AUTOLOGOUS TUMOR-SPECIFIC CYTOTOXIC T LYMPHOCYTES IN THE INFILTRATE OF HUMAN METASTATIC MELANOMAS

Activation by Interleukin 2 and Autologous Tumor Cells, and Involvement of the T Cell Receptor

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Despite the statistical evidence that a more favorable prognosis is associated with a greater magnitude of lymphocyte infiltration, it remains uncertain whether tumor infiltrating lymphocytes (TIL) play any significant role in tumor immunity. We initially reported that TIL from metastatic melanoma were predominantly CD8+ T cells that differentiated in the presence of rIL-2 into CTL, with cytolysis restricted to autologous tumor cells (1). Immunotherapy with rIL2 alone or rIL-2 and lymphokine-activated killer (LAK) cells resulted in marked tumor regression in some metastatic melanoma patients for whom there is no other effective therapy (2, 3). Identification of effector cells responsible for tumor regression is essential to understanding host-tumor interactions, and may be important for increasing the therapeutic efficacy of the treatment and decreasing toxicity and complexity. We and others have reported that most of LAK activity is derived from blood NK cells (4-9). TIL from metastatic melanoma usually do not contain NK cells (1, 10). However, T cells predominate in TIL from metastatic melanomas (1, 10), and may be activated by rIL-2 to CTL specific for autologous tumor targets (1, 10). For these reasons, TIL, rather than rIL-2-activated NK cells (LAK cells), may be more appropriate as effector cells for tumor regression in melanoma patients. Furthermore, the target specificity of rIL-2-activated TIL makes them superior to LAK cells in adoptive immunotherapy (1, 4-12). A serious obstacle in adapting rIL-2-activated TIL for immunotherapy is the difficulty in obtaining large numbers of effector cells. The difficulty resides in (a) limited numbers of TIL recovered from surgical specimens and (b) limited knowledge about the nature and activation of TIL. In this study, we report that TIL from metastatic melanoma increased in number by >1,000-fold when cultured with rIL-2 alone. Activated TIL displayed potent autologous tumorcell-specific CTL activity and the TCR-α/β was involved in target cell recognition. IL-2 receptor...
expression on rIL-2-activated TIL is upregulated by stimulation with fresh autologous melanoma cells.

**Materials and Methods**

**Preparation of Cells.** Single-cell suspensions of TIL and tumor cells were prepared from 12 different surgical specimens from 12 patients with metastatic melanoma, 5 tumors from 5 primary sarcoma patients, and 3 tumors from 3 primary renal cell carcinoma patients using collagenase, hyaluronidase, and DNase as reported previously (1). Pathology examination of the tumor specimens confirmed the diagnosis of melanoma, sarcoma, or renal cell carcinoma in all tumor specimens used in this study. Necrotic areas or tissues surrounding the tumor were carefully removed before the preparation of the cells. Macroscopic examination revealed that the specimens used in this study contained only tumor mass. Ranges of the weight of melanoma tumor specimen were <1 g in patients 3, 5, 7, 8, 10, and 11, between 1 and 10 g in patients 4, 6, 9, and 12, and more than 10 g in patients 1 and 2. Lymph nodes (LN) with melanoma metastasis from six patients were processed similarly. None of the patients had prior chemotherapy or radiation. Cell viability exceeded 80%. Lymphoid cells were discriminated from tumor cells by cell size, morphology, and the brownish cytoplasmic pigmentation of the melanoma cells (1). mAb specific for human lymphocytes (anti-CD3, -CD4, -CD8) or anti-CD16 mAb were used to confirm the identity of these cells. Heparinized peripheral blood was obtained from patients with metastatic melanoma or from healthy volunteers. PBMC were harvested over a Ficoll-Hypaque density cushion as described (4).

**Cell Cultures and Cytokines.** Suspensions of TIL and tumor cells were washed twice and resuspended at 2-10 × 10⁵ cells/ml in RPMI 1640 medium supplemented with 10% FCS (GIBCO Laboratories, Grand Island, NY) (medium) and 20 U/ml or 200 U/ml of rIL-2, and cultured at 37°C with 5% CO₂ in 24-well tissue culture plates (Corning Glass Works, Corning, NY; 2 ml/well). Half the volume of each well was replenished with fresh medium containing rIL-2 every 3-5 d, and the density of cells was maintained at <1-2 × 10⁶ cells/ml during the entire culture period. In some experiments, cells cultured with rIL-2 were applied to a Ficoll-Hypaque density cushion and live cells were collected from the interface and subsequently incubated with rIL-2. Human rIL-2 and rIFN-γ were kindly provided by Biogen Corp. (Boston, MA) and had sp act of 3.6 × 10⁶ U/mg and 1.2 × 10⁶ U/mg of protein, respectively (4, 5). rIL-1, rIL-3, and rIL-4 were purchased from Genzyme, Inc. (Boston, MA).

**Culture of PBMC with Autologous Tumor Cells and rIL-2.** PBMC from patients with metastatic melanoma (n = 4) were incubated with autologous tumor cells at a responder/stimulator ratio of 2:1 in the presence of 20 U/ml rIL-2. Half the volume of medium was replenished with fresh media containing 20 U/ml rIL-2 every 3-5 d. Tumor cells were not irradiated. PBMC were not restimulated with autologous tumor cells.

**Treatment of Cells.** rIL-2-activated TIL were treated with mAb for 30 min at 4°C, followed by incubation with low-toxicity rabbit complement (Cedarlane Lab., Ontario, Canada) (15% in medium) for 30 min at 37°C as reported previously (4).

**Partial Purification of TIL.** For the experiments to detect cytotoxicity in freshly isolated TIL, TIL were partially purified from tumor cells by a discontinuous Percoll density gradient centrifugation as reported previously (1). Briefly, cell suspensions were applied to a Percoll discontinuous gradient (five different concentrations, 38.6, 43.1, 47.6, 52.1, and 56.6%) and centrifuged for 45 min at 400 g. Partially purified TIL (70-85% purity) were recovered from the interface of 52.1 and 56.6% Percoll solution, and used as effector cells to detect cytotoxicity against autologous tumor cells.

**Stimulation of Long-term Cultured TIL.** Long-term (>60 d) cultured TIL were stimulated at an effector/stimulator ratio of 1:1 with fresh melanoma tumor cells that were irradiated (10,000 rad) using a Mark I Cesium 137 source (Nuclear Associates, Glendale, CA).

**mAbs.** Murine hybridoma cells producing anti-CD3, -CD4, -CD8, and anti-HLA-DR mAb (L243) were purchased from American Type Culture Collection (ATCC), Rockville, MD. Murine hybridoma cells producing an anti-HLA-A, -B, -C mAb were kindly supplied by Dr. C. Y. Wang. Ascitic fluid containing mAbs was prepared by injecting hybridoma cells
into BALB/c mice, as reported previously (4). Purified IgG of anti-TCR-αβ mAb (WT31 mAb) (1 mg/ml) was kindly provided by Dr. W. J. M. Tax (13).

Analysis of Lymphocyte Surface Markers. FITC-conjugated Leu series of mAb (anti-Leu-4, Leu-3a, Leu-2a, Leu-7, and Leu-11a) and phycoerythrin (PE)-conjugated anti-Leu 4 and anti-IL-2 receptor mAb (anti-Tac mAb) were purchased from Becton Dickinson Co. (Mountain View, CA). Lymphocytes were incubated with mAb at 4°C for 30 min, and then antigen expression was analyzed using flow cytometry (EPICS C; Coulter, Hialeah, FL) as reported previously (4, 5). To detect the percentage of WT31+ cells, TIL were incubated with 10 μg/ml of anti-WT31 mAb for 30 min at 4°C, followed by staining with FITC-conjugated F(ab′)2 fragments of goat anti-mouse IgG (Cooper Laboratories, Cochranville, PA).

