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T-Cell Receptor Gene Structures of HLA-A26-restricted Cytotoxic T Lymphocyte Lines against Human Autologous Pancreatic Adenocarcinoma

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We isolated two cytotoxic T lymphocyte (CTL) lines, which were independently obtained by mixed lymphocyte-tumor cell culture from tumor-infiltrating lymphocytes of a patient with pancreatic adenocarcinoma. Both lines behaved identically in all the functional aspects tested and appeared to be HLA-A26-restricted. We analyzed their T cell receptor (TCR) gene structures, including V-(D)-J junctional sequences, which are unique to each T-cell clonotype and contribute to TCR diversity. Each line consisted of a clonal T-cell expressing Vα18 and Vβ7. The α chain gene was composed of Vα18/JαF/Cα and the β-chain gene, of Vβ7.1/Dβ/Jβ1.4/Cβ2. The sequences were all in-frame and therefore should yield functional transcripts. The junctional sequences were identical between the two lines. These data suggested that the two CTL clones having the same CDR3 had descended from a common precursor lymphocyte. The clonal expansion of CTL lines with the identical CDR3 implies that they are directed against the same tumor antigen, which seemed to be immunologically dominant in the specific interaction between the CTL and the autologous pancreatic adenocarcinoma.

Key words: Tumor-specific CTL — Autologous human tumor — T cell receptor gene

Certain T-cells derived from tumor-bearing patients appear specifically to lyse autologous tumor cells. These cytolytic T-lymphocytes (CTLs) can be generated from peripheral blood, malignant effusions, lymph nodes and tumor-infiltrating lymphocytes (TILs) by mixed lymphocyte-tumor cell culture (MLTC). The majority of these CTLs present $CD8^+$ and TCR α/β phenotype. They specifically recognize autologous tumor cells mediating T-cell receptors (TCRs) in an MHC class I-restricted fashion.[1-4]

TCR is a heterodimer consisting of two polypeptide chains, designated as α and β chains. Both chains have variable (V), joining (J), and constant (C) regions. In the case of the β chain, a diversity (D) segment exists. The V-J and V-D-J junctions of the α and β chains, respectively, code for the third complementarity determining region (CDR3), which is considered to recognize complexes of antigenic peptide and self MHC molecule. Thus, the gene structures of CDR3 reflect properties of the tumor antigens.[5] Restricted TCR usage has been reported in autoimmunity,[6] allograft rejection,[7] and murine autoimmune encephalomyelitis,[8] though such restriction in tumor immunity is still controversial. In some studies, limited TCR V gene usage was found in CTLs and TILs,[9,10] while other reports demonstrated a diverse TCR usage.[11,12]

In the current study, we established independently two CTL lines against autologous pancreatic adenocarcinoma and analyzed their TCR gene structures. Each line consisted of a single T-cell clone expressing Vα18 and Vβ7. The gene structures including the V-J junction in the α-chain and the V-D-J junction in the β-chain appeared to be identical between the two lines. The reproducible and clonal expansion of the CTL with the equivalent CDR3 implies that the two lines had descended from a common precursor lymphocyte; they were directed against the same antigen. We consider that the molecular nature of the antigenic peptide to the CTLs is stable and possibly immunologically dominant in the specific interaction between the CTL clones and the autologous pancreatic adenocarcinoma.

MATERIALS AND METHODS

Culture of cell lines The procedure for establishment of pairs of CTL and autologous tumor cell lines was described in our previous paper.[1,3] Briefly, PUN human pancreatic carcinoma line was obtained from the malignant ascitic effusion by culturing for approximately 2 months after the initiation of primary cell culture. This line grew as monolayer cultures with a doubling time of approximately 36–48 h. Subcutaneous inoculation of 1×10^6 PUN cells into nude mice resulted in a 100% incidence of tumors with the histological features of moderately differentiated adenocarcinoma. T cells were separated and grown in a serum-free culture medium AIM-V (GIBCO, New York, NY) with 350 U/ml recombinant interleukin-2 (rIL-2; Shionogi Pharmaceutical Co., Ltd.,

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Tokyo). This condition permits the selective outgrowth of T-lymphocytes.

