Interleukin 2 Expanded Tumor-infiltrating Lymphocytes in Human Renal Cell Cancer: Isolation, Characterization, and Antitumor Activity

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ABSTRACT

We have described a method for the generation of, from fresh human renal cell cancers, of lymphoid cells that are capable of exhibiting significant antitumor reactivity when tested in short term ^51Cr release assays. Tumor cell suspensions obtained from 37 consecutive fresh human renal cell cancer specimens (35 patients) could be separated by using enzymatic techniques and culturing in medium containing recombinant interleukin 2 (IL-2). The total cell recovery was 1.5 x 10^7 ± 2.2 (SE) per tumor with a range of 1 x 10^6 to 5 x 10^6 cells. The percentage of tumor cells in the suspension ranged from 6 to 75% with a mean of 39.1 ± 3.3%. The remaining cells were predominantly lymphocytes. Viability of mononuclear cells was greater than 90%. Activated tumor-infiltrating lymphocytes (TIL) within these tumors expanded and by 10 to 14 days after initiation of culture a 5- to 15-fold increase in the number of lymphocytes could be achieved with elimination of all autologous tumor cells. Lymphocytes were recultured in fresh medium containing IL-2 and continued to expand between 2- and 10-fold every 4 to 6 days for an average of 33.7 ± 4.5 days, resulting in greater than 50,000-fold increase in the total number of lymphocytes. The average number of splits was 4.9 ± 0.8, with a range of 0 to 21. In 11 of 11 cases tested, TIL exhibited a far better expansion capability in vitro compared to that of peripheral blood lymphocytes obtained from the same patient and grown under identical conditions. The majority of TIL were T cytotoxic/suppressor cells (Leu 2* Leu 4*). With continued in vitro expansion (up to 50 days) there was a concomitant increase in the helper T (Leu 3*) and pan T populations (Leu 4*) and decrease in Leu 2* and HLA-DR* cells. Compared with expanded peripheral blood lymphocytes, these cells demonstrated higher levels of IL-2* receptors and HLA-DR* antigens.

Renal TIL effectors expanded in IL-2 could lyse almost all autologous tumor targets in 4-h chromium release assays. Allogeneic renal as well as nonrenal targets were equally lysed. TIL lysis of cultured tumor targets K562 and Daudi was significantly better than lysis of autologous, allogeneic-renal, and nonrenal targets. No statistically significant difference in the cytotoxic activity of renal TIL or peripheral blood lymphocyte effectors in killing autologous or allogeneic targets could be demonstrated. The best expansion and cytotoxicity of TIL were achieved in the first 3 weeks in culture, followed by a gradual decrease in both growth rate and lytic potential of the cells. Cryopreserved tumor preparations maintained good autologous cytotoxic activity as well as stable surface markers and could be expanded in about two-thirds of the tumors. This opens the possibility of thawing, activating with interleukin 2, expanding, and reinfusing the patient with his own cells once distant metastases appear.

Murine studies have demonstrated that the adoptive transfer of TIL is from 50 to 100 times more potent than therapy with LAK cells in mediating tumor regression in a variety of animal models (1-4) and human tumors as well (5, 6). This approach, however, requires the transfer of large numbers of sensitized fresh lymphocytes, i.e., more than 10^11 immune cells, into tumor bearing humans, along with the systemic administration of relatively high doses of RIL-2 (100,000 units/kg body weight i.v. every 8 h).

Many human tumors are infiltrated with chronic inflammatory cells, including lymphocytes. We have recently identified a population of lymphoid cells infiltrating murine tumors that could be expanded in vitro in IL-2 and, when adoptively transferred, were capable of totally eliminating 3-day established pulmonary metastases (7). When compared to LAK cells, these TIL cells were at least 50 times more potent in mediating the therapy of established micrometastases. The simultaneous administration of IL-2 enhanced the in vivo therapeutic effectiveness of the adoptive transfer of TIL, although high doses of TIL alone were also effective. The greater therapeutic efficacy of TIL compared to LAK cells in the treatment of established metastases in mice raises the possibility that TIL isolated from human tumors and expanded in vitro in IL-2 may similarly be effective for the treatment of human cancer.

RCC in humans is a highly invasive and metastatic cancer that is resistant to known cyotoxic agents. RCC appears to be highly sensitive to adoptive immunotherapy in the murine system (8) and to LAK lysis by using fresh human RCC targets in vitro. Metastatic RCC in humans appears to be particularly susceptible to adoptive immunotherapy with LAK cells plus IL-2 as well (5).

