{51}Cr were plated in triplicate in 96-well U-bottomed tissue culture plates and mixed with various numbers of effector cells. Supernatants were harvested after 6-h cytotoxicity assays and the radioactivity was measured. The spontaneous release was usually less than 20–25% as compared to the maximum release. The result was calculated as: % cytotoxicity = (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100. In order to prevent LAK activity of CTL, rIL-2 was depleted from the culture 24 h before the cytotoxicity assays.[3]

In the blocking experiment using mAbs, the CTL line was treated with various concentrations of anti-CD3 (clone 38.1), anti-CD4 (TD-4C5) or anti-CD8 (TD-3A2) for 30 min at 37°C, and then the cytotoxicity assays were performed for 6 h. In some experiments, ^{51}Cr-labeled target cells were treated with a saturating amount of anti-HLA class I (W6/32), anti-HLA class II DP (BRAFB6), anti-HLA class II DQ (SPVL3), anti-HLA class II DR (TC-8B1), anti-HLA-DR52 (0635HA) or anti-HLA-DR8/12 (HU-39) for 30 min at 37°C, and the effector cells were added at various effector/target ratios. After 6 h, the supernatant was harvested and the radioactivity was measured. All determinations were made in triplicate, and the data were represented as the mean ± SE. Some results are given as the percent inhibition of cytotoxicity. The percent inhibition was calculated as: % inhibition = (% cytotoxicity without mAb − % cytotoxicity with mAb) × 100/ % cytotoxicity without mAb. To clarify the involvement of Fas antigen in the cytotoxicity, TcOSC-20 cells were treated with 25 and 50 μg/ml of anti-Fas ligand NOK-1 and control LC4 mAbs for 30 min at 37°C, and cytotoxicity assays against ^{51}Cr-labeled OSC-20 cells were performed for 6 h.

RESULTS

Cytotoxicity profiles of CD4$^+$ TcOSC-20 CTL line reacting against OSC-20 The patient's PBL were stimulated three times by MLTC with autologous OSC-20 cells, and CD3$^+$, CD4$^+$, CD8$^-$, TCR-α/β^+ T cell lines, designated as TcOSC-20, were established. Our preliminary study showed that TcOSC20 lines are composed of oligoclonal T cells (data not shown) as determined by reverse transcriptase-PCR of TCR $\alpha\beta$, which was described elsewhere.[27,28]

TcOSC-20 was then tested in a 6-h ^{51}Cr-release assay using autologous or several allogeneic tumor cell lines. As shown in Fig. 1, it exerted a cytotoxic potential on OSC-20 in a dose-dependent manner. The cytotoxicity of TcOSC-20 was not strong; it showed approximately 13, 18 and 24% specific cytotoxicity at E/T ratios of 5 : 1, 10 : 1 and 20 : 1, respectively. It also demonstrated more than 50% cytotoxicity at the E/T ratio of 50 : 1 (data not shown). Higher cytotoxicity might be obtained if cloned TcOSC-20 were employed, but single cell cloning has not been successful so far. In our current study, TcOSC-20 also showed moderate cytotoxicity against autologous EBV-transformed B cell line M-EB and very slight cyto-

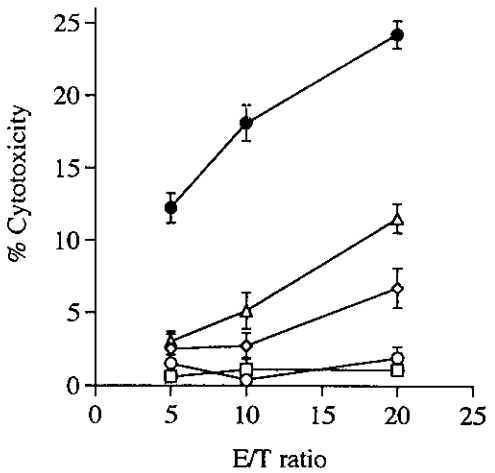

Fig. 1. The cytotoxicity of TcOSC-20 against ^{51}Cr-labeled autologous and allogeneic target cells with various E/T ratios. Bars represent mean±SE from five successive experiments. ● OSC-20, △ M-EB, ◇ OSC-19, ○ Daudi, □ K562.