Establishment of CTL lines Freshly isolated TILs (ascites lymphocytes) at 5×10^6 were cocultured in RPMI 1640 without rIL-2 with 1×10^5 mitomycin C-treated or irradiated tumor cells as stimulators. After 24 h, these activated lymphocytes were harvested, and then cultured with an AIM-V medium containing rIL-2 of 350 U/ml. The cells were split as needed to maintain a concentration of approximately 1×10^5 cells/ml. We performed the MLTC every 10 days. HLA haplotypes of the established lines were determined for further analysis of the HLA-restriction element.

FACS analysis and monoclonal antibodies Cells were incubated with murine monoclonal antibodies (mAbs) at saturating concentrations for 30 min on ice, washed and stained with polyclonal goat anti-mouse antibody coupled with fluorescein isothiocyanate for another 30 min on ice. Samples were analyzed by FACStar (Becton Dickinson, Mountain View, CA). Cells were characterized by use of mAbs. Anti-MHC class I (W6/32) and anti-CD3 (38.1) were prepared by using hybridomas HB95 and HB231, respectively, purchased from American Type Culture Collection (Rockville, MD). Anti-CD4 (TD4C5), anti-CD8 (TD3A2) and anti-class II (TC8B1) were prepared in our laboratory. Anti-TCR-α/β (TCR pan-α/β) was purchased from T Cell Science, Inc. (Cambridge, MA). Anti-HLA-A2 (264HA-1), Anti-HLA-A11 (84HA-2), and anti-HLA-A26 (119HA-1) were purchased from One Lambda, Inc. (Canoga Park, CA).

Cytotoxicity assay and inhibition of cytotoxicity by mAbs or multiparous sera The ^{51}Cr release assay was performed as described previously.[1,3] For inhibition by mAbs, ^{51}Cr-labeled target cells in 50 μl of medium/well in 96-well plates were preincubated for 30 min at 37°C with 50 μl of anti-MHC class I, anti-HLA-A2, anti-HLA-A11, anti-HLA-A26 or anti-class II. The effector cells were also preincubated with anti-CD3, anti-CD4 or anti-CD8 for 30 min at 37°C, and cytotoxicity assays were performed for 12 h at 37°C. Since mAbs against HLA-B39, B54, C1 and C7 were not available, we used multiparous sera for the blocking assay. The multiparous sera were kindly provided by Shiraimatsu Pharmaceutical Co., Ltd. (Mizukuchi-cho, Shiga-ken).

Preparation of total RNA and cDNA Total RNA from lymphocytes was prepared in the presence of guanidium thiocyanate. For the polymerase chain reaction (PCR), the first-strand cDNA was synthesized from 5 μg of total RNA using reverse transcriptase. Briefly, RNA was heated in a reaction mixture containing 200 pmol of random hexamer at 70°C for 10 min and then quickly chilled on ice. The cDNA was synthesized at 37°C for 1 h in a final volume of 30 μl, containing 50 m*M* Tris-HCl, 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, 300 μM deoxynucleotide triphosphates, 17 μM dithiothreitol, and 500 units of Moloney murine leukemia virus reverse transcriptase (United States Biochemical, Cleveland, OH).

PCR procedure V-gene subfamily-specific PCR primer sequences were designed on the basis of known TCR Vα and Vβ gene sequences.[13,14] Sequences of the individual primers, including Vα1-Vα18, Vβ1-Vβ20, Cα and Cβ, were described in our previous paper.[10] All oligonucleotides were prepared on a PCR-MATE 391 DNA Synthesizer (Applied Biosystems, Inc., Foster City, CA). DNA fragments of TCR were amplified from cDNA using PCR as described previously. Briefly, 1% of the first-strand cDNA synthesized from CTL was subjected to amplification in a 50 μl reaction volume with 1 unit of Tth-DNA polymerase (Toyobo Co., Ltd, Tokyo), 200 μM deoxynucleoside triphosphates, 1 μM Vα or Vβ subfamily-specific primer, and 1 μM of the corresponding Cα or Cβ region primer. Amplifications were performed using 35 cycles of 95°C denaturation for 1 min, 55°C annealing of primers for 1 min, and 72°C extension for 1 min.

