Human Cytotoxic T-Cell Lines with Restricted Specificity for Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Human cytotoxic T-lymphocyte (CTL) lines with specificity restricted for autologous squamous cell carcinoma of the head and neck (SCCHN) were established from peripheral blood lymphocytes obtained at the time of surgery and again at two different times after surgery from a patient with cancer of the tongue. The CTL lines were cultured in the presence of interleukin (IL) 2, IL-4, and autologous tumor (AuTu) cell monolayers. All three lines were CD3+CD8+CD11b+HLA-DR+ T-cell receptor α/β+. They were tested in 4-h 51Cr release assays against SCCHN cell lines (n = 5) and a variety of nonsquamous human tumor (n = 5) and normal (n = 5) cell targets and was found to lyse only AuTu (PCI-50) and three allogeneic SCCHN cell lines. Lysis of AuTu and the three allogeneic SCCHN targets by the established CTL lines appeared to be major histocompatibility complex class I restricted, since it was blocked by monoclonal antibodies to class I histocompatibility complex antigens. The CTL lines proliferated in vitro in response to autologous PCI-50 or an allogeneic SCCHN cell line (PCI-1). The lines have been maintained in culture in the presence of AuTu monolayers and retained cytotoxicity against AuTu for over 20 weeks.

The AuTu (PCI-50) cell line was tested for in vitro sensitivity to cytotoxic or cytostatic effects of various effector cells, including the CTL lines. PCI-50 targets were resistant to lysis by resting human mononuclear cells but sensitive to IL2-activated natural killer cells in 4-h 51Cr release assays. In comparison with IL2-activated natural killer cells, the CTL line mediated lower levels of lysis against AuTu. Growth of PCI-50 cells in culture was significantly inhibited by a combination of γ-interferon and IL2 or by high concentrations of tumor necrosis factor α. While supernatants of IL2-activated natural killer cells were growth inhibitory, those of the CTL lines were not. On the other hand, lysis of AuTu targets by the CTL line was increased by preincubation of the tumor cells with tumor necrosis factor α or γ-interferon. These cytokines augmented expression of HLA-class I, HLA-class II, and intercellular adhesion molecule I, but not squamous cell carcinoma-associated antigens, E7 and A9, on PCI-50 cells. The CTL lines described are the first with restricted specificity for autologous SCCHN ever reported and their availability will facilitate studies of the AuTu T-cell response in head and neck cancer.

INTRODUCTION

SCCHN2 cell lines or fresh tumor cells are generally resistant to lysis by nonactivated immune effector cells such as peripheral blood NK cells or T-lymphocytes obtained from normal volunteers (1). Also, fresh PBL, lymph node lymphocytes, or TIL obtained from patients with SCCHN have not been able to lyse SCCHN targets in 4-h 51Cr release assays (2-4). However, these tumor cells have been shown to be quite sensitive in vitro to lymphokine-activated killer cells, IL2-activated human effector cells, conditioned media of A-NK cells (5) or cytotoxins such as IL2, IFNγ, or TNFα (6, 7). In a nude mouse model of human SCCHN, growth of the tumor could be effectively inhibited by cytokine-activated human effector cells or their soluble products (5, 8). Furthermore, we have recently demonstrated that SCCHN are able to activate fresh or cultured human NK cells (5). As a result of such activation in vitro, NK cells were shown to up-regulate expression of activation antigens, levels of mRNA for various cytokines, antitumor cytotoxicity, and proliferation. These results suggest that non-MHC-restricted immune responses elicited by human SCCHN can play a major role in tumor growth inhibition both in vitro and in vivo in a nude mouse xenograft model.

The role of CTL in antitumor immune response to SCCHN is not known. Although these tumors are generally well infiltrated by T-lymphocytes, it has not yet been possible to demonstrate the presence of AuTu-specific CTL among the TIL, lymph node lymphocytes or PBL-T-cells in patients with SCCHN. This is in contrast to patients with other types of cancer, where the presence of CTL specific for AuTu in peripheral blood or tumor has been recently demonstrated in a variety of human carcinomas, including metastatic melanoma (9, 10), ovarian cancer (11, 12), renal cell carcinoma (13), gastric cancer (14), glioblastoma (15), or pancreatic cancer (16). These antitumor CTL have been mainly CD3+CD8+TCRαβ+ and MHC class I restricted. In melanoma, and perhaps in renal cell carcinoma, antitumor-reactive CTL appear to be important for successful adoptive immunotherapy with TIL (17, 18). In vitro ability to lyse AuTu has been shown to correlate with clinical responses to TIL therapy in patients with metastatic melanoma (18). From previous studies, it is unknown whether SCCHN are sufficiently immunogenic to induce a CTL response in vivo or whether immune T-cells play any role in resistance to the growth of such tumors. To the best of our knowledge, it has not been thus far possible to generate effector cells specific for AuTu from lymphoid cells of patients with head and neck cancer.