51Cr-release Assay and Assay for Proliferative Response. A 4-h 51Cr-release assay was used to detect cytotoxic activity as described previously (1, 4–6). Cryopreserved, noncultured tumor cells, K562 targets or unstimulated PBMC from melanoma patients were used as target cells. The spontaneous release did not exceed 25% of maximum release. The percentage of specific lysis was calculated as described (1). The level of cytotoxicity was also expressed as lytic units (LU) per 106 effector cells. One LU was defined as the number of effector cells that lysed 30% of 104 target cells as reported previously (5). The proliferative response was measured by culturing 105 lymphocytes/well for 3 d in flat-bottomed microtiter plates with and without rIL-2. Tritiated thymidine (0.5 μCi/well; New England Nuclear, Boston, MA) was added 6 h before harvesting.

Limiting Dilution Analysis. Limiting dilution analysis of TIL was carried out according to the methods described by Moretta et al. (14), with a slight modification. Briefly, different numbers (8, 4, 2, 1, and 0.5) of fresh TIL per well were incubated in 96-well U-bottomed microwells in RPMI medium supplemented with 10% FCS, 200 U/ml rIL-2, 10 μg/ml PHA (Sigma Chemical Co., St. Louis, MO) and 10 ng/ml PMA (Sigma Chemical Co.) in the presence of irradiated (7,000 rad) PBMC from two different healthy donors (2 × 105 PBMC/well). 7 and 14 d later, half of the medium was replaced with RPMI medium supplemented with 10% FCS and 200 U/ml rIL-2, and 2 × 105 irradiated PBMC per well from two different healthy donors. 3–4 wk after the initial incubation, TIL from the proliferating microcultures were transferred into 24-well culture plates in the presence of 105 irradiated autologous tumor cells per well. Stimulation with irradiated autologous tumor cells was repeated every 10–14 d. Double color analysis of surface antigens was performed with FITC-conjugated anti-CD16 mAb and PE-conjugated anti-Leu 4 mAb, or with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb as reported previously (6).

Results

Kinetic Study of rIL-2-induced Expansion. In all 12 metastatic melanomas tested, a substantial proportion of TIL was present in tumor cell suspensions (Table I). The ratio of lymphocytes to tumor cells ranged from 0.03 to 1.25 with an average of 0.40 ± 0.37. By fluorescence analysis, TIL consisted of 78 ± 11% CD3+ T cells, 33 ± 10% CD4+, and 49 ± 17% CD8+ (Table I). Their CD4/CD8 ratio was 0.67. TIL from all 12 patients increased in number when cultured with rIL-2 (200 U/ml) alone, and reached maximum propagation at an average of 43 d (Table I). The mean n-fold increase was 1,543, ranging from 840 to 3,675. The number of resident TIL isolated from melanoma tumors was in the range of 1.0 × 106 to 2.4 × 106 cells, and thus the expected total numbers of rIL-2-expanded TIL ranged from 1.0 × 109 to 2.0 × 1012 cells (mean 3.0 × 1011 cells) (Table I). These rIL-2-expanded TIL consisted of 97 ± 3% CD3+, 39 ± 5% CD4+, and 59 ± 25% CD8+ cells (CD4/CD8 ratio = 0.60).

TIL from 9 of 12 patients with metastatic melanoma rapidly and consistently increased in number from the initiation of culture with rIL-2 alone. A representative
| Tumor specimen | Patient Site | Lymphocytes/tumor cells | Surface marker (CD3, CD4, CD8) | Proliferation* (Day, n-Fold increase) | Number of resident TIL^3 | Expected total number of rIL2-expanded TIL^3 |
|----------------|--------------|-------------------------|---------------------------------|-------------------------------------|-------------------------|------------------------------------------|
| Metastatic melanoma | 1 Iliac (s.c.) | 0.43 | 84, 28, 56 | 37, 840 | 2.4 x 10^9 | 2.0 x 10^12 |
| 2 Extremities (s.c.) | 0.23 | 86, 27, 71 | 43, 960 | 1.0 x 10^6 | 9.6 x 10^11 |
| 3 Iliac (s.c.) | 0.25 | 87, 25, 60 | 44, 1,052 | 1.2 x 10^7 | 1.2 x 10^10 |
| 4 Axilla | 1.25 | 77, 35, 40 | 59, 3,675 | 8.6 x 10^7 | 3.1 x 10^11 |
| 5 Brain | 0.04 | 54, 43, 30 | 60, 1,600 | 1.6 x 10^6 | 2.6 x 10^9 |
| 6 Axilla | 1.0 | 85, 18, 72 | 27, 3,150 | 2.5 x 10^6 | 7.9 x 10^10 |
| 7 Extremities (s.c.) | 0.28 | 65, 45, 23 | 37, 950 | 1.0 x 10^7 | 9.5 x 10^9 |
| 8 Brain | 0.56 | 90, 49, 49 | 38, 951 | 2.0 x 10^6 | 1.9 x 10^9 |
| 9 Groin (s.c.) | 0.03 | 70, 29, 29 | 42, 1,052 | 1.4 x 10^6 | 1.5 x 10^11 |
| 10 Thigh (s.c.) | 0.10 | 80, 34, 53 | 51, 1,843 | 3.0 x 10^6 | 5.5 x 10^9 |
| 11 Rectum | 0.29 | ND, ND, ND | 41, 990 | 1.0 x 10^6 | 1.0 x 10^9 |
| 12 Lung | 0.35 | 85, 25, 58 | 33, 1,451 | 5.5 x 10^6 | 8.0 x 10^10 |
|                  | Mean ± SD                                      |
|------------------|-----------------------------------------------|
| Metastatic LN (n = 6) | 0.40 ± 0.37 78 ± 11 33 ± 10 49 ± 17 43 ± 10 1,543 ± 932 |
| Sarcoma (n = 5)    | 84 ± 75 64 ± 21 30 ± 16 10 ± 3 14 ± 3 2.7 ± 1.3 |
| Renal Cell Carcino(n = 3) | 0.46 ± 0.42 47 ± 24 7 ± 6 41 ± 4 51 ± 3 5,413 ± 1,899 |
| Mean ± SD         | 0.72 ± 0.5 77 ± 7 43 ± 18 45 ± 15 46 ± 10 1,065 ± 246 |

Cell suspensions of TIL and tumor cells from 12 patients with metastatic melanoma, five patients with sarcoma, three patients with renal cell carcinoma, and those of lymphocytes and tumor cells from LN with melanoma metastases from six patients were incubated in RPMI 1640 medium supplemented with 10% FCS and 200 U/ml rIL-2 for up to 4 mo. The cultures were supplemented every 3 d by replacing approximately half of the culture medium with fresh medium containing rIL-2. The cell density of lymphocytes was maintained at less than 1 to 2 × 10^6 cells/ml during the entire culture period. Viable numbers of TIL and tumor cells were determined every 7-10 d.

* n-fold proliferation was calculated from the original number of plated TIL. The day of maximum propagation was reported. The number of rIL-2-stimulated TIL declined after culturing for longer periods with rIL-2 alone.

† Resident TIL represent the number of lymphocytes recovered from the tumor specimen.

‡ A portion of resident TIL was stimulated with IL-2, whereas the remaining portions were cryopreserved. The expected total number of TIL was determined by multiplying the number of resident TIL by the fold of the expansion.

§ Tumors are from subcutaneous lesions.