Southern blot hybridization PCR products were separated on 1.5% agarose gels along with molecular size standards. Expression of Vα or Vβ genes was visualized with ethidium bromide staining. The amplified product was Southern-blotted onto transfer membranes. Filters were prehybridized at 42°C for 24 h and then hybridized for an additional 48 h. The oligonucleotide sequence of the Cα probe was 5′-GAACCCTGACCCTGCCGTGT-ACC-3′, and that of the Cβ probe was 5′-CCCGA-GGTCGCTGTGTTTGAGCCATCAGAA-3′. For hybridization, the oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4-polynucleotide kinase (Toyobo Co., Ltd.).

Cloning and sequencing of PCR products The amplified products of PCR were electrophoresed in a 1.5% low-melting-point gel. Visualization of a band of the appropriate size on an ethidium bromide-stained gel indicated the presence of that TCR subfamily. The appropriate band was excised from the gel and ligated to the pBluescript II vector, which was subsequently used to transform *Escherichia coli* XL-1-Blue competent cells. Single plaques were picked up and grown. The cDNA was blunt-ended with Klenow fragment of *E. coli* DNA polymerase I (Toyobo Co., Ltd.) for the ligation experiment. Next, the 5′ ends of the cDNA were phosphorylated by T4-polynucleotide kinase. The recombinant plasmid DNA was purified for DNA sequence determination. Sequence reactions were performed with Sequenase 2.0 (United States Biochemical) by use of the dideoxy chain termination procedure. Sequences were compared with gene data bank entries (EMBL, Heidelberg; GenBank, Los Angeles, CA) and with previously published V, D, and J TCR gene segments.

RESULTS

Characterization of the two independent CTL lines: TcPUN line 1 and line 2 The tumor cell line (named PUN) was inoculated into nude mice and grew into highly differentiated pancreatic adenocarcinoma. After repeated *in vitro* stimulation with the PUN autologous pancreatic tumor cell line, two CTL lines (TcPUN line 1 and line 2) were generated independently from freshly isolated TILs in malignant peritoneal effusion (ascites). TcPUN line 1 was obtained two months earlier than TcPUN line 2.

TcPUN line 1 showed a phenotype of CD3(+), CD4(−), CD8(+), TCR-α/β(+), and exhibited specific cytotoxicity for PUN. In contrast, it did not lyse autologous ascitic fibroblasts. TcPUN line 1 was also not cytotoxic against Daudi B cell lymphoma line, K562 myeloid leukemia cell line, or HeLa human uterine cervical cancer cell line (data not shown).

The lysis of PUN by TcPUN line 1 was specifically and dose-dependently blocked by anti-CD3 and anti-CD8 mAb (Fig. 1a), but was not blocked by anti-CD4 mAb. The HLA haplotype of TcPUN line 1 was determined to be A11, A26, B39, B54, C1, C7, DR2, DR4.2, DRw53, DQ6, DQ3. Anti-class I and anti-HLA-A26 mAb specifically blocked the lysis, but anti-HLA-A2 (as a negative control) and anti-HLA-A11 mAbs had no effect. In addition, TcPUN line 1 did not lyse the HOT allogenic HLA-matched (HLA-A26) human ovarian cancer cell line (Fig. 1b). Multiparous serum of the other HLA elements (B39, B54, C1, or C7) did not inhibit the cytotoxicity at all (data not shown). Therefore TcPUN line 1 appeared to be an autologous tumor-specific HLA-A26-restricted CTL. TcPUN line 2 presented the same profile as that of line 1 in immunofluorescence phenotyping. Furthermore, line 2 behaved identically in all functional aspects tested and was concluded to be HLA-A26-restricted CTLs, as shown in line 1 (data not shown).

TCR gene usage of Vα and Vβ chains including their nucleotide sequences in the two TcPUN lines We studied TCR gene usages of TcPUN line 1 and line 2. cDNAs obtained from the two lines were amplified by PCR using Vα1 to Vα18 and Vβ1 to Vβ20 specific primers in combination with a Cα or a Cβ primer, respectively, as described in our previous paper.[10] Fig. 2a shows the ethidium bromide-stained gel. The PCR products were then hybridized with internal oligonucleotide probes for Cα and Cβ in order to ascertain the specificity of amplification. Basically the two lines showed the same hybridization signals in TCR α and β chains. Namely, in the Vβ series, TcPUN line 1 expressed a hybridization signal in only Vβ7, and no other signal was detected in any usage upon further exposure (Fig. 2b). This was also true for TcPUN line 2 (data not shown). In the Vα series, we