In the present study we have examined the immunobiology of IL-2-activated TIL, obtained from 37 consecutive freshly resected human renal cell cancer specimens (35 patients), and have tested their effectiveness in mediating antitumor reactivity in short term ^51Cr release assays.

MATERIALS AND METHODS

Specimens. From October 1985 to October 1986, 37 specimens (35 patients) containing RCC were obtained directly from the operating room in a sterile fashion. The patients with RCC included 24 males and 11 females with a median age of 55 years, ranging from 32 to 74 years. The surgical specimens included 31 primary kidney tumors and 8 tumors metastatic to: liver, 1; bone, 1; hilar lymph nodes, 1; ascites fluid, 3; and pleural fluid, 2. In two patients (patients 9 and 19) a mixture of the primary and metastatic tumors were used. Pathology report confirmed the diagnosis of RCC in all patients.

Culture Medium. Complete medium consisted of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% human AB serum, 2

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2 The abbreviations used are: IL-2, interleukin 2; RIL-2, recombinant interleukin 2; TIL, tumor-infiltrating lymphoid cells; RCC, renal cell cancer; CM, complete medium; HBSS, Hanks' balanced salt solution; NK, natural killer; PBL, peripheral blood lymphocytes; LAK, lymphokine-activated killer; LU, lytic units (number of effector cells required to produce 30% lysis of 5 x 10^7 target cells); FACS, fluorescence-activated cell sorter.

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mm glutamine (NIH Media Unit), 50 units/ml penicillin (NIH Media Unit), and 50 g/ml gentamicin sulfate (MA Bioproducts, Walkersville, MD). AB serum lots were not prescreened prior to use in the experiments.

Recombinant Interleukin 2. RIL-2 has been previously purified to apparent homogeneity and extensively characterized (9). RIL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA), with a specific activity of 3 to 4 × 10^6 units/mg.

Cryopreservation. Cells were cryopreserved in 90% AB serum plus 10% dimethyl sulfoxide. Vials containing 2 to 5 × 10^7 cells in 1 ml were placed in 95% ethanol in a freezer at −70°C. After 16 h, the vials were transferred to a liquid nitrogen freezer for long term storage.

Preparations of Tumor Cell Suspension. Single cell suspensions of TIL and renal tumor cells were obtained by enzymatic or mechanical (tumors 3 and 10 only) digestion. Resected tumor was collected from surgery and transported in HBSS. Necrotic tumor, fatty tissue, and apparent normal tissue were removed and the remaining specimen was minced into small pieces with surgical blades. The chopped tissue was then dissociated by mechanical stirring for 16 h in a flask containing RPMI 1640 (without AB serum) with 0.01% hyaluronidase type V (1500 units/g), 0.1% collagenase type IV (163–230 units/g), and 0.002% deoxyribonuclease type I (100 units/mg) (Sigma Chemical Co., St. Louis, MO) at 37°C. In two tumor preparations, 4, 8, and 16 h of enzyme treatment were compared. In subsequent preparations, 16-h enzyme treatments were used. The resulting mixture was then filtered through steel mesh, washed twice in HBSS without calcium and magnesium, and separated on differential Ficoll-Hypaque gradient (LSM, Litton Bionetics, Kensington, MD) at 900 × g for 20 min, then washed, counted, and either incubated or cryopreserved. An aliquot was sent for cytopathologic analysis. Cell viability was determined by using trypan blue. The fresh tumor targets utilized in our cytotoxicity assays included 28 different renal cell cancers, 2 spindle cell sarcomas, an osteogenic sarcoma, a malignant melanoma, and an ovarian cancer.

Cultured Tumor Targets. K562, the NK cell-sensitive erythroleukemia cell line, and Daudi, the NK cell-resistant cell line, were cultured in CM and used as targets directly from culture.