In this paper, we report the generation and characterization of CTL with specificity restricted to AuTu and a small number of allogeneic SCCHN. Such CTL could be repeatedly generated from the peripheral blood of a patient with SCC of the tongue, indicating that the CTL precursors were present in the patient’s peripheral blood for at least 2 years after surgery. Our data indicate that specific T-cell responses to AuTu can be detected in at least some patients with SCCHN.

MATERIALS AND METHODS

Patient.

The patient was a 92-year-old male with SCC of the tongue (T2N0M0), who underwent partial glossectomy at the University of Pittsburgh Medical Center, Eye and Ear Hospital (Pittsburgh, PA). Histologically the tumor was a well differentiated SCC.

PBL Isolation.

PBL from the patient were isolated by Ficoll-Hypaque centrifugation prior to surgery and then at two other times, at 18 months and 20 months, after surgery. PBL were examined for viability, counted, and cryopreserved.

Establishment of Autologous SCCHN Cell Line (PCI-50).

The fresh tumor specimen obtained under sterile conditions was washed 3 times in Hank’s balanced salt solution (Gibco, Grand Island, NY) containing streptomycin (100 μg/ml), penicillin (100 IU/ml), and amphotericin B (1 μg/ml). Washed tumor tissue was trimmed of fat and necrotic material and finely minced with scalpels.

Received 10/22/92; accepted 1/11/93.

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1. The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; NK, natural killer; PBL, peripheral blood lymphocytes; TIL, tumor-infiltrating lymphocytes; IL, interleukin; IFN, interferon; A-NK, IL2-activated NK; TNF, tumor necrosis factor; MHC, major histocompatibility complex; AuTu, autologous tumor; TCR, T-cell receptor; SCC, squamous cell carcinoma; TCM, tissue culture medium; LU, lytic units; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ICAM-1, intercellular adhesion molecule I; IL2R, IL2 receptor; TAA, tumor-associated antigen(s).
into 1-2-mm³ fragments in a sterile Petri dish (Costar, Cambridge, MA). The fragments of tissue in TCM were transferred to T-25 tissue flasks and were maintained undisturbed for 1 week at 37°C in a humidified atmosphere of 5% CO₂ in air. The TCM used was Eagle’s minimal essential medium supplemented with 1% (v/v) nonessential amino acid mixture, 2 mM glutamine, 100 μg/ml of streptomycin, 100 IU/ml of penicillin, and 15% (v/v) fetal calf serum (Gibco) prescreened for Mycoplasma and viruses. Flasks containing tumor tissue fragments were observed weekly for evidence of growth in an inverted-phase microscope. Fibroblasts outgrowing from explants were removed at weekly or biweekly intervals, using a cell scraper (Costar No. 3010) and differential trypsinization with 0.05% (w/v) trypsin (Gibco) in 0.02% (w/v) EDTA, as described earlier (1). Cultures of cells with the epithelial morphology were extensively washed with TCM following each trypsinization and incubated further in the presence of mitogen-prestimulated and irradiated PBL to generate a population of PCI-50 cells and submitted for histologically examined.

**Growth of PCI-50 in Nude Mice.** Athymic 6-week-old female BALB/c mice were obtained from Taconic Farm, Germantown, NY, and maintained under pathogen-free conditions in the animal facility. The mice were splenectomized 2 weeks before tumor injections. One day prior to inoculation of tumor cells, all mice were given i.p. injections of cyclophosphamide (200 mg/kg; 4 mg/0.1 ml/mouse; Sigma Chemical Co., St. Louis, MO) and anti-asialo GM1 antibody (10 mg/kg; 0.2 mg/0.2 ml/mouse; Wako, Dallas, TX). To establish the tumor, 5 x 10⁶ PCI-50 cells were injected s.c. in the right flank of each mouse in a group of five animals. The tumors were harvested 6 days after injection of PCI-50 cells and submitted for histologically examined.

**Generation of CTL against PCI-50.** The patient’s cryopreserved or fresh PBL were used as a source of CTL precursors. The cells were counted and checked for viability using trypan blue dye. The lymphocytes were cultured in serum-free AIM-V (Gibco) medium in the presence of IL2 (300 IU/ml; Cetus Corp., Emeryville, CA), IL4 (300 units/ml; Immunex Corp., Seattle, WA), and phytohemagglutinin P (5 μg/ml; Sigma) for 2 weeks in 24-well plates (Costar). After 2 weeks of primary culture, PBL were cocultured either with autologous irradiated (8000 R), which were added to cultures once a week, or with viable PCI-50 cells at the lymphocyte:tumor ratio of 5:1 in AIM-V medium supplemented with 300 IU/ml of IL2 and 300 units/ml of IL4. Fresh medium supplements were added twice a week. In cultures containing viable tumor cells, PBL appeared to grow well and when their number was greater than 3 x 10⁶/ml, lymphocytes were transferred to a T-25 flask (Costar) containing partially confluent (30-50%) viable tumor cell monolayers. The lymphocyte:tumor ratio was maintained at 5:1. Cultures were monitored for the cell number, phenotype, and cytotoxicity at regular intervals.