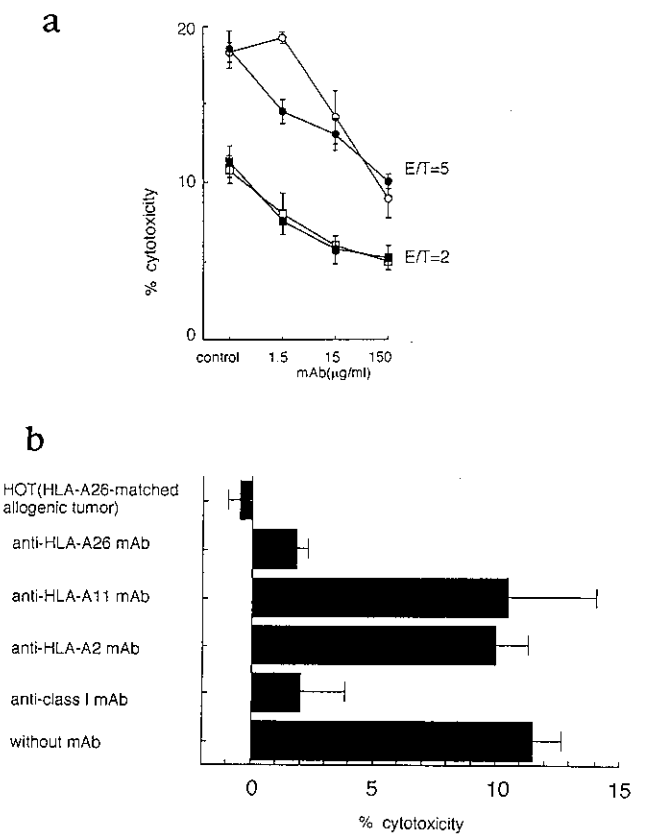

Fig. 1. Inhibition of specific CTL lysis by various mAbs. a: The cytolytic activity of TcPUN line 1 was inhibited by anti-CD3 (38.1) and anti-CD8 (TD3A2) mAbs, depending on the antibody dilution. Open circles and open squares represent anti-CD3 with effector/target (1×10^4 cells/well) ratio=5 and that with E/T=2, respectively; closed circles and closed squares represent anti-CD8 with E/T=5 and that with E/T=2, respectively. b: Anti-class I (W6/32) and anti-HLA-A26 (119HA-1) mAbs inhibited the cytolytic activity of TcPUN line 1. However, anti-HLA-A2 (264HA-1) and anti-HLA-A11 (84HA-2) had no influence on cytotoxicity. TcPUN line 1 did not affect the HOT, HLA-matched allogenic ovarian tumor cell line. E/T was 5 in each experiment. The final concentration of mAbs used in this experiment was 100 μg/ml.

observed hybridization signals of TcPUN line 1 with the expected length in a total of five Vα usages: Vα8, Vα9, Vα14, Vα15, and Vα18 (Fig. 2b). However, on an ethidium bromide-stained gel, definite counterpart bands of the appropriate size were seen in Vα 14 and 18 (Fig. 2 a). This was also the case for TcPUN line 2 (data not shown). These facts indicated that the two lines were highly clonal.

We analyzed nucleotide sequences of the TCR genes to identify a functional transcript. In the Vα series, the PCR products of Vα8, Vα9, Vα14, and Vα15 were finally