Growth of TIL. Cell suspensions from tumors containing both TIL and tumor cells were extensively washed and resuspended at a final concentration of 2.5 × 10^5 cells/ml in CM. The suspension was then placed into 175-cm^2 (750 ml) flasks (Falcon Labware, Oxnard, CA) in 150 ml of CM with 150,000 units of RIL-2. The flasks were incubated, lying flat at 37°C, in 5% CO_2 for approximately 8 days. After 3 to 4

### Table 1 Tumor infiltrating lymphocytes isolated from human renal cell cancer

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<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Source</th>
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<th>% of tumor cells</th>
<th>Total fold expansion</th>
<th>Total days in culture</th>
<th>No. of culture splits</th>
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<td>29</td>
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Fig. 1. Growth and expansion of tumor infiltrating lymphocytes from renal cancer patient 7 in vitro. Cultures were generated at a total concentration of $2.5 \times 10^6$ cells/ml in CM containing 1000 units RIL-2/ml (A). Note the mixture of lymphoid and tumor cells as well as remaining RBC. By day 7 (B) predominantly enlarged, elongated, and convoluted lymphoid cells were seen consistent with IL-2 stimulation. The debris in the culture represents remnants of dead tumor cells and RBC. Cells were recultured in fresh medium on days 10 and 19 at a concentration of $2.5 \times 10^6$/ml. By day 22 (C) numerous lymphoid cells were seen occurring singularly or in loosely cohesive aggregates. In passage 5 after 40 days in culture (D), while still expanding, the cells changed their morphological IL-2 stimulated appearance and by days 45–50 lost lytic activity and stopped growing.

days, small colonies of lymphoid cells could be seen in a bed of tumor cells. On successive days, the number of lymphoid cells increased and the number of tumor cells decreased until by days 8–10, clusters of lymphoid cells were seen while most tumor cells had disappeared. At approximately day 10 the cells were harvested from the flasks by gentle shaking and pipetting, and they were counted and diluted in fresh medium. These cells were placed back in culture at a concentration of $2.5 \times 10^6$/ml and allowed to grow. After approximately 5 days the cultures were ready to be split again.

In patients 1 to 5, we used culture medium which consisted of 80% CM and 20% conditioned medium obtained from the incubation of PBL from normal donors cultured at $10^6$ cells/ml with 1000 units/ml of RIL-2 for 3 to 4 days. In all subsequent patients no conditioned medium was used.

Generation and in Vitro Growth of LAK Cells. Blood samples were obtained before surgery from the same RCC patients. PBL were isolated from heparinized blood on Ficoll-Hypaque gradients as previously described (10). PBL were harvested, washed two times, and either incubated in CM or cryopreserved. LAK cells were generated by placing $2.5 \times 10^5$ cells/ml into 750-ml tissue culture flasks in 150 ml of CM containing 150,000 units/ml of RIL-2 for 3 to 4 days. In all subsequent patients no conditioned medium was used.

Chromium Release Assay for Cytotoxicity. Cytotoxic activity of TIL and PBL were tested in vitro in a standard 4-h $^{51}$Cr release assay against multiple fresh tumor target cells. Target cells were labeled with 200 Ci of Na$_2$CrO$_4$ for 60 min at 37°C. After washing two times, the target cells were incubated at 37°C for another 30 min, washed, filtered through Nytex 110 (Lawshe Instrument Co., Rockville, MD), counted, and resuspended at $10^5$/ml. The target cells were added at a concentration of $5 \times 10^5$ target cells/well in triplicate to various numbers of effector lymphocytes in 96-well round-bottom microtiter plates. Target cells incubated in medium alone and with 0.1 N HCl were used to determine spontaneous and maximum release of chromium. The plates were incubated for 4 h at 37°C, then harvested with a Skatron-Titertek System (Skatron AS, Lierbyen, Norway), and counted in a gamma counter. The percentage of lysis was calculated by:

$$\text{Experimental cpm - spontaneous cpm}$$
$$\text{Maximum cpm - spontaneous cpm}$$

Data are reported in LU/$10^6$ cells. The spontaneous release of chromium from fresh tumor targets generally varies between 30 and 50% in this laboratory. Fresh tumor targets characteristically have higher spontaneous release rates than cultured tumors or lymphoid target cells, although the advantages of working with fresh, noncultured target cells outweighs this disadvantage.