**Generation of A-NK Cells.** A-NK cells were prepared from allogeneic PBL obtained from normal donors as previously described by us (19). Briefly, monocyte-depleted PBL at a concentration of 5 x 10⁶ cells/ml were incubated in TCM containing 6000 IU/ml of recombinant IL2 (Cetus) in plastic flasks for 24 h. At the end of the incubation period, the plastic-adherent cells were supplemented with TCM containing 6000 IU/ml IL2 and cocultured in the presence of mitogen-pretreated and irradiated PBL to generate a population highly enriched in A-NK cells. These cells were maintained in culture at a concentration of 1.5-2 x 10⁶ cells/ml by supplying fresh TCM containing 6000 IU/ml IL2 as needed. The cell cultures and supernatants were harvested between days 10 and 14 of growth.

**Target Cells.** In addition to the autologous SCCHN cell line (PCI-50), 4 allogeneic SCCHN cell lines [PCI-1, -2, -4A, -4B (1)], as well as K562, a chronic myelogenous leukemia cell line; Daudi, B-cell lymphoma; LP and SW, cholangiocarcinomas (20); HR, gastric cancer (14); normal fibroblasts (n = 2); and normal keratinocytes (n = 3) were used as target cells for cytotoxicity assays. The cell lines were maintained in culture as described earlier (1) and passaged by trypsinization.

**Cytotoxicity Assays.** Cytotoxicity of PBL and CTL was determined using 4-h miniaturized ⁵¹Cr-release assays as described earlier (2). Briefly, 1 x 10⁶ cell targets labeled with ⁵¹Cr (5 μCi/ml; New England Nuclear, Boston, MA) were plated in triplicate in wells of a 96-well V-bottomed plate (Costar) and mixed with effector cells at effector:target cell ratios ranging from 25:1 to 3:1. Cells were centrifuged at 1000 rpm for 5 min and incubated for 4 h at 37°C in a CO₂ incubator. The amount of ⁵¹Cr released into the supernatant (20 μl) was measured using a beta counter (LKB, Pharmacia, Gaithersburg, MD). Maximal radioactive release was determined in wells containing target cells only after addition of 5% (v/v) Triton X-100. The percentage of specific lysis was calculated as:

\[
% \text{of specific lysis} = \frac{\text{Mean experimental cpm} - \text{mean spontaneous cpm}}{\text{Mean maximal cpm} - \text{mean spontaneous cpm}} \times 100
\]

The LUs of cytotoxicity were calculated according to the method of Pross et al. (21). One LU was defined as the number of effector cells required for 20% lysis of 1 x 10⁶ target cells, and the number of lytic units present in 10⁷ effector cells was calculated.

In blocking experiments, CTL were preincubated with various concentrations of anti-CD3 (Leu4), anti-CD8 (Leu2a), anti-CD4 (Leu3a), anti-CD56 (Leu19), anti-TCR α/β (WT31), or mouse IgG (isotype control), all from Becton Dickinson, Mountain View, CA, for 30 min at 4°C before their addition to ⁵¹Cr-labeled target cells. In some cases, target cells were incubated with anti-HLA class I mAb (W6/32, provided by Dr. Olivia Finn, Pittsburgh Cancer Institute); anti-HLA-DR mAb (obtained from Dr. Massimo Trucco, Pittsburgh Cancer Institute); antibodies against SCC-associated antigens, A9 (22) and E7 (kindly donated by Dr. Thomas Carey, University of Michigan (23)); or E48, U36, K928, K984, or K931 antibodies (kindly provided by Professor Gordon Snow, Free University, Amsterdam, the Netherlands (24-26)), before cytotoxicity assays. Inhibition was calculated as:

\[
% \text{of inhibition} = 1 - \frac{\text{% of specific lysis in mAb-treated wells}}{\text{% of specific lysis in control wells}} \times 100
\]

In some experiments, PCI-50 cells were incubated with 1000 units/ml IFNγ (Biogen, Cambridge, MA) or 1000 units/ml TNFα (Knoll Pharmaceuticals, Whippany, NJ) for 72 h prior to cytotoxicity assays to examine the effects of cytokines on target cell susceptibility to lysis by the CTL line.

**Effects of Cytokines or Conditioned Media on Growth of the PCI-50 Cell Line.** The effects of various cytokines on growth of the PCI-50 cell line were determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay (27). Briefly, doubling dilutions of each cytokine, a combination of cytokines, or conditioned media from CTL or A-NK cell cultures were obtained in a 96-well flat-bottomed plate (Costar), using TCM as a diluent. Tumor cells (5 x 10⁵/well) were added to a final volume of 200 μl/well. The plate was incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. On day 3 of culture, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide solution (0.5% w/v; Sigma) were added to each well, and the plates were incubated for 4 h. Following incubation, supernatant (150 μl) was removed from each well and replaced with 150 μl of dimethyl sulfoxide (Sigma) to dissolve the formazan crystals. The plates were placed on a shaker for 15 min and read in a plate reader (Dynatech Laboratories, Inc., Chantilly, VA), using a wavelength of 570 nm. Results from four wells for each dilution of the cytokines tested were expressed as percentage of control ± SD.

