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Functional and T Cell Receptor Gene Usage Analysis of Cytotoxic T Lymphocytes in Fresh Tumor-infiltrating Lymphocytes from Human Head and Neck Cancer

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Twenty-one cytotoxic T lymphocyte (CTL) clones or lines that killed autologous tumor cells, but not allogeneic tumor, K562, or Daudi cells, were established from fresh tumor-infiltrating lymphocytes of two individuals (HP-1 and HP-2) with head and neck cancer by limiting dilution in the presence of recombinant interleukin-2. Sixteen (76%) of these 21 clones or lines comprised $CD4^+$ CTLs and the other five comprised $CD8^+$ CTLs. These observations suggest that autologous tumor cell-specific $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ CTLs are present *in vivo* at the tumor site in head and neck cancer. Analysis of T cell receptor (TCR) gene arrangements in 20 of the 21 CTL isolates with reverse transcriptase and the polymerase chain reaction revealed that five of 12 and five of eight isolates from HP-1 and HP-2, respectively, were clones, the other isolates being lines comprised of two or more clones. Each CTL clone showed a different combination of Vα and Vβ gene expression, suggesting that more than five different tumor-associated antigens may be expressed on head and neck cancer cells. In spite of the diversity of TCR αβ combinations, TCR Vα1, Vα3, Vα8, Vα10, Vβ8, Vβ9, and Vβ17 were also frequently expressed in both patients. These data suggest that specific CTLs proliferate oligoclonally and contribute to the specific immune response against head and neck cancer *in vivo*.

Key words: T cell receptor — Cytotoxic T lymphocyte — Squamous cell carcinoma — Tumor-associated antigen — Gene usage

Cytotoxic T lymphocytes (CTLs) specific for autologous tumor cells have been identified in tumor-infiltrating lymphocytes (TILs) from a variety of human carcinomas, including melanoma,[1,2] ovarian cancer,[3] and gastric cancer.[4,5] These CTLs are largely composed of T cells expressing the CD3 αβ T cell receptor (TCR) and specifically recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules. CTL-mediated responses to autologous head and neck squamous cell carcinoma (SCC) have been difficult to demonstrate, although evidence suggests that head and neck SCCs induce non-MHC-restricted effector mechanisms both *in vivo* and *in vitro*.[6,7] Recently, however, human CTL lines with restricted specificity for autologous human SCC were established from the peripheral blood lymphocytes (PBLs) of an individual with cancer of the tongue.[8] Advances in molecular biology have greatly contributed to elucidation of the mechanism of the interaction between the CTL TCR and tumor-associated antigens (TAAs) presented by MHC molecules. For example, a human tumor rejection antigen has been identified from melanoma.[9–11] This antigen is expressed on various tumor cells in the context of human leukocyte antigen (HLA) A1.[9,11–14] Furthermore, analysis of TCR gene usage has revealed preferential expression of the Vα7 gene segment in TILs associated with melanoma,[15] and gastric signet ring cell carcinoma.[4] In contrast, no preferential TCR gene expression was detected in TILs from metastatic lesions of cutaneous melanoma.[16]

We recently showed that TILs freshly isolated from 15 individuals with head and neck cancer preferentially expressed TCR Vα1, Vα8, and Vα10 genes.[17] Though T cells infiltrating the sinus mucosa of chronic sinusitis, studied as a control, also frequently expressed the Vα10 gene, expression of Vα1 and Vα8 genes was not detected in these cells. These results suggested that Vα1 and Vα8 expression may be relatively specific to TILs in head and neck cancer. However, a direct role of Vα1 and Vα8 in the antitumor response in head and neck cancer was not demonstrated because cytotoxic function studies were not performed. We have now isolated autologous tumor cell-specific CTLs from TILs associated with hypopharyngeal carcinoma in two individuals and have examined the functional characteristics and TCR gene usage of these cells.