FACS Analysis. A FACS IV microfluorometer (Becton Dickinson, Mountain View, CA) equipped with argon and krypton lasers and interfaced with a Consort 40 computer was used for quantitative fluorescence analysis. All washings and staining were performed in HBSS without phenol red (MA Bioproducts) containing 2% bovine serum albumin (Pathocyte; Miles Laboratory, Naperville, IL) and 0.1% NaN$_3$. 208
TIL IN RENAL CELL CANCER

Expansion Index — x 10,300

10
20
30
40
50
60
70
80

NUMBER OF CELLS \times 10^9/ml
10
20
30
40
50
60
70
80

DAYS IN CULTURE

Expansion Index — x 2800

10
20
30
40

DAYS IN CULTURE

Expansion Index — x 30 (+)

10
20
30
40

Expansion Index — x 11,000 (+)

10
20
30
40

Fig. 2. Pattern of growth of IL-2 expanded tumor-infiltrating lymphocytes isolated from 2 patients with renal cell cancer (A and B representing patients 1 and 7, respectively). Cultures were initiated at a concentration of 2.5 \times 10^5 cells/ml in CM in the presence of 1000 units/ml of recombinant IL-2. The final lymphocyte concentrations for patients 1 and 7 were 0.8 and 1.25 \times 10^6/ml, respectively (see text). By 5 days after initiation of culture, expanding lymphocytes were evident. On successive days, the number of lymphoid cells increased and the number of tumor cells decreased until by approximately day 10 clusters of lymphoid cells were seen while most tumor cells had disappeared. Cells were split every 4–8 days as needed. Expansion index was calculated by multiplying the total fold increase of cells in each split.

Fig. 3. Pattern of growth of lymphocytes of patient 26 simultaneously obtained directly from the renal tumor (A), from PUL (B), and from PBL grown in CM containing irradiated autologous tumor cells (C), at concentrations similar to those in the TIL preparations. The presence of autologous tumor cells in the medium did not significantly alter the growth or expansion of the PBL population in the first 30 days in culture. TIL exhibited a far better expansion capability in vitro compared to that of PBL grown under identical conditions, 81,000- versus 25-fold expansion between days 1 and 35, respectively.

Weights of the tumors used for preparation of the single cell suspensions were not obtained. In the first 13 patients only relatively small parts (25–40%) of the surgical specimen containing the tumor were obtained for our study, which accounted for the low total cell recovery. In the next 15 patients, more generous portions of the tumor (60–80%) were available and the cell yield ranged between 2 and 5 \times 10^9 per tumor. Necrotic and/or hemorrhagic tumors had a significantly lower cell yield with fewer viable cells than firm solid tumors. The percentage of tumor cells in the suspension ranged from 6 to 75% with a mean of 39.1 ± 3.3% (Table 1). The remaining cells were predominantly lymphocytes (53.8 ± 2.8%) and a small percentage (2–7%) of plasmacytes, histiocytes, and eosinophils. Viability of mononuclear cells recovered was greater than 90%.

Results

Cell Yield. Cell suspensions were prepared from 37 consecutive specimens containing renal cell tumors (35 patients). The total cell recovery was 1.5 \times 10^9 ± 2.2 (SE) cells per tumor with a range of 1 \times 10^8 to 5 \times 10^9 cells, depending upon the tumor size and initial composition of the dispersed tumor. (Sigma). Approximately 5 \times 10^6 cells were stained with 15 ml of diluted antibody (Becton Dickinson & Co.) at 4°C for 30 min, washed twice, and resuspended in 0.5 ml for FACS analysis. The staining reagents included anti-Leu 2 (class I reactive T cytotoxic/suppressor cells), anti-Leu 3 (class II reactive T helper/inducer cells), anti-Leu 4 (pan T-cells), anti-Leu 7 (NK cells), anti-Leu 11 (NK/K cells), antitransferrin receptor (lymphoblasts, monocytes), anti-HLA-DR (Ia-bearing cells), anti-Tac (directed against IL-2 receptor), and as a negative control, Thy-1.1 (murine T-cells). Two-color staining analyses were also performed.

Statistical Analysis. The significance of differences in number of lytic units in assay was determined by the Wilcoxon rank sum test. Two-sided P values are presented in all experiments. The same test was used to analyze the significance of differences in incidence of surface markers.

Results

Weights of the tumors used for preparation of the single cell suspensions were not obtained. In the first 13 patients only relatively small parts (25–40%) of the surgical specimen containing the tumor were obtained for our study, which accounted for the low total cell recovery. In the next 15 patients, more generous portions of the tumor (60–80%) were available and the cell yield ranged between 2 and 5 \times 10^9 per tumor. Necrotic and/or hemorrhagic tumors had a significantly lower cell yield with fewer viable cells than firm solid tumors. The percentage of tumor cells in the suspension ranged from 6 to 75% with a mean of 39.1 ± 3.3% (Table 1). The remaining cells were predominantly lymphocytes (53.8 ± 2.8%) and a small percentage (2–7%) of plasmacytes, histiocytes, and eosinophils. Viability of mononuclear cells recovered was greater than 90%.