‖ Significantly higher than that of metastatic melanoma (p < 0.01, student's two-tailed t test).
result is shown in Fig. 1A. TIL from patient 4 increased by 17-fold at 14 d in the presence of rIL-2 (200 U/ml), by 148-fold at 27 d, by 588-fold at 36 d, by 1,470-fold at 47 d, and by 3,675-fold at 59 d. These rIL-2-expanded TIL displayed high levels of autologous tumor lysis as early as 5 d in culture (Fig. 1A). Cytotoxic activity was consistent for as long as 59 d in culture. In contrast, there were very low levels of allogeneic melanoma lysis, and only during the first 5-17 d in culture (Fig. 1A). After maximum expansion with rIL-2, the number of TIL decreased gradually in culture with a concomitant decrease in cytotoxicity. Coexisting tumor cells were completely eliminated within 10 d in culture with rIL-2 (data not shown). In control cultures without rIL-2, the number of TIL decreased and disappeared within 10 d while the number of tumor cells essentially remained the same for 7-10 d (data not shown).

TIL growth from the other three patients with metastatic melanoma (2, 5, and 9) was delayed when compared with that of the nine patients. The kinetics of one of the three is shown in Fig. 1B. TIL from patient 2 increased in number in the presence of rIL-2 by fivefold at 17 d, by 96-fold at 31 d, and by 960-fold at 43 d with consistently high levels of CTL activity restricted to autologous tumor cells (Fig. 1B). There was weak allogeneic tumor lysis that disappeared after 5 wk in culture. After maximum expansion, the number of TIL decreased gradually, accompanied by decreased cytotoxicity. Coexisting tumor cells from these three patients proliferated in culture with or without rIL-2. Further, there were substantial numbers of dead TIL and tumor cells in culture with rIL-2. Therefore, TIL and certain tumor cells that were not adherent to the culture plate were applied to a Ficoll-Hypaque density cushion to isolate live cells. The procedure was repeated several times during the first few weeks of the culture, and the cells were continued
in culture with rIL-2 (200 U/ml). Thereafter, TIL consistently increased in number in culture with rIL-2 alone, whereas the proportions of tumor cells decreased and disappeared within 4 wk of culture. In the control cultures without rIL-2, tumor cells vigorously increased in number, while TIL disappeared within 10 d (data not shown).

rIL-2-activated TIL from all patients with melanoma (n = 12) primarily lysed only autologous tumor cells. None of the other targets tested, such as freshly prepared autologous PBMC, allogeneic melanoma or renal cell carcinoma cells or K562 targets, showed higher susceptibility to rIL-2-activated melanoma TIL as compared to autologous melanoma tumor cells. Representative results are shown in Table II.

Lymphocytes from LN with melanoma metastasis poorly responded to rIL-2 (2.7-fold maximum expansion at 14 d in culture) (Table I). In contrast, the number of TIL from sarcoma increased in culture with rIL-2 by more than 5,000-fold (3,059–7,700, mean; 5,413), which is significantly higher (P < 0.01) than that of TIL from metastatic melanoma (Table I). rIL-2-activated TIL from three renal cell carcinomas increased in number by ~1,000-fold in culture with rIL-2.

Kinetic Study of Cytotoxicity. Freshly isolated TIL preparations did not lyse autologous melanoma tumor cells (data not shown). rIL-2-activated TIL from all 12 melanoma patients tested in this study demonstrated stable and potent CTL activity against autologous tumor cells. Cytotoxicity against allogeneic tumor cells was at least 10 times lower than against autologous tumor cells. Representative results from nine patients are shown in Fig. 2. During 2–3 or 4–6 wk in culture with rIL-2, TIL from all patients were consistently cytotoxic for autologous targets (mean LU/10⁶ effectors during 2–3 wk: 44, range of 4–400; mean LU during 4–6 wk: 44, 9–100) (Fig. 2). There was very low (in five of nine patients) or no (in four of nine patients) cytotoxicity against allogeneic melanoma cells. Tumor cells were no longer present in culture with rIL-2 after 3 wk. Thus, rIL-2-activated TIL can be propagated during 4–6 wk in culture with rIL-2 alone in the absence of autologous tumor cells without loss of their specific CTL activity. During the 7–9 wk in culture with rIL-2, the levels

### Table II

**Autologous Tumor-specific Cytotoxicity Mediated by rIL2-activated Melanoma TIL**

| TIL from patient | Autologous tumors | Autologous PBMC | Allogeneic tumor cells |
|------------------|-------------------|-----------------|-----------------------|
|                  |                   |                 | Melanoma A | Melanoma B | RCC² | K562 |
| 1                | 55.7 ± 5.5*       | 0               | ND         | 2.5 ± 1.0 | 3.0 ± 1.0 |
| 2                | 35.2 ± 3.45       | 3.1 ± 1.0       | 4.3 ± 2    | ND        | 2.5 ± 1.1 |
| 3                | 39.4 ± 6.45       | 0               | 0          | 6.1 ± 2.1 | ND    |
| 4                | 44.9 ± 8.5*       | ND              | 8.2 ± 3.1  | 6.8 ± 1.2 | ND    | 10.1 ± 3.5 |
| 5                | 35.3 ± 4.1*       | ND              | 0          | ND        | 4.5 ± 3.1 |
| 6                | 49.3 ± 3.0*       | ND              | 10.1 ± 4.1 | 5.2 ± 3.0 | ND    |

TIL from melanoma patients (n = 6) were cultured with rIL-2 (200 U/ml) for 3–4 wk, and then were tested for their cytotoxicity against a panel of targets shown above at an E/T ratio of 10:1 in triplicate. Values represent the mean ± SD of percent lysis.

* Freshly prepared PBMC were used as targets.

† Renal cell carcinoma.

§ Significantly (p < 0.001) higher than those of the others (student’s two-tailed t test).
FIGURE 2. Kinetic analysis of cytotoxicity. TIL from nine metastatic melanomas were incubated with rIL-2 (200 U/ml) for 9 wk, and tested every one or two weeks for cytotoxicity against fresh autologous (□) and allogeneic (□) melanoma cells in 4-h ⁵¹Cr-release assays. The levels of cytotoxicity were expressed as LU/10⁶ effectors. The means of LU during 2-3, 4-6, and 7-9 wk from each patient are depicted. One or two allogeneic targets were used to evaluate cytotoxicity against allogeneic tumor cells, and the mean levels of lysis of allogeneic tumor cells at the three different periods were shown. The levels of autologous tumor lysis were at least 10 times higher than allogeneic tumor lysis during the entire incubation period.

of cytotoxicity against autologous tumor cells decreased gradually (mean LU: 21.9-35) accompanied by a reduction of the number of cells (Figs. 1 and 2). None of the TIL in 0 of 7 patients lysed allogeneic tumor cells during the period of 7-9 wk in culture (Fig. 2). It should be emphasized that rIL-2-activated TIL from all 12 patients studied here displayed significantly higher cytotoxicity against autologous tumor cells than that against allogeneic melanoma tumor cells. Allogeneic melanoma cells from three to six different patients were used as targets to investigate allogeneic tumor cell lysis during the entire culture period for 9 wk. Therefore, it appears that melanoma TIL exhibit specific cytotoxicity after culture with rIL-2.

CTL activity restricted to fresh autologous melanoma cells largely resided in the CD8⁺ CD4⁻ T cells, since treatment of effectors from three out of three patients with anti-CD3(OPT3) mAb or anti-CD8(OPT8) mAb plus rabbit complement abolished or significantly decreased the cytotoxicity, respectively (data not shown). Treatment with anti-CD4(OPT4) mAb plus complement did not significantly decrease the cytotoxicity.