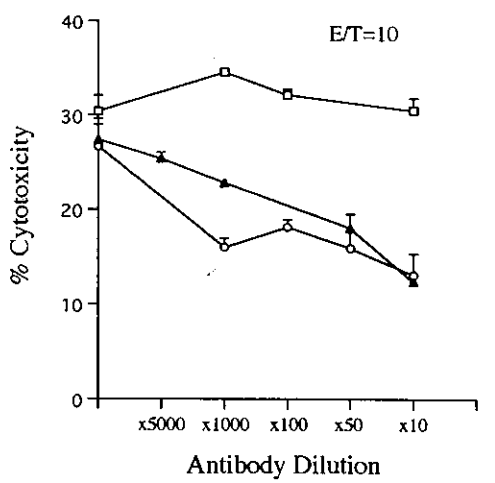

Fig. 2. Inhibition of TcOSC-20 cytotoxicity against OSC-20 cells by using various mAbs reacting against TcOSC-20. TcOSC-20 were preincubated with various dilutions of originally 1 mg/ml of purified anti-CD3 (○) mAb, anti-CD4 (▲) mAb, and anti-CD8 (□) mAb for 30 min prior to ^{51}Cr-release assay at the E/T ratio of 10 : 1. Bars represent mean ±SE.

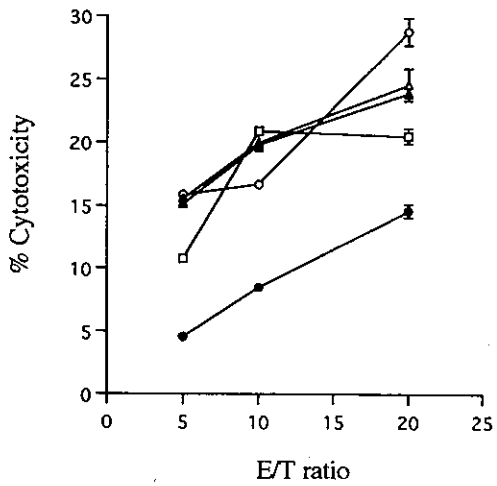

Fig. 3. Inhibition of TcOSC-20 cytotoxicity against OSC-20 cells by using various mAbs reacting against OSC-20. ^{51}Cr-labeled OSC-20 cells were preincubated with or without (▲) a saturating amount (10 μg/ml) of anti-HLA class I (□) mAb, anti-HLA class II DP (△), DQ (○) or DR (●) mAb for 30 min prior to ^{51}Cr-release assay at various E/T ratios. Bars represent mean±SE.

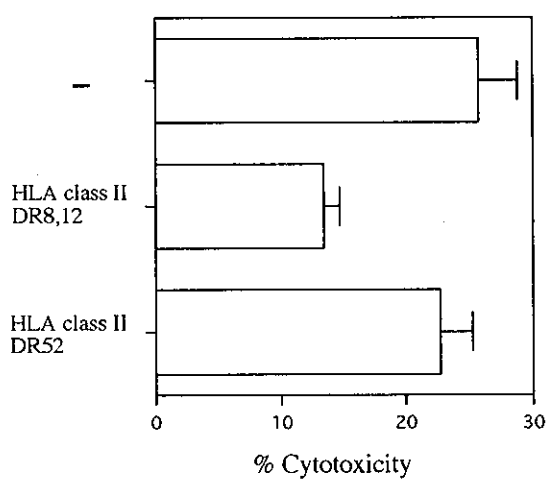

Fig. 4. Inhibition of TcOSC-20 cytotoxicity against OSC-20 cells with anti-HLA DR haplotype mAbs. ^{51}Cr-Labeled OSC-20 cells were preincubated with a saturating amount (10 μg/ml) of anti-HLA-DR52 and DR8, 12 mAb for 30 min prior to ^{51}Cr-release assay at the E/T ratio of 10 : 1. Bars represent mean±SE.