Growth and Expansion of TIL. The characteristic patterns of

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growth and expansion of TIL are shown in Figs. 1 and 2. Cultures were initiated at a total concentration of 2.5 × 10^5 cells/ml in CM (as described above) in the presence of 1000 units/ml of RIL-2. As compared to the same experiment to PBL from a healthy donor cultured for 7 days (as a positive control for fresh tumor lysis) in identical conditions to those of patient 24, all three effectors exhibit marked cytotoxic activity against autologous, allogeneic, nonautologous, and cultured 5^1/Cr labeled tumor targets when tested in a 4-h 5^1/Cr release assay. There was no difference between autologous PBL and autologous TIL lysis or TIL lysis of allogeneic targets. Percentages of spontaneous release (spontaneous/maximum × 100) of 5^1/Cr were: target, 24-34.8% (636 of 1827); target, 23-34.1% (296 of 868); target 7-21.7% (718 of 3298); sarcoma, 47.3% (839 of 1773); K562, 31.8% (420 of 1322), and Daudi, 29.9% (542 of 1811).

Fig. 4. Pattern of lysis in patient 24 of tumor infiltrating lymphocytes (4) and PBL (B) grown for 13 days in CM containing 1000 units/ml of RIL-2, as compared to the same experiment to PBL from a healthy donor cultured for 7 days (as a positive control for fresh tumor lysis) in identical conditions to those of patient 24. All three effectors exhibit marked cytotoxic activity against autologous, allogeneic, nonautologous, and cultured 5^1/Cr labeled tumor targets when tested in a 4-h 5^1/Cr release assay. There was no difference between autologous PBL and autologous TIL lysis or TIL lysis of allogeneic targets. Percentages of spontaneous release (spontaneous/maximum × 100) of 5^1/Cr were: target, 24-34.8% (636 of 1827); target, 23-34.1% (296 of 868); target 7-21.7% (718 of 3298); sarcoma, 47.3% (839 of 1773); K562, 31.8% (420 of 1322), and Daudi, 29.9% (542 of 1811).

expressed the Leu 4^+ surface marker. Lymphocytes were usually recultured in fresh medium containing IL-2 at approximately day 10, and subsequently continued to expand between 2- and 10-fold every 4 to 6 days for an average of 33.7 ± 4.5 days (range, 8-130 days), resulting in 67,873 ± 24,986 (range, 0 to >500,000)-fold increase in the total number of lymphocytes (Table 1). The average number of splits was 4.9 ± 0.8 with a range of 0–21. No clear advantage was found by adding the conditioned medium used in the first five experiments and it was thereafter discontinued. Cultures from six consecutive patients (patients 14 to 19) grown at the same time were terminated because of precipitates generated from a poor lot of AB serum. Four of these six specimens were subsequently recultured from cryopreserved preparations. All four cultures achieved a greater than 100-fold total expansion. Six of the eight preparations initially terminated due to poor growth were later recultured from cryopreserved specimens. Five of the six were grown for over 3 weeks and all achieved greater than 50-fold total expansion. It appears therefore that 20 specimens (54%) obtained from freshly prepared tumor cell suspensions and 9 additional specimens from cryopreserved preparations, a total of 29 of 37 or 78%, had a greater than 50-fold total expansion after 14 days or more in culture. Our success in growing TIL has improved as experience has increased. The last 10 tumor specimens (patients 24 to 29 and 31 to 34) were successfully grown (mean, 60.1 ± 10.0 days; range, 24-130), recultured (10.0 ± 16.0 times; range, 5–21), and expanded (225,189 ± 69,153-fold; range, 90 to >500,000), in vitro all 10 specimens had a total expansion greater than 50-fold. There was no correlation between the proliferation of TIL from different renal cell carcinomas and the percentage of lymphoid infiltrate in the tumor, the tumor burden or the origin of these lymphoid cells from primary versus metastatic lesions. In our first several tumor preparations we compared the in vitro expansion and cytotoxic activity of mechanical versus enzymatic tumor digest, and various digestion periods (4, 8, and 16 h).
These groups were found to be similar (data not shown), and 16-h incubations were used subsequently. Exposure to enzymatic digestion did not alter the lytic potential of these cells nor their phenotype.