MATERIALS AND METHODS

Subjects TILs and tumor cells were obtained from the primary lesions of two individuals (HP-1 and HP-2) with hypopharyngeal carcinoma, who had undergone surgery.

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The patients had received no anticancer drug or radiation therapy before surgery. In both subjects, the malignancy was diagnosed by histological examination. HP-1 (HLA haplotype: A26, A33, B61, B44, Cw3) was a 46-year-old man with a moderately differentiated SCC (T2N2bMO). HP-2 (HLA haplotype: A11, B39, B51, Cw7) was a 70-year-old man with a poorly differentiated SCC (T3N2bMO).

Isolation of TILs and tumor cells TILs and tumor cells were isolated as described previously.[18] Briefly, tumor tissue was placed in sterile saline containing penicillin G (100 units/ml) (Meiji, Tokyo), streptomycin (1000 μg/ml) (Meiji), and amphotericin B (500 μg/ml) (Squibb, Tokyo). After at least 30 min, the tissue was cut into pieces 1 to 2 mm^3 in size with scalpels, and the pieces were digested in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing protease (800 to 1000 units/ml) (Dispase; Godo Shusei, Tokyo). The digested tissue was filtered through a 200-gauze stainless steel mesh and washed twice with RPMI 1640, after which TILs and tumor cells were separated by centrifugation on a discontinuous (75 and 100%) Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. The isolated TILs were incubated at 37°C in RPMI 1640 containing 10% (v/v) human heat-inactivated AB serum from a healthy male blood donor, 10 m*M* Hepes, 2 m*M* L-glutamine (Linbro, Flow Laboratories, Irvine, UK), 0.1 m*M* nonessential amino acids (Linbro, Flow Laboratories), 0.05 m*M* β-mercaptoethanol, penicillin G (100 units/ml), and streptomycin (100 μg/ml) (complete medium) before limiting dilution analysis. Tumor cells and TILs not used immediately were suspended in RPMI 1640 containing 10% (v/v) dimethyl sulfoxide and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Kyoto Biken, Kyoto) and stored in liquid nitrogen as described.[19] When used as stimulator or target cells, tumor cells were thawed rapidly, diluted in RPMI 1640 containing 20% FBS, washed twice, and checked for viability by trypan blue exclusion; 80–95% of the viable cells were identified as tumor cells under a light microscope.

Limiting dilution analysis Limiting dilution analysis of TILs was performed as described[20] with minor modifications. Fresh TILs were seeded in 96-well round-bottomed culture plates (Linbro, Flow Laboratories) at densities of 0.5, 1, 2, and 5 cells per well. Each well contained irradiated (5000 rad) PBLs (10^5/well) from three different healthy donors as "feeder" cells in a final volume of 200 μl of complete medium supplemented with recombinant interleukin-2 (rIL-2) (200 units/ml) (Shionogi, Osaka) and phytohemagglutinin (10 μg/ml) (Difco, Detroit, MI). After 7 and 14 days, half of the medium from each well was replaced with complete medium supplemented with rIL-2 (200 units/ml) and 10^5 irradiated allogeneic PBLs. After 3 weeks of culture, the cells in each well were transferred to 24-well culture plates (Nunc, Roskilde, Denmark) containing 10^5 irradiated allogeneic PBLs and 5×10^4 irradiated (10,000 rad) autologous tumor cells per well. The growing lymphocytes were restimulated with 5×10^4 irradiated autologous tumor cells at weekly intervals in 2 ml of complete medium supplemented with rIL-2 (200 units/ml).

Flow cytometric analysis The growing lymphocytes were incubated with fluorescein isothiocyanate-conjugated monoclonal antibodies (mAb) to CD4 (Leu 3a) (Becton Dickinson, Mountain View, CA) or CD8 (Leu 2a) (Becton Dickinson) for 30 min on ice and washed twice. All incubations and washes were performed in phosphate-buffered saline (PBS) containing 0.1% NaN_3 and 2% FBS. Cells (10^4) were analyzed on a FACScan (fluorescence-activated cell sorter) (Becton Dickinson).