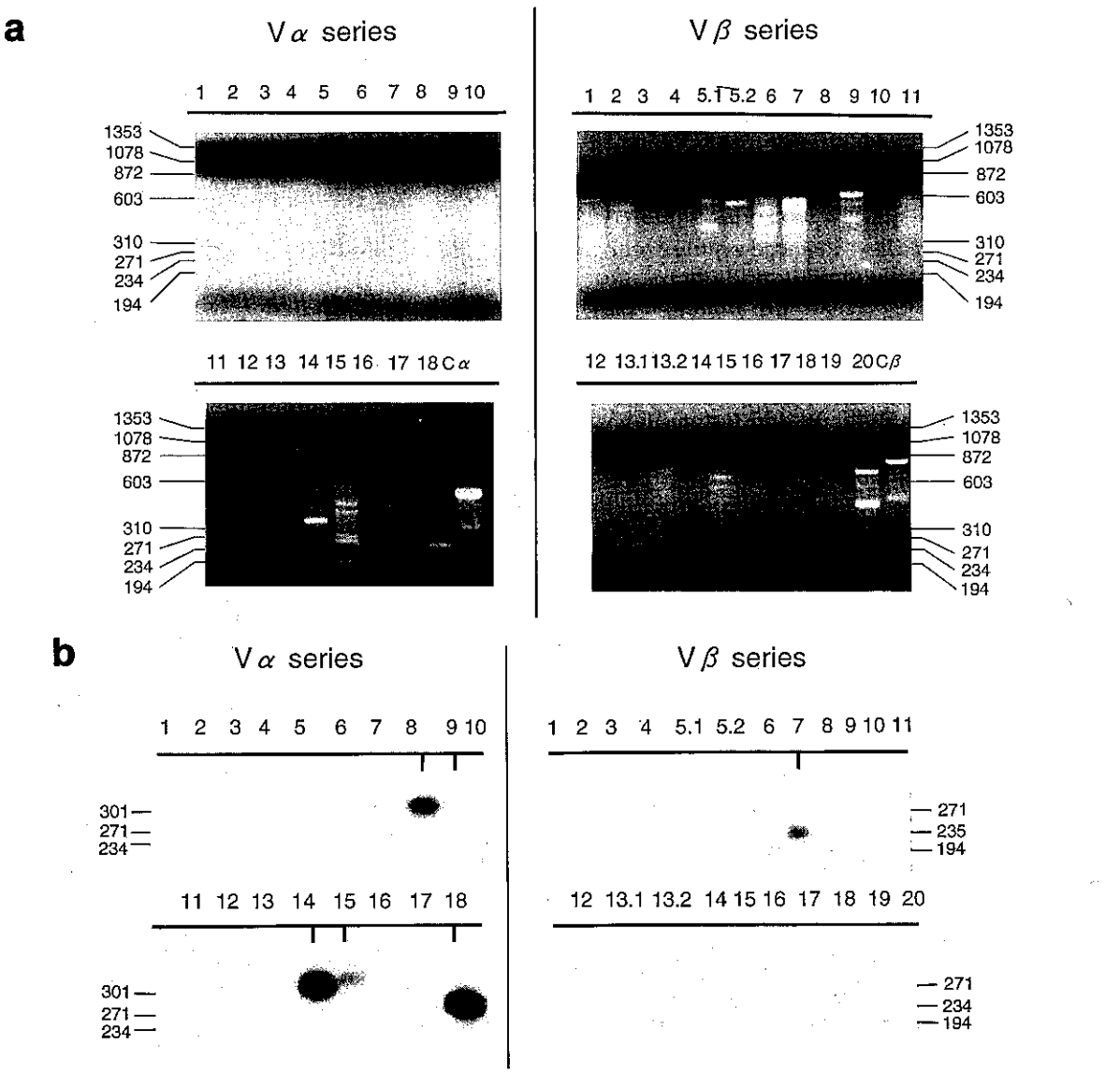

Fig. 2. a: Ethidium bromide staining of PCR-amplified cDNA of TcPUN line 1 with TCR Vα1-Vα18 and Vβ1-Vβ20 primers. b: TCR Vα and Vβ repertoire of TcPUN line 1 as detected by Southern blot analysis of the above PCR products. The experimental procedures were similar to those described in our previous paper.[10] TcPUN line 2 also presented the same hybridization signals. Ordinate, DNA sizes in base pairs.

determined to be out-of-frame, in spite of repeated attempts to determine the nucleotide sequences. The PCR product of Vα18, however, was found to be in-frame and had a productively rearranged nucleotide sequence: Vα18/JαF/Cα (Fig. 3). The nucleotide sequences, including V-J junction, were identical in the two lines.