Growth and Expansion of PBL. To contrast TIL with cultures of lymphocytes grown from PBL, simultaneous cultures of TIL and PBL from our renal cancer patients were initiated at a concentration of $2.5 \times 10^5$ cells/ml in IL-2-containing medium, and grown in conditions identical to the fresh tumor targets. When growing PBL meticulous attention was necessary to obtain sustained growth and they regularly grow slower than TIL. The cultures required splitting at a median of 4- to 5-day intervals. Fig. 3 illustrates the growth curves of lymphocytes simultaneously obtained directly from the renal tumor (A), from PBL (B) and from PBL grown in CM containing irradiated autologous tumor cells (C) at concentrations similar to those in the TIL preparations. The presence of autologous tumor cells in the medium did not significantly alter the growth, expansion, or lytic potential of the PBL population in the first 30 days in culture. There was a 30-fold increase in the number of PBL grown in CM containing irradiated autologous tumor cells versus a 25-fold increase in CM only group. Lymphocytes from the tumor (TIL), however, expanded 81,000-fold. Growth curves in Fig. 3, B and C, represent one of our best expansion of PBL. In 11 of 11 specimens of PBL obtained from patients with advanced RCC and grown in culture between 14 and 45 days we achieved a total expansion of less than 40-fold. In all 11 cases tested, TIL exhibited a far better expansion capability in vitro compared to that of PBL grown under identical conditions.

Pattern of Lysis by TIL. TIL from surgical specimens and PBL from the corresponding patients were cultured in medium containing IL-2 containing medium, and grown in conditions identical to the fresh tumor cell preparations.

Table 3 Fresh versus cryopreserved tumor cell suspensions from patient 25

<table>
<thead>
<tr>
<th>Tumor cell suspension</th>
<th>Day</th>
<th>Lysis of autologous target</th>
<th>Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total -fold expansion</td>
<td>1–30</td>
<td>356</td>
<td>378</td>
</tr>
<tr>
<td>Lysis of autologous target</td>
<td>10</td>
<td>8.5</td>
<td>7.0</td>
</tr>
<tr>
<td>26</td>
<td>4.5</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>1.5</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td></td>
</tr>
</tbody>
</table>

* Tumor cell suspension was thawed 6 h after cryopreservation, expanded in IL-2-containing medium, and grown in conditions identical to the fresh tumor cell preparations.

** LU/ml $10^6$ cells.
TIL IN RENAL CELL CANCER

Fig. 7. Cell surface antigen expression of IL-2 activated tumor infiltrating lymphocytes from renal cancer patients 1 (A) and 24 (B) at various days in culture as measured by fluorescence-activated cell sorter analysis. Both patients exhibited high percentage of Leu 4+ cells, a tendency of increase in Leu 3+, and a decrease in Leu 2+ and HLA-DR+ cells. IL-2 receptor levels varied.

The accumulation of lymphocytes, predominantly T-cells, at tumor sites may reflect an immunologic-aid recognition of the neoplasm by the host, suggesting a specific function of TIL directed against antigenic determinants present on tumor cells. The study of the nature of cells infiltrating the tumor may thus be considered to be a valid tool for the study of the mechanisms of tumor rejection.

The continuous in vitro expansion of TIL was correlated with the ability to maintain cytotoxic activity against various tumor targets. Two representative patients are shown in Fig. 5. Lymphoid cells obtained from these patients exhibited a similar in vitro pattern. The best expansion and cytotoxicity were achieved in the first 3 weeks in culture followed by a gradual decrease in both growth rate and lytic potential of the cells. The accumulation of lymphocytes, predominantly T-cells, at tumor sites may reflect an immunologic-aid recognition of the neoplasm by the host, suggesting a specific function of TIL directed against antigenic determinants present on tumor cells. The study of the nature of cells infiltrating the tumor may thus be considered to be a valid tool for the study of the mechanisms of tumor rejection.

The continuous in vitro expansion of TIL was correlated with the ability to maintain cytotoxic activity against various tumor targets. Two representative patients are shown in Fig. 5. Lymphoid cells obtained from these patients exhibited a similar in vitro pattern. The best expansion and cytotoxicity were achieved in the first 3 weeks in culture followed by a gradual decrease in both growth rate and lytic potential of the cells. The accumulation of lymphocytes, predominantly T-cells, at tumor sites may reflect an immunologic-aid recognition of the neoplasm by the host, suggesting a specific function of TIL directed against antigenic determinants present on tumor cells. The study of the nature of cells infiltrating the tumor may thus be considered to be a valid tool for the study of the mechanisms of tumor rejection.