Cytotoxicity assays Cytotoxic activity was evaluated by means of a standard 4-h ^{51}Cr release assay. In addition to fresh autologous tumor cells, an allogeneic maxillary SCC cell line (HLA haplotype: A9, A28, Bw48, Bw54, Cw1, Cw4), a chronic myelogenous leukemia cell line (K562), and a B cell lymphoma cell line (Daudi) were used as targets. Target cells were incubated with 100 μCi of $Na^{51}CrO_4$ (Daiichi, Tokyo) for 60 min at 37°C, washed four times, and plated in triplicate at a final concentration of 5000 cells/well in 96-well round-bottomed microtiter plates (Linbro, Flow Laboratories) together with effector cells (effector:target ratio, 20:1). The plates were centrifuged at 200*g* for 5 min and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The culture supernatants were then collected with the Titertek collecting system (Linbro, Flow Laboratories) and the amount of radioactivity in each supernatant was determined with a gamma scintillation counter. The percent cytotoxicity (percent specific ^{51}Cr release) was determined as: [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100%. Maximum ^{51}Cr release was determined in the presence of 1% Triton X-100. Spontaneous ^{51}Cr release was assessed by incubation of tumor target cells with medium alone and did not exceed 28 or 20% of maximal release for fresh tumor cells and cultured tumor cells, respectively.

Preparation of RNA and cDNA synthesis Total cellular RNA was prepared from fresh TILs and growing lymphocytes by the guanidinium thiocyanate method with ISOGEN (Nippon Gene, Tokyo). RNA was detected by electrophoresis in a 1% agarose gel. Single-stranded cDNA was prepared from 4 μg of total RNA with reverse transcriptase. The RNA was incubated for 10 min at 70°C with random hexamers (Gibco BRL), and then for 50 min at 42°C and 5 min at 95°C in 20 μl of 1× synthesis buffer [50 m*M* KCl, 20 m*M* Tris-HCl (pH 8.4), and 2.5 m*M* $MgCl_2$] containing 0.01 *M* dithio-

threitol, 0.5 m*M* deoxynucleotide triphosphates, and 200 units of Superscript reverse transcriptase (Gibco BRL). The reaction mixture was finally incubated with 2 units of *Escherichia coli* ribonuclease H (Gibco BRL) for 20 min at 37°C.

Amplification of cDNA by polymerase chain reaction A 1-μl sample of cDNA was mixed with a polymerase chain reaction (PCR) mixture containing deoxynucleotide triphosphates (50 μM each), primers (5′Vα, 3′Cα, 5′Vβ, 3′Cβ), and 1×PCR buffer [50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.8), 2.5 m*M* $MgCl_2$, and 0.01% gelatin] in a final volume of 48 μl. Sequences of the individual primers were described previously.[17] The mixture was heated for 10 min at 98°C before addition of 1 unit of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). The PCR procedure, consisting of 35 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s, followed by final extension at 72°C for 10 min, was performed in a DNA thermal cycler (Perkin Elmer Cetus). PCR products were analyzed by 2% agarose gel electrophoresis.

Southern blot analysis The amplified products were confirmed by Southern blot analysis with specific oligonucleotide probes for Cα (5′-GAACCCTGACCCTGCCGTGTACC-3′) or Cβ (5′-AGCGACCTCGGTTGGGAACAC-3′). The PCR products were separated by electrophoresis in a 2% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham, UK). The membrane was then incubated for 30 min at 45°C in 0.1×standard sodium citrate (SSC) and 0.5% sodium dodecyl sulfate (SDS), and hybridized for 2 h at 40°C with ^{32}P-labeled Cα or Cβ probe. It was washed twice for 10 min at room temperature with 2×SSC and 0.1% SDS, and then with 6×SSC and 0.1% SDS, and exposed to X-ray film for 1, 12, or 24 h at −70°C.