**Flow Cytometry.** The phenotype of fresh or cultured lymphocytes was determined by two-color flow cytometry, as described previously (2). Briefly, cells were adjusted to a concentration of 1 x 10⁶/ml in PBS-0.1% (v/v) sodium azide solution, and 0.2 ml of this cell suspension was incubated with 5 μl of fluorescein- or phycoerythrin-labeled mAbs at 4°C for 30 min. The cells were then washed three times in PBS-sodium azide and fixed in 2% (w/v) paraformaldehyde solution in PBS. Two-color analysis was performed on a FACSscan (Becton Dickinson). The mAbs used were purchased from Becton Dickinson and included the following specificities: Leu4 (anti-CD3); Leu2a (anti-CD8); Leu3a (anti-CD4); Leu19 (anti-CD56); Leu15 (anti-CD11b); anti-IL2Ra (anti-CD25); anti-IL2RB (p75); anti-HLA-DR; anti-TCR α/β. As controls, mouse isotypes IgG1 and IgG2 were used in all experiments. The mAb to p75 IL2R was purchased from Endogen.

In addition, expression of HLA antigens, ICAM-1 or SCC-associated antigens on PCI-50 cells was examined. The tumor cell suspensions (2 x 10⁶/tube) were first incubated with 5 μl of anti-HLA class I or several concentrations of one of the following mAbs, E7, A9, E48, U36, K928, K984, or K931, at 4°C for 30 min, washed, and then incubated with fluorescein-labeled goat anti-

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mouse IgG (TAGO, Burlingame, CA) at 4°C for 30 min. Fluorescein-conjugated anti-ICAM-1 (AMAC, Westbrook, MA) and anti-HLA DR mAbs (Becton Dickinson) were also used in this study.

**Proliferation Assay.** Proliferative responses of the CTL line were examined by coculturing 10⁴ responder cells with irradiated (8000 R) autologous PCI-50 cells (at responder:stimulator ratios of 1:1:1-8), allogeneic tumor cells or normal keratinocytes in 96-well round-bottomed plates at 37°C for 3 days in AIM-V medium supplemented with 300 IU/ml of IL2 and 300 units/ml of IL4. The cells were pulsed with 1 μCi of [³H]thymidine 16 h before cell harvest and [³H]thymidine incorporation was measured.

**Statistical Analysis.** The significance of differences between experimental and control groups was analyzed using Student's t test or Wilcoxon's signed rank test, as appropriate. The level of significance was set at P < 0.05.

**RESULTS**

**Establishment and Characteristics of the PCI-50 Cell Line.** PCI-50 cell line was established from fresh SCCHN tumor explants. Once established, the tumor cell line grew rapidly (doubling time, 27.3 h). In culture, the PCI-50 line grew as sharply demarcated, compact islands of cells with a distinct epithelial morphology (Fig. 1A). The conditioned medium of the cell line contained small amounts of prostaglandin E₂ (up to 6 pg/10⁶ cells) and almost no TGFβ (up to 0.03 pg/10⁶ cells). Histologic examination of hematoxylin and eosin-stained sections obtained from the patient’s tumor showed a well-differentiated SCC, containing numerous keratinized epithelial pearls (Fig. 1B). The tumor line was tumorigenic in nude mice. Following a s.c. injection of tumor cells (5 × 10⁶ per mouse), tumors (7–10 mm in diameter, V = 250–300 mm³) developed within 4 weeks. On the other hand, these tumors grew s.c. in nude mice and had a poorly differentiated morphology (Fig. 1C).