Cytotoxicity of TIL from Metastatic Melanoma and from other rIL-2-activated Lymphocytes. The mean ± SD of autologous melanoma cell lysis mediated by rIL-2-activated TIL from patients (1-9) tested in this study was 44 ± 40 LU/10⁶ effectors at 2-3 wk as shown in Fig. 2, 44 ± 30 at 4-6 wk (Fig. 3) and 21 ± 11 at 7-9 wk. Against allogeneic melanoma cells values were 2.3 ± 4.9 (at 2-3 wk), 0.9 ± 1.7 (at 4-6 wk) (Fig. 3), and <0.1 (at 7-9 wk). Thus, autologous melanoma cytotoxicity by rIL-2-activated TIL remained consistently high during the entire culture period with rIL-2 (Figs. 2 and 3). In contrast, rIL-2-activated lymphocytes from melanotic LN had both diminished autologous (0.2 LU/10⁶ effectors) and allogeneic (0.7 LU) tumor cell lysis during 2-3 wk in culture with rIL-2 (Fig. 3). Thereafter, lymphocytes decreased in number and ceased growing in culture with rIL-2. rIL-2-activated TIL from sarcomas displayed only moderate autologous (2.9 LU) and allogeneic (3.3 LU) tumor cell lysis (Fig. 3). rIL-2-activated TIL from renal cell carcinoma were potently cytotoxic against both autologous (35 LU) and allogeneic (35 LU) tumor cells. rIL-2-activated PBMC (LAK cells) from patients with metastatic melanoma were not very cytotoxic for allogeneic melanoma cells after 3 d in culture with rIL-2 (2.8 LU) (Fig. 3). As previously reported (15), LAK activity against autologous mela-
Cytotoxicity by rIL-2-activated lymphocytes from different sources. rIL-2-activated lymphocytes from the sources shown were tested for cytotoxicity against fresh autologous (□) or allogeneic (□) tumor cells. Incubation period with rIL-2 (200 U/ml) was 4-6 wk for TIL from melanoma, sarcoma, or renal cell carcinoma, 2-3 wk for lymphocytes from LN, or 3 d for PBMC from patients with metastatic melanoma. Levels of cytotoxicity were expressed as mean of LU/10⁶ effectors.

Levels of autologous tumor cell lysis by effectors from melanomas were significantly higher than those of the others except with those by rIL-2-activated TIL from renal cell carcinoma (P < 0.001; Student's two-tailed t test).

MHC-nonrestricted Cytotoxicity by PBMC Cultured with Autologous Tumor Cells and rIL-2.

We investigated whether PBMC exhibit autologous tumor-specific CTL activity when initially cultured with fresh autologous tumor cells followed by incubation with a low dose of rIL-2 (Table III). PBMC were incubated with fresh autologous tumor cells was not high (mean ± SD of LU 10⁶ effectors; 1.4 ± 2.0 at 3 d in culture with 20 U/ml rIL-2) and was not significantly different from allogeneic tumor cell lysis (16). These results suggest that TIL from metastatic melanomas have unique characteristics different from lymphocytes obtained from other sources and thus can be propagated in vitro with rIL-2 alone in larger numbers with high levels of cytolyis restricted to fresh autologous melanoma cells.

### Table III

| Patients | Lymphocytes | Incubation time (d) | Autologous melanoma cells 10 | Autologous melanoma cells 2.5 | Allogeneic melanoma A 10 | Allogeneic melanoma A 2.5 | Allogeneic melanoma B 10 | Allogeneic melanoma B 2.5 | K562 10 | K562 2.5 |
|----------|-------------|---------------------|-----------------------------|-----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|---------|---------|
| A        | PBMC        | 6                   | 19 6                        | 20 7                        | ND ND                    | 25 10                    |                            |                            |                 |         |
|          | PBMC        | 20                  | 12 23                       | 12 4                        | ND ND                    | 45 15                    |                            |                            |                 |         |
|          | PBMC        | 30                  | 2 0                         | 6 4                         | 0 0                      | ND ND                    |                            |                            |                 |         |
| B        | PBMC        | 20                  | 3 1                         | 11 5                        | 7 4                      | 29 18                    |                            |                            |                 |         |
|          | TIL         | 20                  | 25 18                       | 12 6                        | 5 3                      | ND ND                    |                            |                            |                 |         |
| C        | PBMC        | 9                   | 38 17                       | 20 8                        | 15 4                     | ND ND                    |                            |                            |                 |         |
|          | TIL         | 9                   | 35 19                       | 5 4                         | 0 0                      | ND ND                    |                            |                            |                 |         |
| D        | PBMC        | 11                  | 11 6                        | 42 13                       | 16 8                     | 41 25                    |                            |                            |                 |         |
|          | TIL         | 11                  | 29 18                       | 14 5                        | 8 5                      | 8 3                      |                            |                            |                 |         |

PBMC from patients with metastatic melanoma (n = 4) were incubated with autologous tumor cells at an effector/stimulator ratio of 2:1 in the presence of 20 U/ml rIL-2 for 30 d. Cell suspensions of melanoma tumors from the same patients (n = 3) were incubated with 20 U/ml rIL-2. At different times of incubation periods, cells were washed and were tested for cytotoxicity against autologous and two different allogeneic melanoma cells and the K562 targets at E/T ratios of 10:1 and 2.5:1 in a 4-h ⁵¹Cr-release assay. Half the volume of the medium was replenished every 3-5 d with RPMI 1640 medium supplemented with 10% FCS and 20 U/ml rIL-2. Tumor cells in culture disappeared within 7-14 d. PBMC were not restimulated with autologous tumor cells. Values represent mean of percent-specific lysis from the triplicate determinations.
cells at a ratio of 2:1 in the presence of 20 U/ml rIL-2. Cell suspensions of TIL and tumor cells from the same patients were incubated with 20 U/ml rIL-2. PBMC or TIL were replenished with fresh medium with rIL-2, but were not restimulated with autologous tumor cells. Only in one of the four patients, PBMC exhibited higher levels of autologous tumor cell lysis rather than those of allogeneic melanoma cells. PBMC from the other three patients exhibited MHC-nonrestricted cytotoxicity even when cultured for >20–30 d. PBMC exhibited lower cytotoxicity when cultured for longer periods. PBMC from two of the four patients (Exp. C and D) did not increase in number in culture with rIL-2 after 9 (Exp. C) or 11 (Exp. D) d. Thus, the cytotoxicity was not measurable. These results suggest that PBMC primarily contain few T cells capable of exhibiting specific CTL activity after the initial stimulation with fresh autologous tumor cells followed by incubation with rIL-2. In contrast to PBMC, rIL-2 (20 U/ml)-activated TIL from the same patients (n = 3) displayed much higher cytotoxicity against autologous tumor cells than that against allogeneic melanoma cells (Table III). Autologous tumor cells were present in the initial culture of TIL with rIL-2, and rIL-2-activated TIL were not restimulated with autologous tumor cells. The stimulation conditions of PBMC and TIL were almost identical.