RESULTS

TCR Vα and Vβ gene expression in fresh TILs TCR V gene expression was examined in fresh TILs from the two patients with hypopharyngeal cancer. Vα-Cα and Vβ-Cβ gene arrangements were visualized by ethidium bromide staining of PCR-amplified V gene cDNA separated on agarose gels and by hybridization on Southern blots with Cα- or Cβ-specific probes. PCR products corresponding to TCR Vα1, α2, α3, α4, α6, α8, α10, α12, α14, α15, and all Vβ segments were detected for patient HP-1 (Fig. 1). PCR products corresponding to TCR Vα1, α3, α6, α8, α10, α12, α14, α15, α18, and all Vβ segments except Vβ10 and Vβ12 were detected for patient HP-2 (data not shown).

Cytotoxicity and phenotype of CTLs TILs from subjects HP-1 and HP-2 were cloned by limiting dilution and tested for cell surface antigen expression and cytotoxicity against fresh autologous tumor cells and cultured allogeneic maxillary SCC, K562, and Daudi cells. Sixty-one proliferating microcultures were obtained by limiting dilution from fresh TILs, 21 of which showed significant specific cytotoxicity against autologous tumor cells (Table I). Nine of the 12 autologous tumor cell-specific CTL clones from HP-1 were $CD4^+$, and three were $CD8^+$; seven of the nine clones from HP-2 were $CD4^+$ and two were $CD8^+$ (Table I). The other 40 proliferating microcultures showed little or no cytotoxicity against autologous tumor cells or high cytotoxicity against allogeneic tumor cells, K562, or Daudi (data not shown).

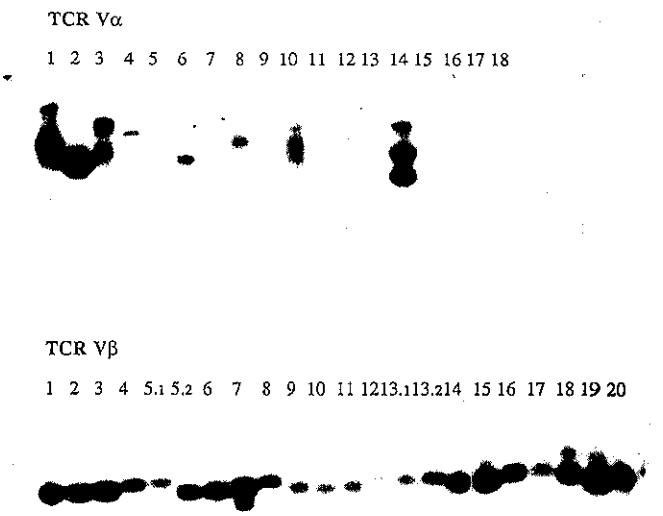

Fig. 1. TCR Vα and Vβ repertoire of fresh TILs from subject HP-1 as detected by Southern blot analysis of PCR products amplified from cDNA with TCR Vα(1–18)-Cα and Vβ(1–20)-Cβ primers. Hybridization was performed with Cα or Cβ internal oligonucleotides.

TCR Vα and Vβ gene expression in autologous tumor cell-specific CTL clones Five of the 12 HP-1 autologous tumor cell-specific CTL clones and five of eight specific HP-2 clones showed only one Vα and one Vβ rearranged transcript (Table II). (Clone HP-2-9 could not be examined because of an insufficient number of cells.) Each of these CTL clones showed a different Vα and Vβ combination. The other 10 CTL clones showed two or more Vα and Vβ transcripts and therefore actually represented oligoclonal lines. T cells expressing Vα10 were the most frequent among both HP-1 and HP-2 clones: eight of 12 (67%) and three of eight (38%), respectively. T cells expressing Vα1, Vα3, and Vα8 were also dominant among the autologous tumor-cell specific CTLs. Vβ8, Vβ9, and Vβ17 were also frequently expressed.