The PCR product of Vβ7 also gave the same nucleotide sequences for the two lines except for a change at position 54 from A in line 1 to G in line 2. The sequences were in-frame and therefore should represent functional transcripts. Comparisons among published nucleotide sequence data suggested that the TCR β-chain gene was composed of Vβ7.1/Dβ/Jβ1.4/Cβ2 (Fig. 4). The V-D-J junctional sequences, which are unique to each T-cell clonotype and contribute to TCR diversity, were identical in the two lines.

Thus, in the two independently established CTL lines, each line consisted of a single T-cell clone expressing Vα18 and Vβ7. The V-J junction in the α chain and V-D-J junction in the β chain, which were supposed to be

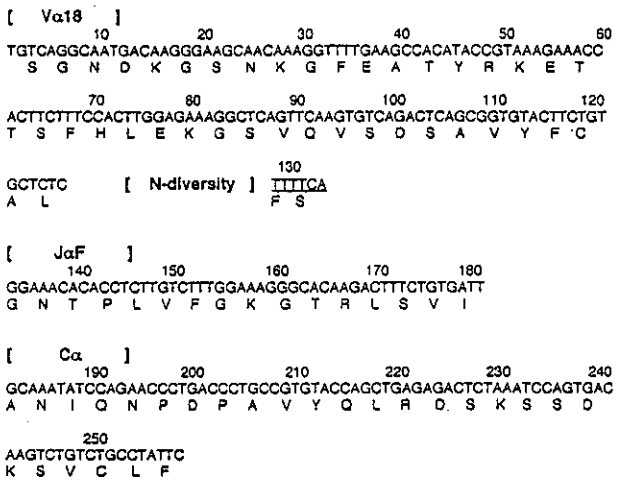

Fig. 3. Alignment of nucleotide sequences and corresponding protein sequences of TCR α chain from TcPUN line 1. The N-region is underlined. The nucleotide sequence of line 2 was completely identical with that of line 1.

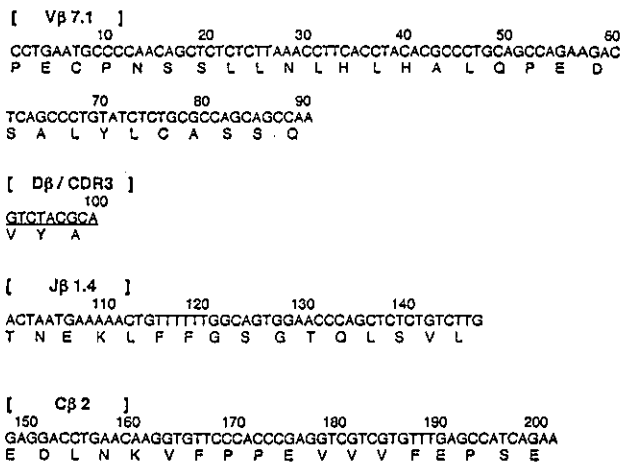

Fig. 4. Alignment of nucleotide sequences and corresponding protein sequences of TCR β from TcPUN line 1. The short D-segment including junctional sequences is underlined. In line 2, the nucleotide at position 54, CCA, was changed to CCG, but the coded amino acid (proline) was unchanged. Except for this nucleotide, all sequences were identical in these two lines.

the CDR3 equivalent portion, appeared to be identical in the two. These data indicated that clonal CTL lines with certain CDR3 were the dominant effector cells against the autologous pancreatic tumor cells.

DISCUSSION

Understanding of antigen recognition by CTLs was greatly advanced by the discovery of the self-MHC restriction. CTLs recognize peptide fragments bound to MHC class I molecules on cells that are targets for their lytic action. Recently, there have been increasing numbers of reports describing MHC class I-restricted recognition by CTL clones specific to various human autologous cancer cells.[1-4] These CTLs usually express TCR α/β type, and generally anti-CD3 mAb inhibited the cytolytic action. This fact suggests that CD3-TCR α/β complexes actually participate in the specific recognition of target tumor cells. Transfection of the TCR gene would allow us to prove the involvement of TCR α/β heterodimer in both the antigen specificity and the self-MHC restriction in anti-tumor immunity. A TCR gene transfection experiment was reported in a cytochrome *c*-specific, I-E^k restricted murine T cell clone.[15]