toxicity against allogeneic SCC OSC-19 when the effector/target ratio was increased. Perhaps this observation may reflect the oligoclonality of the TcOSC-20 line. However, other allogeneic target cells tested, including Daudi and K562, were not lysed. Furthermore, selectively high cytotoxicity of TcOSC-20 against OSC-20 cells remained even when OSC-20 cells were cultured in medium containing 10% human AB serum instead of 10% FCS (data not shown). This indicated that the recognition of TcOSC-20 was not due to artifacts di-

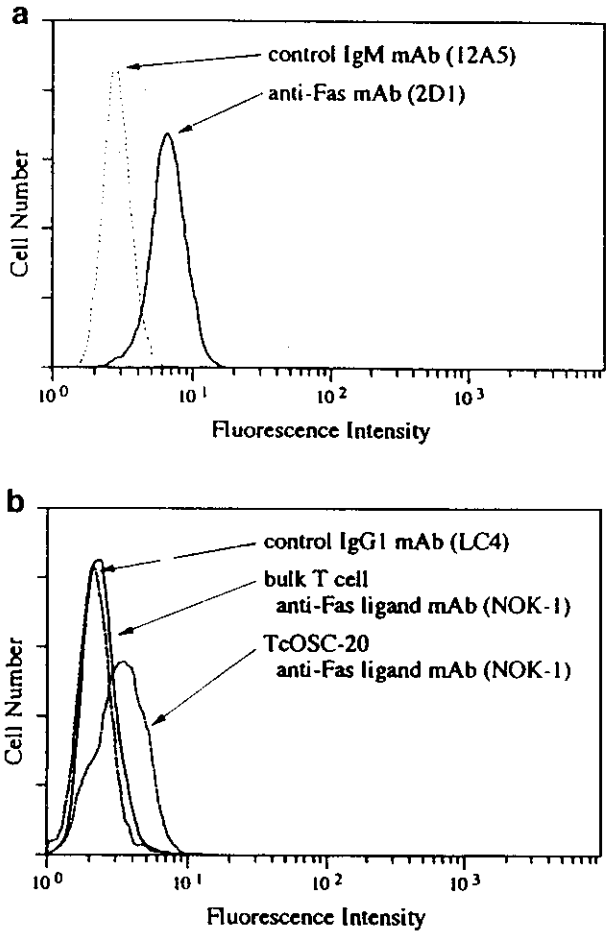

Fig. 5. Expression of Fas antigen on OSC-20 cells (a) and Fas ligand on TcOSC-20 (b). OSC-20 and TcOSC-20 cells were stained with a saturating amount (1 μg/ml) of anti-Fas 2D1 and anti-Fas ligand NOK-1 mAb, respectively. MLTC (−) bulk T cells were also stained with NOK-1 mAb, and compared with TcOSC-20.

rected against xenogeneic FCS components. These data suggest that OSC-20 predominantly expressed the antigen(s) that was recognized by TcOSC-20.

Analysis for the presentation molecule in the cytotoxicity To define the cytotoxic mechanism of TcOSC-20 against OSC-20, a blocking experiment was done using several mAbs which react with the cell surface antigen expressed on effectors or targets. First, TcOSC-20 was treated with various concentrations of anti-CD3, anti-CD4 and anti-CD8 mAbs, and the cytotoxic inhibition was assessed. As shown in Fig. 2, dose-dependent inhibition of the cytotoxicity against OSC-20 was observed only with anti-CD3 and anti-CD4 mAbs, indicating that TcOSC-20 are conventional $CD4^+$ CTLs. Second, we treated OSC-20 cells with a saturating amount of mAbs reacting with the

Table I. The Involvement of Fas-Fas Ligand in the Cytotoxicity of TcOSC-20 against Autologous OSC-20 Tumor Cells[a)]

	E/T ratio (% cytotoxicity±SE)	
	20 : 1	5 : 1
NOK-1 concentration		
(−)	39.6±2.16	7.1±0.89
25 μg/ml	33.3±0.52 (15.9)[b)]	5.2±0.11 (26.8)
50 μg/ml	ND[c)]	−0.2±1.45 (102.8)
LC4 concentration		
(−)	41.0±0.39	7.0±1.01
25 μg/ml	42.6±0.46 (−3.9)	7.8±0.55 (−11.4)
50 μg/ml	ND	7.1±0.81 (−1.4)

a) TcOSC-20 cells were preincubated with 25 or 50 μg/ml of anti-Fas ligand NOK-1 and control LC4 mAbs for 30 min prior to ^{51}Cr-release assay at E/T ratios of 20 : 1 and 5 : 1.
b) The values in parenthesis are % inhibition of the cytotoxicity.
c) ND, not determined.