Table I. Limiting Dilution Analysis of TILs from Two Individuals with Hypopharyngeal Carcinoma

Subject	Clone	Phenotype	Cytotoxicity (%)			
			Autologous tumor	Allogeneic tumor	K562	Daudi
HP-1	1	CD4	30.1	7.4	4.1	3.1
	2	CD8	27.2	−1.2	1.2	6.1
	3	CD4	55.4	8.9	8.0	4.4
	4	CD4	22.4	7.4	2.4	7.7
	5	CD4	27.1	0.3	1.7	5.8
	6	CD4	19.1	2.0	1.5	4.7
	7	CD8	30.2	6.6	8.8	9.7
	8	CD4	23.5	0.3	3.6	4.4
	9	CD8	27.8	4.9	5.5	4.8
	10	CD4	29.0	8.5	4.2	5.1
	11	CD4	24.7	3.9	6.8	8.8
	12	CD4	19.4	0.7	2.1	4.4
HP-2	1	CD4	17.7	7.9	2.7	1.6
	2	CD8	15.3	8.8	5.0	4.7
	3	CD4	26.6	6.6	11.1	10.4
	4	CD4	13.8	7.0	4.2	4.4
	5	CD8	12.6	2.3	4.3	2.6
	6	CD4	13.3	7.7	9.1	7.6
	7	CD4	12.5	2.7	9.5	5.8
	8	CD4	17.3	3.4	3.5	2.5
	9	CD4	20.4	7.3	6.1	4.9

The cytotoxicity of TIL clones against autologous and allogeneic tumor cells, K562, and Daudi was measured by a 4-h ^{51}Cr release assay. Percentage cytotoxicity is presented as the mean of triplicate determinations. Analysis of surface antigens was performed with fluorescein isothiocyanate-conjugated mAbs to CD4 or CD8.

Table II. Analysis of TCR V Gene Expression in Autologous Tumor Cell-specific CTLs

Clone	Vα	Vβ
HP-1-1	10	5.2
HP-1-2	10	8
HP-1-3	3	2
HP-1-4	1	17
HP-1-5	14	3
HP-1-6	8, 10	3, 5.1
HP-1-7	3, 15	9, 15
HP-1-8	10, 14	4, 5.2, 9
HP-1-9	3, 10	6, 8, 13.1
HP-1-10	10, 15	2, 4, 7, 8, 16, 18
HP-1-11	1, 10, 15	9, 13.1, 13.2
HP-1-12	3, 8, 10, 15	2, 4, 5.2
HP-2-1	10	17
HP-2-2	8	6
HP-2-3	8	17
HP-2-4	3	7
HP-2-5	1	8
HP-2-6	9	6, 9
HP-2-7	3, 10	8, 17
HP-2-8	1, 8, 10	3, 8, 16

TCR V gene expression of each CTL clone was determined by reverse transcription, PCR amplification, and Southern blot analysis with Cα- or Cβ-specific oligonucleotide probes.

DISCUSSION

TILs consist mainly of $CD4^+$ and $CD8^+$ T lymphocytes. Clonal analysis of TILs from metastatic melanoma and renal cell carcinoma has demonstrated that both $CD4^+$ and $CD8^+$ T lymphocytes exhibit cytolytic activity against autologous tumor cells.[1] Unlike TILs from melanoma, all TIL clones from renal carcinoma also lysed allogeneic tumor cells and some lysed K562 cells. On the basis of these results, Itóh *et al.*[1] suggested that TILs from metastatic melanoma differ from those isolated from other solid tumors in that they show autologous tumor cell-specific cytotoxicity. We have now shown that TILs associated with hypopharyngeal cancer also contain autologous tumor cell-specific CTLs. Our procedure for limiting dilution analysis of TILs was similar to that of Itoh *et al.*,[1] with the exceptions that we did not use phorbol myristate acetate and that we replaced 10% FBS with 10% human AB serum. Thus, differences in experimental conditions are unlikely to be responsible for the different results obtained with TILs from renal carcinoma and hypopharyngeal carcinoma. Because Itoh *et al.*[1] performed limiting dilution analysis of TILs from only one individual with renal cell carci-