rIL-2-activated CD16+ NK Cells in TIL. We investigated if CD3-CD16+ NK cells were present in the infiltrate of solid tumors and responsible for autologous tumor lysis. Freshly prepared TIL from metastatic melanoma (n = 11), contained 80% CD3+ T and <2% CD16+ NK cells. Those from sarcoma (n = 5) or renal cell carcinoma (n = 3), contained 40% CD3+ T and 6% CD16+ NK cells, or 75% CD3+ T and 5% CD16+ NK cells, respectively. rIL-2-propagated TIL consisted of >98% CD3+ T and <0.1% CD16+ NK cells in metastatic melanoma (n = 12), 50% CD3+ T and 21% CD16+ NK cells in sarcoma (n = 4), or 75% CD3+ T and 25% CD16+ NK cells in renal cell carcinoma (n = 3) after 2–4 wk in culture. There were <2% CD3+CD16+ T cells as determined by two-color immunofluorescence analysis. After 4–6 wk in culture with rIL-2, TIL from sarcoma or renal cell carcinoma consisted of >96% CD3+ T cells and <2% CD16+ NK cells in rIL-2-activated TIL (data not shown). CD16+ NK cells and CD16- (CD3-) T cells from rIL-2-activated TIL (cultured for 3 wk) from a patient with renal cell carcinoma were purified using a cell sorter, and the individual populations were tested for cytotoxicity (Table IV). Purified CD16+ NK cells were potently cytotoxic for autologous renal cell carcinoma, allogeneic melanoma, and K562 target cells (Table IV), and allogeneic renal cell carcinoma cells (data not shown). CD16+ CD3+ T cells from renal cell carcinoma also displayed MHC-nonrestricted cytotoxicity. Cytotoxicity by CD16+ CD3+ T cells was lower than purified CD16+ cells or unseparated effector cells, but higher than rIL-2-activated PBMC. Both CD16+ NK and CD16- CD3+ T cells proliferated in response to rIL-2 (Table IV). The results suggest that there may be no contribution from NK cells in melanoma to autologous tumor cell lysis, but rIL-2-activated CD16+ NK cells from renal cell carcinoma have the capacity to lyse both autologous and allogeneic tumor cells.

Clonal Analysis of TIL. To investigate immunological properties of TIL at the clonal level, we carried out limiting dilution analysis (Table V). TIL from a patient with metastatic melanoma and from a patient with renal cell carcinoma were cloned by limiting dilution and tested for cell surface antigen expression and cytotoxicity against fresh autologous or allogeneic tumor cells, or K562 targets (Table V). TIL
Table IV
MHC-nonrestricted Cytotoxicity by rIL2-activated CD16+ NK or CD16-CD3+ T cells
Obtained from the Infiltrate of Renal Cell Carcinoma

| rIL-2-activated cells | Percent positive cells | Proliferation (CPM 10^4 cells/well) | Cytotoxicity (LU/10^6 cells) against |
|-----------------------|------------------------|-------------------------------------|------------------------------------|
|                       | CD3 | CD16 | Autologous RCC | Allogeneic Melanoma | K562 |
| Unseparated TIL from RCC#1 | 71  | 30  | 28,586 ± 4,291 | 30  | 22  | 140 |
| CD16+ NK cells from RCC#1 | 4   | 99  | 32,755 ± 625  | 34  | 26  | 1,670 |
| CD16-(T3+) T cells from RCC#1 | 98  | 0   | 31,462 ± 160  | 16  | 12  | 57  |
| PBMC (LAK cells)       | 93  | 5   | ND            | 2   | 1   | 33  |

TIL from a patient with renal cell carcinoma (RCC#1) were cultured with rIL-2 (200 U/ml) for 3 wk. PBMC from a healthy donor were cultured with rIL-2 (200 U/ml) for 3 d. rIL-2-activated TIL were stained with FITC-conjugated anti-Leu-lla (CD16) mAb for 30 min at 4°C followed by sorting into positive and negative fractions using a cell sorter (EPICS C). These sorted fractions and unseparated TIL were incubated overnight in culture with 200 U/ml rIL-2 and then tested for surface antigen expression, proliferation, and cytotoxicity against autologous fresh RCC cells, allogeneic fresh melanoma cells, and K562 target cells in a 4-h 51Cr-release assay. [3H]TdR (0.5 μCi/well) was added to wells containing 10^4 cells/well/0.2 ml 6 h before harvesting, and values represented the mean ± SD of triplicate determinations. Cytotoxicity was expressed as LU per 10^6 cells.

were plated at 8–0.5 cells/well. Proliferating microcultures were <10%. All these TIL expressed the CD3 antigen, and either CD4 or CD8 antigens on the cell surface. These results assured high probability of clonality. 8 of the 10 clones from metastatic melanoma displayed significant lysis only against autologous tumor cells (>10% lysis at an E/T ratio of 8), while only 2 of the 10 exhibited more than 10% lysis against autologous and allogeneic tumor cells (TXRC3, renal cell carcinoma cells). None of them showed NK activity against K562 targets. 3 of 10 clones vigorously proliferated in culture with rIL-2. The means ± SD of percent lysis from these three clones at E/T ratios of 20:1, 10:1, and 5:1 against autologous tumor cells were 31.8 ± 7.2, 25.9 ± 5.6, and 22.2 ± 3.6, respectively. Those against allogeneic melanoma cells were 2.6 ± 1.8, 1.7 ± 0.6, and 1.0 ± 1.0, respectively, and they did not lyse allogeneic renal cell carcinoma targets at any E/T ratios tested. In contrast, all 13 cloned TIL from renal cell carcinoma lysed autologous renal carcinoma cells and allogeneic melanoma tumor cells equally well (Table V). 3 of the 13 displayed NK activity against K562 targets. 1 of the 13 cloned TIL vigorously proliferated in culture with rIL-2, and this cloned T cell exhibited cytotoxicity against allogeneic renal cell carcinomas as well as autologous renal carcinoma and allogeneic melanoma cells (data not shown).

Role of TCR-α/β. Addition of anti-CD3 mAb (OKT3 mAb) or anti-TCR-α/β mAb (WT31 mAb) to the 4-h 51Cr-release assay significantly decreased CTL activity against autologous tumor cells mediated by TIL from all six melanoma patients tested, while anti-CD4 mAb did not affect the cytotoxicity in any (Fig. 4A). Anti-CD8 mAb inhibited CTL activity by 70% in one of six patients (patient 8) and modestly in patient 2 (Fig. 4A). None of the mAb inhibited LAK activity mediated by PBMC when added to the assay (data not shown). These results suggest that TCR-α/β on rIL-2-activated TIL is involved in autologous tumor cell-specific CTL activity. rIL-2-dependent proliferation of cultured TIL was also inhibited by adding
Clonal analysis of TIL from a patient with metastatic melanoma or renal cell carcinoma was evaluated by limiting dilution by a modification of the method described by Moretta et al. (14). Briefly, different numbers (8, 4, 2, 1, and 0.5) of fresh TIL per well of 96 U-bottomed microplate were incubated with RPMI medium supplemented with 10% FCS, 200 U/ml rIL-2, 10 ng/ml PHA, and 10 ng/ml phorbol 12-myristate 13-acetate (PMA), in the presence of x-irradiated (7,000 rad) PBMC from two different healthy donors (2 x 10^5 PBMC/well). 7 and 14 d later, half of the medium was replaced with RPMI medium supplemented with 10% FCS and 200 U/ml rIL-2, and 2 x 10^5 x-irradiated PBMC per well from two different healthy donors. 3-4 wk after the initial incubation, proliferating TIL were transferred into 24-well culture plates in the presence of 10^5 x-irradiated autologous tumor cells per well. Stimulation with x-irradiated autologous tumor cells was repeated every 10-14 d. Double-color analysis of surface antigens was performed with FITC-conjugated anti-CD3 mAb and PE-conjugated anti-CD8 mAb, or with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb as reported previously (6). Cytotoxicity against autologous, allogeneic tumor cells, or K562 was repeatedly investigated in a 4-h ^51^Cr-release assay. Representative results are shown.