nonpolymorphic determinant of HLA class I, HLA class II DP, DQ and DR, and the influence of these mAbs on the cytotoxicity was determined. The data showed that the cytotoxicity was blocked only with anti-HLA-DR mAb (Fig. 3). Thus, TcOSC-20 appeared to recognize preferentially the antigen(s) presented by HLA-DR on OSC-20 through the TCRα/β-CD3 complex.

The HLA-DR phenotype of the OSC-20 patient's PBL was HLA-DR8, DR12, and DR52. We determined which phenotype acted as the antigen-presenting molecule in the cytotoxicity. FACS analysis showed that OSC-20 cells do not express HLA-DR52, but express mAb-HU39-defined HLA-DR8/12 molecules (data not shown). Next, we determined DR at the DNA level of HLA-DR8/12 by using the modified PCR-restriction fragment length polymorphism method with DRB1 group-specific primers as described Kaneshige *et al.*[29] The data indicated that OSC-20 showed DRB1*08032, but the DRB1*1201 sequence was deleted in OSC-20 cells (data not shown). This suggested that mAb HU-39 detected HLA-DR*08032 expressed on the cell surface of OSC-20. Based on these observations, we examined whether mAb HU-39 could inhibit the cytotoxicity of TcOSC-20 against OSC-20 targets. As shown in Fig. 4, this mAb inhibited the cytotoxicity, although only partially. Taken together, the results suggested that the presenting molecule in the cytotoxicity between this pair of CTL and tumor cells may be HLA-DRB1*08032.

Involvement of Fas-Fas ligand interaction in the cytotoxicity CTL may exert their cytotoxic activity via Fas-Fas

ligand interaction in virus-specific CTL and tumor-specific CTL in animal models. To determine whether such a mechanism operates in the cytotoxicity between TcOSC-20 and OSC-20 cells, we assessed the expression of Fas and Fas ligand. As shown in Fig. 5a, FACS analysis indicated that OSC-20 expresses mAb 2D1-defined Fas molecule on the cell surface. Furthermore, the expression of mAb NOK-1-defined Fas ligand was clearly induced on TcOSC-20 as compared to PBL without MLTC (Fig. 5b).

We next performed a blocking experiment using NOK-1. TcOSC-20 was treated with 25 and 50 μg/ml of NOK-1 and isotype-matched control mAb LC4, washed, and mixed with ^{51}Cr-labeled OSC-20 at E/T ratios of 5 : 1 and 20 : 1. As shown in Table I, lysis of OSC-20 by TcOSC-20 was inhibited by NOK-1 treatment in a dose-dependent manner at an E/T ratio of 5 : 1, and was partially inhibited at 20 : 1. An isotype-matched control LC4 had no effect. These results indicate that the Fas-mediated cytotoxic mechanism may be predominantly involved in the cytotoxicity between TcOSC-20 and OSC-20.

DISCUSSION

Understanding of the antigen recognition by CTLs was greatly advanced by the discovery of the presentation mechanism of antigenic peptides by HLA molecules. CTLs, mostly CD8$^+$ TCRα/β types, recognize tumor rejection antigenic peptides bound to HLA class I molecules to exert their lytic action, and such antigens have been isolated from human melanomas by several laboratories.[9, 11–13, 15, 30–32]

In comparison to these CD8$^+$ CTLs, little is known about the cytotoxic mechanism of CD4$^+$ CTLs that can selectively lyze human autologous tumor cells. It is generally believed that CD4$^+$ T cells recognize exogenous antigenic peptides presented by HLA class II molecules. However, there are several recent reports suggesting the existence of a pathway by which HLA class II-restricted CD4$^+$ T cells also recognize endogenously derived antigens.[33] Further, Topalian *et al.* reported recently that the product of the tyrosinase gene was recognized by HLA class II-restricted CD4$^+$ T cells.[16]

It has not yet been clarified, however, whether CD4$^+$ CTLs have any effect on non-melanoma tumors. We therefore attempted to establish HLA class II-restricted CD4$^+$ CTL lines reacting against autologous tumors. Such CTL lines, TcOSC-20, were successfully established from PBL of the patient by MLTC. Although TcOSC-20 lines were thus generated with the aid of a rather extensive stimulation program, the present study strongly suggests that CD4$^+$ CTLs or their progenitor cells might have been circulating in peripheral blood, and might have played a role in the patient's anti-tumor immunity.