**Generation of the CTL Lines Reactive to PCI-50.** To induce AuTu-reactive effector cells, irradiated (5000 R) or viable tumor cells were coincubated with PBL obtained from the patient at a ratio of 5:1 in AIM-V medium in the presence of 300 IU/ml IL2 and 300 units/ml IL4. The PBL obtained prior to surgery or those obtained at two times after surgery and cultured with irradiated PCI-50 cells generally stopped growing by week 5. Cryopreserved fresh TIL did not proliferate at all. In contrast, PBL cocultured with nonirradiated viable tumor cell monolayers grew exponentially, and their cytotoxicity against AuTu continued to increase. Initially, these PBL had high levels of cytotoxicity against K562 or Daudi and showed no cytotoxicity against PCI-50 targets (Fig. 2A). By week 6 in culture, the effector cells no longer lysed K562 or Daudi targets but had substantial anti-PCI-50 cytotoxicity. The CTL line lysed PCI-50 cells (e.g., 1295 LU/10⁶ cells, at week 10) and was maintained in long-term culture for over 20 weeks in the presence of viable tumor cells, IL2, and IL4. As shown in Fig. 2B, by week 10–12 of culture, nearly all proliferating cells were CD3⁺DR⁺ and CD8⁺, while the proportion of CD4⁺ T-cells was less than 10%. However, by week 20, the CD4⁺/CD8⁺ ratio of cultured cells shifted to about 1, and the CTL lost cytotoxic activity. When the same patient's PBL were obtained at 18 and 20 months after surgery, CTL lines again were generated, with reactivity against PCI-50 AuTu. These other CTL lines were similarly studied for phenotypic and functional characteristics and found to be identical to the first CTL line. These CTL lines also retained cytotoxic activity against PCI-50 targets for about 12 weeks in culture. No sign of tumor recurrence was observed in the patient during 2 years after surgery. This is of interest, since it indicates that CTL precursors for autologous SCCHN were present in the blood for at least 20 months in the absence of all detectable tumor. In mixed cultures containing tumor cells and CTL, rosettes of CTL surrounding the tumor cells were consistently observed at 24 h after tumor stim-

ulation (Fig. 3). The presence of rosettes indicated that lymphoid cells were able to bind to AuTu cells in culture.

**Phenotypic and Functional Characteristics of the CTL Line.** To determine phenotypic properties of the CTL lines, flow cytometry studies were performed at various times in culture. As shown in Fig. 2B, the culture established from PBL obtained before surgery contained more than 90% of CD3⁺CD8⁺ T-cells by week 6. At week 8, the phenotype of the CTL line was CD3⁺ (99%), CD8⁺CD11b⁻ (90%), TCR α/β⁺ (93%), HLA-DR (90%), CD56⁺ (72%), CD25⁺ (52%), and IL2R p75⁺ (93%). The presence of IL2R on SCCHN cell lines and normal keratinocytes was described by us earlier (28). In contrast to many other SCCHN cell lines, which usually express low levels of CD25 (28), the level of expression of CD25 (the IL2Rα chain) on PCI-50 was considerably greater, approaching that seen on keratinocytes in primary culture (28). The CTL lines established sub-

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**Fig. 1.** (A) Microscopic characteristics of the PCI-50 line, which grows as a compact monolayer. × 315. (B) Histological sections of the original tumor removed at the time of surgery. H & E. × 315. (C) Histological sections of the tumor established by s.c. injection of PCI-50 cell line derived from this human tumor. H & E. × 315.
CTL FOR HUMAN SQUAMOUS CELL CARCINOMA

Fig. 2. Changes in cytotoxic activities against K562 (□), Daudi (▲), or PCI-50 (○) cells of PBL cocultured with AuTu in the presence of IL2 and IL4 (A). (B) Changes in the phenotype during culture of these PBL. PBL were obtained from the patient before surgery.

sequently (i.e., from PBL obtained at 18 and 20 months after surgery) also had the same phenotype at week 8 in culture (data not shown).

The original CTL line was tested in 4-h ⁵¹Cr release assays against SCCHN cell lines (n = 4) and a variety of nonsquamous human tumor (n = 5) and normal (n = 5) cell targets but was found to lyse only the autologous SCCHN cell line (PCI-50, 482–1295 L U₂₀/10⁷ cells) and 3 allogeneic SCCHN (PCI-1, -2, -4B, 219, 132, or 141 L U₂₀/10⁷ cells, respectively; see Fig. 4). The CTL did not lyse allogeneic normal keratinocytes (n = 3), fibroblasts (n = 2), or a variety of nonsquamous cell lines tested: gastric carcinoma (HR), 2 cholangiocarcinomas (SW and LP), K562, or Daudi. Thus, our data suggest that this CTL line probably recognizes a SCC-associated antigen(s) expressed on autologous and allogeneic SCCHN lines.

In order to better define cytotoxicity of AuTu by the CTL line, blocking experiments with mAbs were performed. Incubation of CTL with mAbs specific for the TCR α/β, CD3, CD8 (Fig. 5A), or HLA class I antigens (Fig. 5B) resulted in dose-dependent inhibition of cytotoxicity against PCI-50, and similar results were obtained with PCI-1 target cells (data not shown). In contrast, mAbs to CD4 or CD56 (Fig. 5A) or to HLA-DR or SCC-associated antigens (A9 or E7) did not block AuTu cytotoxicity (Fig. 5B). Moreover, five additional mAbs to SCC-associated antigens (E48, U36, K928, K984, or K931) also did not inhibit cytotoxicity of the CTL against PCI-50 (data not shown). These results indicate that recognition of an unknown antigen on autologous and some allogeneic tumor cells by CTL was MHC class I-restricted and involved the CD3-TCR complex. HLA typing indicated that PCI-50 shared B44, DR4, and DQW3 antigens with PCI-4 (A and B refer to cell lines established from the primary and recurrent tumor, respectively). Unfortunately, no HLA typing data were available for PCI-1 or PCI-2 cell lines. Studies are in progress with additional cell lines to confirm the HLA-restricted nature of the CTL line-SCCHN cell interaction.