anti-CD3 mAb or anti-TCR-α/β mAb to the culture (Fig. 4 B). Anti-CD8 mAb (Fig. 4 B) or anti-CD4 mAb (data not shown) did not inhibit rIL-2-dependent proliferation of TIL from any patients tested. Anti-CD3 or anti-TCR-α/β alone did not induce the proliferation of TIL. In contrast, PBMC from one patient showed high levels of anti-CD3- or rIL-2-dependent proliferation, as well as modest levels of anti-TCR-α/β mAb-induced proliferation (Fig. 4 B).
Role of MHC Antigens. To investigate the involvement of MHC antigens in CTL activity, either effector cells or fresh autologous tumor targets from patient 2 were pretreated for 30 min with various mAb, washed, and tested for CTL activity or susceptibility to lysis, respectively (Table VI). Pretreatment of effectors with anti-CD3 or anti-TCR-α/β mAb moderately decreased CTL activity against targets pretreated with control IgG2a, anti-CD3 mAb, or anti-HLA-DR mAb. Pretreatment of targets with anti-HLA-A, -B, and -C mAb, but not with the others, inhibited CTL activity by ~70%. However, it did not affect LAK activity mediated by rIL-2-activated PBMC. Pretreatment of effectors with anti-CD3 or anti-TCR-α/β mAb completely abolished CTL activity against targets pretreated with anti-HLA-A, -B,
and -C mAb but not with anti-HLA-DR mAb. These results suggest that both TCR-α/β on TIL effectors from patient 2 and HLA-A, -B, and -C antigens on fresh autologous tumor targets are involved in specific CTL activity. In contrast, anti-HLA-DR mAb but not anti-HLA-A, -B, and -C mAb inhibited specific CTL activity mediated by rIL-2-activated TIL from patient 1 when added to the 4-h ^{51}Cr-release assay (data not shown).

Role of Autologous Tumor Cells. After maximum propagation, TIL from all 12 melanoma patients responded poorly to rIL-2 with decreased proliferation and CTL activity (Table I, Figs. 1 and 2). However, stimulation of these TIL with both rIL-2 and autologous tumor cells restored cytotoxicity against autologous tumor cells and proliferation in all seven cases tested (Fig. 5). Occasionally, stimulation with autologous tumor cells alone induced CTL activity (in patient 1) and proliferation (in patients 1 and 4). Addition of medium alone or allogeneic melanoma tumor cells to the culture resulted in loss of activity. Stimulation with rIL-2 alone maintained low but substantial levels of lysis of autologous tumor cells accompanied by low levels of proliferation. Addition of rIFNγ, rIL-1, rIL-3, or rIL-4 with or without rIL-2 did not increase either CTL activity or proliferation of long-term cultured TIL (data not shown). Addition of anti-CD3 mAb as well as anti-TCR-α/β mAb to the culture of these TIL with rIL-2 and autologous tumor cells abolished both CTL activity and proliferation (data not shown). These results suggest that both rIL-2 and autologous tumor cells are required for further propagation of long-term cultured TIL, and that TCR-α/β on these TIL is involved in the recognition of fresh autologous tumor cells.

![Figure 5. Role of autologous tumor cells on TIL activation.](image-url)
**IL-2 Receptor Expression on Long-term Cultured TIL.** rIL-2-dependent TIL from all three melanoma patients whose responsiveness to rIL-2 was restored after stimulation with autologous tumor cells (Fig. 5), expressed IL-2 receptor (IL-2R) (Tac antigen) on the surface after stimulation with autologous tumor cells alone (Fig. 6 A). Addition of rIL-2 or allogeneic tumor cells did not induce IL-2R expression. rIL-2 also did not have any synergistic effect on IL-2R expression induced by stimulation with autologous tumor cells (Fig. 6 A). These results suggest that stimulation with autologous melanoma cells is sufficient to induce IL-2R on TIL. A representative histogram is shown in Fig. 6 B. Thus, addition of autologous tumor cells induced IL-2R expression on rIL-2-activated TIL by 65% at 16 h and by 92% at 96 h of incubation. rIL-2 alone or allogeneic melanoma tumor cells (Fig. 6 B) or K562 target cells (data not shown) failed to induce IL-2R expression. The expression of IL-2R induced by adding autologous tumor cells disappeared after 14–18 d in culture containing rIL-2 and without restimulation (data not shown). These results suggest that IL-2R expression of rIL-2-activated TIL from metastatic melanoma is upregulated by fresh autologous tumor cells.

**Discussion**

TIL from metastatic melanomas increased in number by >1,000-fold in culture with rIL-2 alone. Although autologous melanoma cells were initially present in TIL cultures with rIL-2, they were not detectable after 3 wk in culture. Therefore, TIL
proliferated in culture with rIL-2 alone at least for several weeks (4-6 wk). Cytolysis restricted to fresh autologous tumor cells was consistently high during culture with rIL-2, in agreement with our previous observation (1) and those of others (10). Generally, CTL induced in vitro require both the antigenic stimulation and IL-2 for their activation. However, rIL-2-activated TIL from metastatic melanoma have the ability to proliferate for a substantially long period in culture with rIL-2 alone in the absence of autologous tumor cells without loss of their specific CTL activity. Stimulation with autologous tumor cells facilitated further expansion of these TIL with specific CTL activity. This specific cytotoxicity may be a consequence of in vivo priming and therefore may not be induced during in vitro culture with rIL-2. Lymphocytes from melanotic LN neither increased in large numbers nor exhibited specific CTL activity, even after culture with rIL-2 in the presence of autologous tumor cells. rIL-2-activated PBMC (LAK cells) exhibited modest proliferation in response to rIL-2 (17) and did not exhibit MHC-restricted cytotoxicity (4-9, 15, 16). rIL-2-activated TIL from the other solid tumors such as sarcoma (Fig. 3), renal cell carcinoma (Fig. 3; Table IV; Reference 18), lung cancer (19), carcinomas of the head and neck (20) did not exhibit autologous tumor cell-specific CTL activity. All these results suggest that TIL from metastatic melanomas have unique characteristics different from lymphocytes obtained from other sources, and thus can be propagated in larger numbers with autologous tumor cell-specific CTL activity in vitro with rIL-2 alone.

The results obtained from limiting dilution clones were consistent with those obtained from the bulk cultures of TIL. Thus, cloned cells from melanoma TIL displayed cytotoxicity restricted to autologous tumor cells, while those from renal cell carcinoma TIL lysed autologous and allogeneic tumor cells. The major difference in renal cell carcinoma TIL obtained from the limiting dilution and those from the bulk culture is the absence of CD16+ NK cells at the clonal levels. Although the exact mechanisms involved are unknown, different culture conditions may account in part for the differences. Only rIL-2 was used for the bulk culture of TIL, while 10 µg/ml PHA, 10 ng/ml PMA, and irradiated PBMC from two different healthy donors were used for the limiting dilution assays in addition to rIL-2. These reagents may selectively propagate T cells but not NK cells.

One explanation for the unique immunological specificity of TIL from metastatic melanoma is the potential presence of precursor CTL sensitized in vivo to autologous tumor cells. Exposure to rIL-2 alone induces differentiation into mature CTL. Fresh melanoma cells express tumor-associated antigens (TAA) on the surface defined by several mAb such as anti-GD3 or p97 mAb (21, 22). Fresh melanoma cells may be more immunogenic than the other solid tumor cells. TIL from metastatic melanoma may recognize antigens expressed on tumor cells in association with self-MHC, thereby explaining the antigen-specificity and the MHC restriction. Another explanation is the absence of CD16+ NK cells in the infiltrate of metastatic melanoma. CD16+ NK cells have the ability to respond to rIL-2 and subsequently exhibit MHC-nonrestricted cytotoxicity (4-9). In contrast to the absence of NK cells in metastatic melanoma, TIL from sarcoma or renal cell carcinoma contained a substantial proportion of CD16+ NK cells. These NK cells increased in number in culture with rIL-2.