So far, at least two molecular mechanisms of T cell-mediated cytotoxicity have been demonstrated; one is a perforin-dependent pathway and the other, a Fas-dependent pathway.[34, 35] Although perforin is usually detected in IL-2-stimulated CD4$^+$ T cells, it has also been suggested that Fas antigen could be the major target molecule for CD4$^+$ CTL-mediated cytotoxicity.[36] In the Fas-dependent cytotoxic pathway, it is necessary to express both Fas ligand on effector cells and Fas antigen on target cells. In our present system, both of these molecules were detected in FACS analysis. It was also demonstrated that anti-Fas ligand mAb efficiently blocked the cytotoxicity of TcOSC-20 against autologous OSC-20 tumor cells. These data imply that this cytotoxicity is mediated predominantly via Fas-Fas ligand interaction. To our knowledge, this demonstration of CD4$^+$ CTL cytotoxicity against human autologous tumor cells is the first in the literature.

Meanwhile, the recognition and activation of the TcOSC-20 line seemed to be HLA-DR-restricted. The HLA-DR phenotype of this patient's PBL was DR8, DR12 and DR52. However, DR52 was not detected on the cell surface of OSC-20 tumor cells. Further, treatment of mAbs reacting with HLA-DR8/12 resulted in the inhibition of the cytotoxicity by TcOSC-20. Moreover, since it is known that DR52 and DR12 are colocalized in the same allele,[29] there was a possibility that DR12 might have been deleted in OSC-20 tumor cells. Indeed, DNA typing analysis suggested that the HLA-DRB1*08032 DNA sequence was present, but that of DR12 (DRB1*1201 in PBL) was deleted in OSC-20 tumor cells (data not shown). Overall, it seems likely that the presenting molecule is HLA-DRB1*08032.

We made a preliminary examination of several characteristics of the antigenic peptide which is recognized by TcOSC-20. First, there was a possibility that the antigens might have been derived from *in vitro* artifacts such as components of FCS, since MHC class II molecules preferentially bind to peptides derived from bovine albumin *in vitro*.[37] However, our study ruled out this possibility, since TcOSC-20 was similarly cytotoxic against OSC-20 cells even when these target cells were cultured in the presence of human AB serum instead of FCS (data not shown). This indicates that the antigenic peptides were not artifacts, but rather, endogenous cellular antigens. Second, our preliminary data also showed that the antimetabolite brefeldin A, which blocks the association between endogenously derived peptides and MHC molecules,[38] tended to inhibit the cytotoxicity. Thus, it is strongly suggested that TcOSC-20 recognizes endogenous intracellular peptides presented by HLA-DR, perhaps HLA-DRB1*08032. There remains a question mark over this finding, because this is the characteristic of one tumor which may have been selected through culturing. Confirmation is needed. Nevertheless, it is interesting

that MHC class II molecules can use the endogenous pathway of tumor antigen presentation.[33]

In order to verify the HLA-DRB1*08032-restricted cytotoxic activity of TcOSC-20, it would be useful to investigate the cytotoxicity on allogeneic cells expressing the HLA-DRB1*08032 antigen. However, such tumor cell lines are not available. As to the positive killing of autologous EBV-transformed B cell line, M-EB, there is a possibility that M-EB might also express only a low level of OSC-20 antigen recognized by TcOSC-20. We are currently analyzing the antigen(s) recognized by TcOSC-20, using acid elution of antigens and other techniques including reverse-phase HPLC and mass spectrometry. In conjunction with $CD8^+$ CTL epitopes, the determination of $CD4^+$ CTL- epitopes may lead to much more effective protocols for peptide-based vaccinations in SCC.

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