The characterization of TCR expressed by CTLs is one approach to understanding how the TCR recognize an antigen in the context of MHC. The properties of TCR expressed by TILs will also represent these local responses. Restricted TCR-gene expression has been demonstrated in various disorders, including human autoimmune diseases, renal allograft rejection, and murine autoimmune encephalomyelitis. In malignant diseases, TILs of uveal melanoma predominantly expressed T cell receptor Vα7.[9] A repertoire of TCR Vβ genes was used to recognize the antigen-HLA class I complexes on the surface of ovarian tumor cells, and two specific Vβ usages appear to mediate the antitumor activity.[16] In contrast, it was concluded from an analysis of TILs of metastatic melanoma that the T cell infiltrates were extremely diverse, with no preferential TCR gene segment usage.[11] In these studies, however, TCR V gene usage was determined in bulk cultures. Although the frequencies of TCR V gene subfamilies increased, the antitumor reactivity of the T cells presenting these receptors was uncertain. Furthermore, analysis by Southern blotting alone cannot distinguish between productively and nonproductively rearranged TCRs. One should recognize that this procedure has a limitation in determining which TCR mediates specific recognition of tumor antigens.

Thus, we must analyze TCR of clonal and functional CTLs. Three CTL clones, which lyse autologous pancreatic tumor cells in an MHC-restricted fashion, were previously reported.[17] They expressed the same TCR-β chain, indicating that they descended from a common precursor lymphocyte and were directed against the same antigen. In the autologous melanoma system, four independent MLTC cultures of TILs and two independent cultures of peripheral blood lymphocytes appeared to share exactly the same TCR. They had identical α and β V-(D)-J junctional sequences.[18] In this paper, we have

described two independent clonal CTL lines which lyse autologous pancreatic adenocarcinoma in an MHC-restricted fashion. The CTL lines developed from a single patient and presented the same TCR α/β usage with identical CDR3 gene structures. Our data suggest that these functional CTLs with a certain CDR3 were the dominant effector cells against the autologous tumor cells. Furthermore, the highly reproducible and clonal expansion of two independently obtained CTL lines with the same CDR3 suggests that the molecular nature of the antigenic peptide to the clonal CTL lines is stable and immunologically dominant in the specific interaction between the CTL lines and the autologous pancreatic adenocarcinoma.

Although in TcPUN lines strong messages of Vα8 and 14 and weak messages of Vα9 and 15 were observed, and the Vα14 message seems to be even stronger than Vα18, repeated analyses of nucleotide sequences showed that these PCR products, except for Vα18, were out-of-frame. Currently we can not explain this observation. PCR primers for Vα transcripts used in this experiment may pick up transcripts which are unrelated to TCR Vα.

TIL clones isolated from a single melanoma patient, including two HLA-A2 restricted and one HLA-A1 restricted, have been analyzed,[12] and alignment of junctional TCR gene sequences and polypeptide sequences from the HLA-A2 restricted clonotypes revealed no sequence homology or common structural motifs within the CDR3. In this case, the CTLs may have recognized different T-cell epitopes by chance and expanded into two different HLA-A2 restricted CTL clones. It is postulated that at least six distinct melanoma peptides can be presented by HLA-A2.[19] Thus, it is not surprising that multiple CTL clonotypes can be derived from a single patient, since various tumor-associated epitopes are presented in the context of the same restriction element.

Except for melanoma series, it is uncertain whether shared tumor antigens exist or not, since there are only a few reports about T-cell epitopes and TCR gene structures in other types of tumors. We have also found that autologous gastric tumor-specific CTL bearing the identical combination of TCR α/β heterodimer, including the CDR3 region, were expanded in two independent MLTC experiments (Ikeda *et al.*, submitted). Therefore, we speculate that certain tumor antigens expressed on tumor cells may be preferentially recognized by certain TCR. Analysis of such a dominant T-cell epitope will afford critical information for the development of immunotherapy-based cancer treatment.

Finally, we should note that the Vα18 subfamily was first determined in the AC9 clone[14]; we used this clone to make the Vα18-specific oligonucleotide primer in this study. Another study on the human T cell receptor α/β genes has identified additional subfamilies, particularly in Vα subfamilies and Jα gene segments.[20] As a result, AC9 has been renumbered as the Vα22 subfamily in a review.[21] The nomenclature of TCR subfamilies is still quite variable and needs to be systematized.

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