To determine whether the CTL lines also proliferated in response to tumor cells, [³H]thymidine incorporation tests were performed. As shown in Fig. 6, the CTL line established from PBL obtained prior to surgery responded by dose-dependent proliferation to PCI-50 (11,962 ± 1,742 cpm) and PCI-1 (5,418 ± 735 cpm) but not to epithelial gastric cancer cell line, HR (1,544 ± 166 cpm), or to normal keratinocytes (1,642 ± 152 cpm). The CTL had a considerably higher level of response to autologous than to allogeneic SCCHN line.

Effects of Cytokines on Susceptibility of PCI-50 Targets to CTL or Their Supernatants. SCCHN cell lines have been shown by us earlier to be NK cell resistant but sensitive to lymphokine-activated killer cells in 4h ⁵¹Cr release assays (27). The data presented in Table 1 demonstrate that PCI-50 targets were resistant to resting NK cells (normal mononuclear cells) but very sensitive to adherent A-NK cells and that the CTL lines we established selectively lysed autologous tumor but not K562 or Daudi targets. In our previous experiments, we observed that lysis of SCCHN targets by non-MHC-restricted effector cells was often increased in vitro by preincubation of the tumor cells

Fig. 3. Rosette formation between PCI-50 cells and the CTL line established from PBL obtained from the patient before surgery. Rosettes were usually observed within 24–48 h of coculture of the CTL with a viable PCI-50 monolayer. Rosettes shown were photographed after 48 h of coculture.

Fig. 4. Cytotoxicity of the AuTu-reactive CTL line (10-week culture). Cytotoxicity against a panel of tumor or normal cell targets was examined in 4 h ⁵¹Cr release assays. Data are from one of three experiments performed.
cytokines on PCI-50 susceptibility to lysis by CTL lines, we next
anti-HLA class I, anti-HLA class II, or antibodies for the SCC-associated antigen (A9 or
5'Cr release assays. D, control; •¿, anti-CD3; A, anti-CD4; A, anti-CD8; •¿, anti-TCR;
anti-CD8, anti-CD56, anti-TCR αβ, or isotype control antibodies for 30 min prior to 4-h

tentively more effective in augmenting lysis of PCI-50 targets by CTL
however, an additive effect was observed only for class II MHC
expression of all three types of surface antigens; and class II MHC antigens (Fig. 7). A combination of these two
cytokines increased expression of class I MHC anti
t gens and ICAM-1, TNFa primarily up-regulated expression of class I
E7.

While IFNy significantly increased expression of class I MHC anti
gens and IL2Ra and IL2Rβ, IL2 did not inhibit their growth over a wide range of
centations tested (data not shown), as demonstrated by us for other SCCHN targets (28). However, when a combination of IFNγ and IL2 (22 ng/ml) or supernatants of A-NK cells known to contain significant levels of IFNγ and IL2 (5) were tested, growth of PCI-50 was significantly inhibited (Fig. 8, A and C). Similarly, high concentrations of TNFa had profound growth-inhibitory effects on PCI-50 cells in vitro.

These experiments indicated that preincubation of PCI-50 targets with combination cytokines or certain effector cell supernatants could either alter the tumor cell sensitivity to lysis, inhibit tumor growth, or
with exogenous cytokines (5). To determine in vitro effects of various cytokines on PCI-50 susceptibility to lysis by CTL lines, we next preincubated these targets with various cytokines or mixtures of cytokines before cytotoxicity assays. As shown in Table 2, preincubation of PCI-50 targets with 1000 units/ml of IFNγ, 1000 units/ml of TNFa, or a combination of the two cytokines for 3 days prior to 51Cr release assays significantly increased (P < 0.05) lysis of PCI-50 targets by the CTL. Such preincubation was shown to be associated with increased expression of certain surface molecules on PCI-50 (Fig. 7). While IFNγ significantly increased expression of class I MHC antigens and ICAM-1, TNFa primarily up-regulated expression of class I and class II MHC antigens (Fig. 7). A combination of these two cytokines increased expression of all three types of surface antigens; however, an additive effect was observed only for class II MHC molecules (Fig. 7). Pretreatment of PCI-50 with IFNγ was consistently more effective in augmenting lysis of PCI-50 targets by CTL
line than TNFa (Table 2). Neither the two cytokines nor their combination increased expression of SCC-associated antigens, E7 or A9, on PCI-50 targets (data not shown).