It is well known that activation of PBMC from melanoma patients either in secondary MLC in which EBV-transformed B cell lines were used as stimulators, or
by stimulation with autologous tumor cells in mixed leukocyte tumor culture (MLTC) resulted in the generation of cytotoxic T lymphocytes with autologous tumor-specific cytotoxicity (23-28). However, PBMC usually did not exhibit specific CTL activity after a single stimulation with autologous tumor cells followed by IL-2 (Table III). In contrast to PBMC, melanoma TIL develop autologous tumor-specific CTL activity when cultured with rIL-2. Autologous tumor cells were only initially present in TIL cultures with rIL-2. These results suggest that PBMC primarily contain few T cells capable of differentiating into autologous tumor-specific CTL after the initial stimulation with autologous tumor cells followed by rIL-2. An alternative explanation would be that suppressor cells present in PBMC inhibit the differentiation of precursor to effector CTL specific for autologous tumor cells. Repeated stimulation of T cell cultures or cloned T cells with autologous tumor cells is usually required for induction and maintenance of autologous tumor-specific CTL activity when PBMC from melanoma patients are used as effector cells (23-28). Furthermore, only selective T cell cultures or cloned T cells derived from PBMC display specific CTL activity, and the others show MHC-nonrestricted cytotoxicity (23-28). In contrast to PBMC, the majority of T cell cultures and clones derived from melanoma tumors display autologous tumor-specific cytotoxicity and repeated stimulation with autologous tumor cells is not required for induction and maintenance of specific cytotoxicity. All these results suggest that melanoma TIL are different from PBMC, and contain significantly higher proportions of cells sensitized to autologous tumor cells that can differentiate into effector CTL.

rIL-2-activated CD16+ NK as well as CD3+CD16 T cells from renal cell carcinoma exhibited MHC-nonrestricted cytotoxicity (Table IV). It is possible that renal cell carcinoma contains a substantial proportion of blood-derived NK cells, since the kidney has a rich blood supply, and single-cell suspensions of renal cell carcinoma usually contain many red blood cells and neutrophils. However, cell suspensions of metastatic melanoma usually do not contain red blood cells or neutrophils. Blood-derived T cells also exhibit MHC-nonrestricted cytotoxicity when incubated with rIL-2 as reported previously (6), as might be the case in rIL-2-activated CD3+CD16 T cells from renal cell carcinoma. To directly address the difference of immunological properties between TIL and PBMC, we carried out limiting dilution analysis using renal carcinoma TIL and PBMC from the same patients. The majority of PBMC-derived T cell clones with MHC-nonrestricted cytotoxicity expressed CD4+CD8+ antigens, while ~50% of TIL-derived T cell clones expressed CD4-CD8+ antigens indicating that even in renal cell carcinoma, TIL may be different from PBMC (unpublished results). An alternate explanation is that TIL from sarcoma or renal cell carcinoma may contain suppressor cells that inhibit rIL-2-induced differentiation of T cells into autologous tumor-specific CTL.

Normal tissues surrounding the tumor or necrotic areas were carefully removed from the specimen before preparing cell suspensions of TIL and tumor cells. Therefore, except with tumors from axillary lesions (patients 4 and 6) or brain (patients 5 and 8), it is very likely that the lymphocytes isolated from the tumor specimen represent true TIL.

Tumors from patients 1-3, 7, 9, and 10 were from subcutaneous lesions, and therefore they could not be contaminated from lymph node lymphocytes. TIL exhibiting specific cytotoxicity for autologous tumor cells were obtained from all these six patients whose
specimens were from subcutaneous lesions. Therefore, at least in these six patients, TIL with specific cytotoxicity were obtained. Tumors from patients 11 and 12 were from viscera (rectum and lung), and the surrounding tissues were removed before preparation of the cells. Therefore, they could not be contaminated from lymph node lymphocytes or peripheral blood lymphocytes. rIL-2-activated TIL from these two patients also displayed autologous tumor-specific cytotoxicity.

Cell suspensions of metastatic melanoma from axillary lesions might contain both TIL and lymphocytes from lymph nodes (LN). However, the substantial numbers of lymphocytes prepared from these specimens and the majority of rIL-2-activated lymphocytes are very likely TIL because (a) the ratios (1.25 and 1.0) of lymphocytes to tumor cells from these two specimens were significantly lower than those of metastatic LN (84 ± 75) (Table I), (b) ratios of CD4+ lymphocytes to CD8+ lymphocytes (respectively, 0.9 and 0.25) were lower than those found in metastatic LN (3.0); (c) The n-fold increase in response to rIL-2 (3,675 and 3,150, respectively) was higher than that observed in metastatic LN (2.7 ± 1.3) and (d) rIL-2-activated lymphocytes from these two axillary lesions displayed potent autologous tumor-specific cytotoxicity (Fig. 2) while those of metastatic LN did not. Melanoma specimens from brain lesions from two patients were used in this study. These cell suspensions did not contain lymph node lymphocytes; however, they may contain both TIL and lymphocytes from peripheral blood, since tumor specimens from these two patients contained numerous red blood cells, possibly due to hemorrhage at surgery. However, on the basis of (a) lymphocyte to tumor cell ratio, (b) CD4/CD8 ratio, (c) the n-fold increase in response to IL-2, and (d) their cytotoxicity which was restricted against autologous tumor cells; the substantial numbers of lymphocytes from these specimens and the majority of rIL-2-activated lymphocytes appear to be TIL.

Two different patterns of growth kinetics were observed with TIL from metastatic melanoma. The first pattern was observed in 9 of 12 cases, and was characterized by the rapid and consistent growth of TIL from the initiation of culture with rIL-2. The second pattern observed in the other three patients was characterized by the delayed growth of TIL. There was no apparent difference in surface phenotypes, cytotoxic potential restricted to autologous tumor cells, or n-fold propagation of TIL between the former nine and the latter three patients (Table I, Figs. 1 and 2). However, tumor cells from the latter rapidly proliferated in culture without rIL-2, and increased in numbers even in the presence of rIL-2, while tumor cells from the former did not increase in numbers in culture with rIL-2. The ratios of TIL to tumor cells in the latter (0.23, 0.04, and 0.03) were lower than those in the former (0.25–1.25). This difference might facilitate tumor cell growth, since there were few cytotoxic TIL per well in culture of the latter group. The different capabilities of tumor cells to proliferate in vitro may account for the different pattern of growth kinetics of TIL, although the exact mechanism involved is not clear at present.

Antigen-specific CTL activity is usually inhibited by adding anti-CD3 mAb to the effector phase of cytotoxicity (29, 30). However, Muul and coinvestigators reported that anti-CD3 mAb did not inhibit the specific cytolysis of autologous tumor cells mediated by rIL-2-activated TIL from metastatic melanomas (10). The role of TCR on TIL was investigated to elucidate the mechanism involved in the CTL-mediated recognition of fresh autologous melanoma cells. For these experiments, rIL-2-activated TIL from metastatic melanoma were incubated for >30 d with rIL-2 to induce
cytotoxicity restricted to autologous tumor cells. In all five cases, TCR-α/β on rIL-2-activated TIL was involved in specific CTL activity (Fig. 4A), as well as in the rIL-2-dependent proliferation (Fig. 4B). An additional argument suggesting that TCR-α/β might be involved in the recognition of fresh autologous melanoma cells by TIL was the fact that addition of anti-TCR-α or anti-CD3 mAb to the restimulated long-term cultured TIL resulted in complete inhibition of the redevelopment of specific CTL activity and proliferation (Fig. 5 and data not shown). These results strongly suggest that rIL-2-activated TIL from metastatic melanomas primarily recognize fresh autologous tumor cells through TCR-α/β. For the inhibition study, we used 1:20 dilution of 1 mg/ml of WT31 mAb or ascitic fluid (10 mg/ml) of the anti-CD3 mAb or the other mAb. In contrast, Muul et al. used as low as 25 μg/ml anti-CD3 mAb (10). This difference may account for the different results, because high concentrations of anti-CD3 mAb are required for the inhibition of CTL activity, as reported by us (31, 32) and others (33).