noma, it is possible that the nonspecific tumor cell lysis observed with these cells is atypical. Alternatively, the allogeneic tumor cells they tested may have shared common HLA class I antigens with the autologous tumor cells (they did not mention the HLA class I types of the tumors). The allogeneic tumor cells that we analyzed did not share any HLA class I antigens with the autologous tumor cells. Furthermore, Schendel *et al.*[21] recently described a renal carcinoma TIL line that showed autologous tumor cell-specific cytolytic activity. Similarly, head and neck SCC can be sufficiently immunogenic to induce CTL lines with restricted specificity for autologous tumor cells.[8] Taken together, these results support the idea that TILs from hypopharyngeal carcinoma, renal carcinoma, and melanoma contain autologous tumor-specific CTLs.

Cancer cells express multiple antigens that are recognized by CTLs.[3,22-25] Because CTLs recognize TAAs in association with HLA class I molecules through the $\alpha\beta$ TCR, a limited repertoire of TCR V gene expression corresponding to these TAA-HLA complexes would be expected to exist. Peoples *et al.*[26] suggested that a selective repertoire of TCR Vβ gene expression, specifically Vβ2 and Vβ6, is responsible for recognition of the antigen-HLA class I complex on ovarian cancer cells and for mediation of antitumor activity. Similarly, a dominant HLA-A2-restricted T cell clone expressing TCR Vα8 and Vβ2 and directed to a melanoma lineage-associated antigen was isolated from a large set of TILs from an HLA $A2^+$ melanoma patient.[27] Moreover, quantitative PCR revealed that the specific TCRβ chain (Vβ2.1/Dβ1/Jβ1.1/Cβ1) expressed by the dominant TIL clone represented 19 and 18.4% of all $V\beta2^+$ cDNA clones from fresh melanoma TILs or from the surgical tumor specimen, respectively. Our previous analysis of fresh bulk TILs from head and neck SCC suggested that CTLs expressing Vα1 and Vα8 were the major contributors to tumor immunity in these cancers.[17] To examine directly the relation between specific Vα and Vβ gene expression and specific tumor recognition, we have now investigated Vα and Vβ gene usage in tumor-specific CTLs from two patients with hypopharyngeal cancer. We seeded fresh TILs at densities of 0.5, 1, 2 and 5 cells/well for limiting dilution analysis. Analysis of TCR Vα and Vβ gene expression suggested that 10 of 20 CTL cultures were derived from single cells, whereas the remaining 10 were oligoclonal lines. The five single cell-derived CTL clones from each patient all expressed different combinations of Vα and Vβ genes, indicating that each tumor expressed more than five different TAAs. We performed limiting dilution analysis with fresh autologous tumor cells rather than tumor cell lines, which may show less TAA diversity. Despite their different HLA class I phenotypes, T cells expressing Vα10 were predominant among CTL clones and lines from both patients. Vα1, Vα3, and Vα8 were also frequently expressed on CTLs. Together with the results of fresh TIL analysis,[17] our current data suggest that TILs expressing not only Vα1 and Vα8 but also Vα10 directly contribute to tumor immunity in head and neck cancer.

Although preferential TCR Vβ gene expression was not apparent in fresh TILs, limiting dilution analysis revealed preferential usage of Vβ8 (30%), Vβ9 (20%), and Vβ17 (20%). Such dominant expression of TCR Vα and Vβ genes in autologous tumor cell-specific CTLs supports the hypothesis that CTLs clonally or oligoclonally proliferate or accumulate *in vivo* at the site of head and neck SCC growth. TAAs recognized by CTL clones with specificities restricted to head and neck SCC will probably be identified in the near future, given that a melanoma antigen recognized by autologous HLA A2-restricted CTLs has been shown to be encoded by the tyrosinase gene.[28] Such identification will help to elucidate in greater detail the mechanism of interaction between autologous tumor-specific CTLs and tumor cells in patients with SCC of the head and neck.

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