In addition to increasing tumor cell sensitivity to lysis by immune effector cells, cytokines have been shown to mediate cytostatic effects on SCCHN cell lines (7). PCI-50 cell line was not inhibited in growth by IFNγ, low concentrations (<2000 units/ml) of TNFa, or supernatants of the CTL lines (Fig. 8). Although the tumor cells expressed the IL2Ra and IL2Rβ, IL2 did not inhibit their growth over a wide range of concentrations tested (data not shown), as demonstrated by us for other SCCHN targets (28). However, when a combination of IFNγ and IL2 (22 ng/ml) or supernatants of A-NK cells known to contain significant levels of IFNγ and IL2 (5) were tested, growth of PCI-50 was significantly inhibited (Fig. 8, A and C). Similarly, high concentrations of TNFa had profound growth-inhibitory effects on PCI-50 cells in vitro.

Table 1 Lysis of the SCCHN cell line (PCI-50) by immune effector cells

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Target cells</th>
<th>Resting MNC</th>
<th>A-NK cells</th>
<th>CTL line</th>
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<tbody>
<tr>
<td>K562</td>
<td>153 ± 72</td>
<td>5863 ± 872</td>
<td>4 ± 4</td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
<td>9 ± 3</td>
<td>3142 ± 456</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>PCI-50</td>
<td>9 ± 4</td>
<td>2369 ± 580</td>
<td>1125 ± 228</td>
<td></td>
</tr>
</tbody>
</table>

* Cytoxicity data (4-h 51Cr release assays) are means ± SD of lytic units (LU/10^7 effector cells) from three experiments.

Table 2 Effects of cytokines on susceptibility of PCI-50 SCCHN cell line to lysis by the CTL line

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lytic units (LU/10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>853 ± 31</td>
</tr>
<tr>
<td>TNFa (1000 units/ml)</td>
<td>1096 ± 130β</td>
</tr>
<tr>
<td>IFNγ (1000 units/ml)</td>
<td>1624 ± 219α</td>
</tr>
<tr>
<td>TNFa + IFNγ</td>
<td>1268 ± 254β</td>
</tr>
</tbody>
</table>

PCI-50 cells were incubated with medium or cytokines for 3 days prior to cytotoxicity assays. Tumor cells were washed and used as targets in cytotoxicity assays.

The cytotoxicity data (4-h 51Cr release assay) are means ± SD from three experiments. The CTL line established from PBL obtained prior to surgery was used.

P < 0.05, statistically significant difference in cytotoxicity of PCI-50 target incubated with or without cytokines.
Fig. 7. Expression of the HLA antigens, ICAM-1, and SCC-associated antigens on PCI-50 cells in the presence of cytokines. PCI-50 cells were incubated for 72 h in the presence of medium, TNFa, IFNy, or both cytokines and then examined for expression of the HLA antigens, ICAM-1, A7, or E7 by flow cytometry. Abscissa, relative fluorescence intensity (log scale); ordinate, relative cell numbers (linear scale). *, significant (P < 0.05) shift in mean fluorescence intensity from that in medium control.

DISCUSSION

Hallmarks of a T-cell response to, e.g., TAA are its specificity and immunological memory. The cytotoxic CD8+ T-cell recognizes antigens through the TCR in association with a restriction element on the HLA class I peptide (29). The result of such recognition is a signal for activation, cytokine production, and expansion of a T-cell clone, with concomitant generation of memory T-lymphocytes (30). It has been uncertain to what extent antigens on human solid tumors are immunogenic for inducing specific CTL responses. Although CTL with "specificity" for AuTu have been obtained from peripheral blood or TIL in some patients with solid tumors, in most patients, it has not been possible to document the presence of such T-cells in the periphery or at the tumor site. Also, rigorous studies to confirm AuTu specificity of CTL are difficult in humans, as AuTu cells, large panels of allogeneic tumor cells as well as normal cell targets are not readily available. It has been suggested that quantitative rather than qualitative antigenic changes, which occur on the surface of tumor cells, may not be recognized as "nonself" by the immune system (31). For this reason, in vivo development of CTL with AuTu specificity may be a rare event. Also, some tumors may produce immunosuppressive factors (32), while others may be associated with a defective antigen-presenting pathway (33), thus preventing generation of effective immune responses. In the case of SCCHN, humoral responses to TAA have been demonstrated and studied extensively (22-26). Monoclonal antibodies to TAA have been produced, and in some cases, these mAbs have been shown to recognize a unique antigen (e.g., K931) not present on normal squamous epithelium. In contrast, specific T-cell-mediated responses to autologous SCCHN have been difficult to demonstrate, although a large body of evidence exists for the ability of these tumors to induce non-MHC-restricted effector mechanisms both in vivo and in vitro (2, 4).