Our observations on rIL-2-activated melanoma TIL showed much lower ratios of CD4 T cells to CD8+ T cells than those reported by Muul et al. (10). Although exact mechanisms involved are yet unknown, different culture conditions may account for the difference. Thus, we used rIL-2 alone as the stimulus, while Muul et al. used rIL-2 plus supernatants from the culture of autologous PBMC with rIL-2.

Our results suggest that MHC antigens on fresh melanoma cells may be recognized by autologous CTL (Table VI). Anti-GD2, -GD3, or P97 did not inhibit TIL-mediated CTL activity against autologous melanoma targets when added to the cytotoxic assay in certain experiments (data not shown). These results suggest that tumor antigens defined by these mAb may not be involved in tumor cell lysis by autologous tumor-specific CTL. However, this experimental system may provide a model for

### Table VI

MHC Antigens on Fresh Tumor Cells are Involved in the Recognition by rIL-2-activated TIL through TCR

| Effector cells | Pretreatment of effectors | Percent lysis of fresh autologous melanoma cells pretreated with mAb |
|---------------|---------------------------|------------------------------------------------------------------|
| rIL-2-activated TIL | Control IgG2a | 36 ± 6 |
| | Anti-CD3 mAb | 18 ± 3* |
| | Anti-TCR mAb | 14 ± 4* |
| | Anti-HLA-DR mAb | 36 ± 11 |
| | Anti-HLA-A, -B, -C mAb | 38 ± 12 |
| rIL-2-activated PBMC (LAK cells) | None | 18 ± 3 |

Effector cells or ⁵¹Cr-labeled fresh autologous melanoma cells were preincubated for 30 min at 4°C with the mAb shown above. The cells were washed twice and used as effectors or targets in a 4-h ⁵¹Cr-release assay without mAb at an E/T ratio of 10:1 for TIL or 40:1 for LAK cells. Values represent mean ± SD of percent lysis from triplicate determinations. Statistical analysis (Student's two-tailed t test) was done between percent lysis of targets treated with control IgG2a (mediated by effectors treated with control IgG2a) (36 ± 6% lysis) and that of each group.

* p < 0.01.
† p < 0.02.
‡ p < 0.005.
§ p < 0.001.
the determination of the molecular basis of both MHC antigens and tumor antigens recognized by autologous tumor cell-specific CTL.

rIL-2-activated TIL from metastatic melanomas did not express detectable IL-2 receptor (IL-2R) (Tac antigen) on their surface as determined by immunofluorescence after 4–9 wk in culture with rIL-2 (data not shown). Paradoxically, there was rIL-2-dependent proliferation during 4–6 wk in culture with rIL-2 (Fig. 4). Possibly another IL-2R besides Tac antigen such as the p75 protein (34) plays a major role as IL-2R on rIL-2-activated TIL from metastatic melanomas at least after 5 wk in culture with rIL-2. In fact, studies using a chemical crosslinker and 125I-labeled rIL-2 revealed that the p75 protein on the surface of these TIL played a major role as IL-2R (Yagita, M., K. Itoh, M. Tsudo, L. Owen-Schaub, C. D. Platsoucas, C. M. Balch, and E. A. Grimm, submitted for publication).

Stimulation of long-term cultured TIL with autologous tumor cells restored specific CTL activity and proliferation in response to rIL-2 (Fig. 5). The latter was associated with IL-2R (Tac antigen) expression (Fig. 6). rIL-2 itself did not affect induction of IL-2R on rIL-2-activated TIL. These results suggest that IL-2R (Tac antigen) expression on rIL-2-activated TIL from metastatic melanomas is upregulated by autologous tumor cells in an autocrine loop.

NK cells responsible for LAK cell precursors (4–9) are usually not detectable in single-cell suspensions of metastatic melanomas as determined by us (in this report, and reference 1) and others (10), although Kornstein et al. reported the presence of B73.1 mAb positive cells (reactive to NK cells, neutrophils and few blood T cells) in frozen sections of metastatic melanomas using an immunoperoxidase technique (35). However, T cells predominate in TIL from metastatic melanomas and are activated by adding rIL-2 to the culture to differentiate into mature CTL specific for autologous tumor cells (1, 10). Substantial numbers of LAK cells were found in biopsies inside renal cancer tumors when they were injected through major tumor-feeding arteries (36). In contrast, studies of the in vivo distribution of intravenously infused LAK cells suggested that there was little accumulation of the infused LAK cells in tumor sites (37, 38).

This investigation established several critical characteristics of T cell response to melanoma: (a) T cells from metastatic melanomas increased in number by more than 1,000-fold in culture with rIL-2 alone; (b) rIL-2-activated TIL demonstrated autologous melanoma cell-specific CTL activity, whereas those from the other sources displayed MHC-nonrestricted cytotoxicity; (c) stimulation of long-term cultured TIL with fresh autologous melanoma cells restored specific CTL activity and proliferation in response to rIL-2. Based on the results of this and previous studies (1, 10), we postulate that rIL-2-activated TIL, compared with LAK cells, are more appropriate effector cells responsible for tumor regression in metastatic melanoma patients, and furthermore, that they will be superior to LAK cells in adoptive immunotherapy of metastatic melanoma.

Summary

TIL from metastatic melanoma proliferated by >1,000-fold (840–3,675, mean 1,543) after 6 wk in culture of mixtures of TIL and tumor cells with rIL-2 alone. Cytolysis was restricted to autologous tumor cells. CD8+ T cells were the predominant population of TIL before and after expansion, and were primarily responsible...
for autologous tumor-specific CTL activity. No other rIL-2-activated lymphocytes from peripheral blood, lymph nodes with melanoma metastasis, or TIL from sarcoma or renal cell carcinoma had autologous tumor-specific CTL activity. There were few or no CD16+ NK cells in TIL from metastatic melanoma before or after incubation with rIL-2, respectively. However, TIL from sarcoma or renal cell carcinoma contained a substantial proportion of CD3+CD16+ NK cells, which increased in number in culture with rIL-2. Purified CD16+ NK cells as well as CD3+CD16+ T cells from rIL-2-activated TIL of renal cell carcinoma displayed MHC-nonrestricted cytotoxicity. At the clonal level as determined by limiting dilution, 8 of 10 clones from melanoma TIL displayed cytotoxicity restricted to autologous tumor cells, while all 13 clones from renal cancer TIL equally lysed autologous and allogeneic tumor cells. Anti-T cell receptor (TCR)-α/β(WT31) mAb as well as anti-CD3 mAb inhibited autologous melanoma cell-specific CTL activity mediated by rIL-2-activated TIL at the effector phase. These two mAbs also inhibited rIL-2-dependent proliferation of these TIL when added to the culture. Pretreatment of fresh melanoma cells with mAb to MHC antigens followed by washing inhibited specific CTL activity. These results suggest that both TCR-α/β on effector TIL and MHC antigens on fresh tumor cells are involved in the specific immune-recognition. After reaching maximum propagation, TIL from metastatic melanoma responded poorly to rIL-2 alone. However, stimulation with fresh autologous melanoma cells restored both CTL activity and proliferation in response to rIL-2. The latter is associated with IL-2 receptor (Tac antigen) expression on the surface. These results indicate that TIL from metastatic melanomas may have unique characteristics different from lymphocytes obtained from the other sources, and may contain precursor CTL sensitized in vivo to autologous tumor cells, and thus can be propagated in larger numbers with rIL-2 alone while retaining autologous tumor-specific CTL activity.

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