In this paper, we have described the generation and properties of AuTu-reactive CTL lines obtained from PBL of a patient with SCC of the tongue. The one intriguing aspect of the process of CTL generation has been that all three attempts at establishing a CTL line from this patient's PBL over a period of 20 months have been successful. In contrast, we have failed to establish CTL from PBL of 10 other individuals with SCCHN for whom AuTu cell lines and cryopreserved PBL were available, utilizing the same experimental conditions. These individuals either have recurrent disease or have died of disease. The ability to repeatedly generate CTL line from this patient's PBL indicates that CTL precursors have been present in the peripheral circulation of this individual, and that in the presence of the AuTu cell line, these CTL precursors have been able to proliferate and develop into cytotoxic effector cells. The patient, who was surgically treated, has not relapsed in more than 2 years following surgery and remains in good health in spite of an advanced age of 92 years. Clearly, this patient has had a strong antitumor immune response. In contrast, we were unable to establish a CTL line from cryopreserved TIL obtained both. Also, direct interaction of the CTL line with autologous tumor cells appeared to be necessary for its antitumor activity, and CTL supernatants were not cytostatic or cytotoxic for AuTu in vitro.
from this patient's tumor at the time of surgery. This might be due to unfavorable in vitro conditions including considerable contamination of TIL with AuTu cells or production by the tumor of immunoinhibitory factors responsible for poor TIL proliferation in vitro. Even though PCI-50 supernatants contained only low levels of prostaglandin E\textsubscript{2} and no TGFβ\textsubscript{1}, other immunoinhibitory factors might down-regulate TIL response to IL2 (34). Also, levels of immunoinhibitory factors produced in vitro by PCI-50 line might not reflect levels of these factors produced in vivo.

The nature of an antigen(s) responsible for the TCR-mediated recognition of AuTu cells by our CTL lines is unknown. We have attempted to use a selection of available anti-SCC mAbs for inhibition of AuTu cell lysis by the CTL to obtain information about the antigen(s) the CTL recognize. However, none of these mAbs, e.g., to A9 integrin α\textsubscript{β}6 (22) or to the E7 antigen, the expression of which on tumor cells may be related to the chromosome 11 rearrangement in SCC (23), were able to block lysis of AuTu, PCI-50, by the CTL lines. A series of mAbs, E48, U36, K928, or K984, which recognize squamous cell epithelial antigens on both normal and tumor epithelia (24–26) were also tested and found ineffective in blocking experiments. Because the CTL lines had strong lytic activity against AuTu and 3 allogeneic SCCHN, but not against other carcinomas tested thus far or normal keratinocytes, it appears that the CTL recognize an antigen the distribution of which is restricted to some SCCHN cell lines. Such a restricted distribution of the antigenic epitope, which presumably is recognized by the TCR in association with a class I MHC antigen, suggests that it is a common epitope shared by certain SCCHN but not by normal epithelial cells. Studies are in progress to determine both the identity of the MHC-restricting element and biochemical nature of this antigen.

Although interactions between AuTu and CTL are dependent on the TCR-mediated recognition of a putative tumor antigen, other surface molecules appear to be involved as well. For example, preincubation of PCI-50 targets with TNFa or IFN\gamma increased expression of HLA class I, HLA class II and ICAM-I but not the SCC-associated antigens, E7 or A9. At the same time, such preincubation with cytokines also significantly increased susceptibility of AuTu to lysis by the CTL line. Thus, the addition of cytokines to the tumor in vitro and their ability to modulate expression of surface molecules on tumor target cells clearly contributes to antitumor effects mediated by the CTL. In addition to augmenting sensitivity of tumor cells to lysis by CTL, cytokines may also mediate cytostatic effects, and in the case of PCI-50, a combination of IFN\gamma and IL2 as well as TNFa at concentrations higher than 1000 units/ml were able to inhibit tumor growth in vitro. In contrast to culture supernatants of normal allogeneic NK cells, supernatants of the CTL were not growth inhibitory for PCI-50 cells. This observation suggests that direct contact of the CTL with AuTu targets is essential for activation of CTL and their antitumor effects, as also demonstrated by both rosette formation and proliferation in the presence of AuTu. Our preliminary observations regarding in vitro effects of cytokines and cellular supernatants of effector cells on growth or susceptibility to lysis of PCI-50 targets provide a basis for further studies of these effects in vivo.

Previous results from our laboratory indicated that immunotherapy of SCCHN with effector cells and/or cytokines such as IL2 or IFN\gamma or supernatants of A-NK cells may be effective in the control of tumor growth and lead to regression of established tumor in a xenograft model of SCCHN in nude mice (5, 7, 8). The availability of the AuTu cell line, PCI-50, as well as specific (the CTL lines) and nonspecific (A-NK cells) effector cells allows us to compare their antitumor activities first in vitro and later in vivo, in the xenograft model of SCCHN in nude mice established in our laboratory (5, 7, 8). This combination of well characterized effector cells should facilitate further studies of the mechanisms involved in effector-tumor target interactions and possibly lead to identification of tumor antigens which initiate and sustain these interactions. Understanding of these mechanisms is important for future development of novel therapeutic approaches for SCCHN, a relatively common cancer with significant morbidity and very high rates of recurrence (35) when treated with conventional therapies.

ACKNOWLEDGMENTS

The authors wish to thank Alan Moghul for performing bioassays for TGFβ on supernatants of the CTL.

REFERENCES


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