**Table 4 Phenotype of IL-2-expanded T in culture from human renal cell cancer**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Days 8–15</th>
<th>Days 16–29</th>
<th>Days 30–35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of observations</td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>Leu 2</td>
<td>9</td>
<td>52.2 ± 7.7</td>
<td>14–96</td>
</tr>
<tr>
<td>Leu 3</td>
<td>9</td>
<td>37.8 ± 7.4</td>
<td>10–74</td>
</tr>
<tr>
<td>Leu 4</td>
<td>6</td>
<td>62.2 ± 5.8</td>
<td>49–87</td>
</tr>
<tr>
<td>IL-2</td>
<td>9</td>
<td>29.3 ± 4.7</td>
<td>9–62</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>9</td>
<td>59.0 ± 3.2</td>
<td>33–85</td>
</tr>
</tbody>
</table>

*Percentage of positive cells.*
provide insight into the complex issue of tumor-host interactions. We have analyzed 37 specimens of TIL obtained from fresh human renal cell cancers cultured in medium containing recombinant IL-2. Activated TIL could be grown, activated, and unlike most autologous PBL, significantly expanded (average of over 65,000-fold) in medium containing IL-2, in most cases. Cryopreserved tumor preparations maintained good autologous cytotoxic activity as well as stable surface markers and could be expanded in about two-thirds of the tumors.

Renal TIL effectors expanded in IL-2 could lyse almost all autologous tumor targets in 4-h chromium release assays. Allogeneic renal as well as nonrenal targets were lysed equally well. There appears to be no statistical difference in the cytotoxic activity of renal TIL or PBL effectors in killing autologous or allogeneic targets. Data from various laboratories have confirmed that freshly isolated TIL from a wide variety of human tumors demonstrate an immunologically depressed responsiveness, compared with PBL or lymph node lymphocytes, as measured by their proliferative and cytotoxic activity (11-17). The underlying mechanism for the depressed activity of TIL is unknown, although the presence of suppressor cells (18), activation of suppressor cells by tumor cell supernatants (19), production of suppressive factors by tumors (20), and depressed recycling capacity for multiple lytic events (15), have all been proposed as mechanisms. This suppressor activity, however, could be reversed by the addition of IL-2 (21).

In 1980, we first used supernatants containing IL-2 to isolate and expand murine TIL from single cell suspensions of tumors (22). As the lymphocytes grew they appeared to mediate the destruction of tumor cells so that relatively pure cultures of TIL could be obtained. These observations were later extended by studying the ability of expanded TIL to mediate antitumor effects against established growing tumors (7). The TIL exhibited potent antitumor reactivity and were capable of eradicating pulmonary metastases from five of seven transplantable murine tumors tested. These TIL were 50 to 100 times more potent than LAK cells in mediating the elimination of established tumors. These findings have stimulated our interest in the use of these human TIL for the adoptive immunotherapy of human cancer.

We have recently isolated TIL with unique in vitro antitumor reactivity from selected patients bearing malignant melanomas (23). These cells showed lytic specificity for their own autologous fresh melanoma cells but not for autologous fresh normal cells or for fresh allogeneic melanoma cells obtained from other patients. Itoh et al. (24), studying in vitro activity of TIL obtained from nine patients with melanoma, demonstrated that all patients exhibited good lysis against autologous tumor cells. Only five patients exhibited an apparent specificity for autologous melanoma, the others killed allogeneic tumors and K562 as well.

In this paper we have demonstrated significant differences in the growth and expansion patterns as well as the phenotypic surface markers between cultured lymphoid populations that were cultured directly from the kidney tumor site (TIL) versus peripheral blood of the same patients grown in culture side by side under the exact same conditions. Once activated by IL-2, both populations exhibited the same wide range of cytotoxicity which was maintained in culture for 3 to 5 weeks, with a gradual decrease thereafter. These findings are in agreement with those of Vose and Moore (25), who cultured lymphocytes from tumor, lymph node, and blood in medium containing IL-2. We, however, were unable to demonstrate specific antitumor activity in any of the 37 nonenriched preparations containing IL-2-expanded TIL. It therefore appears that culturing TIL in IL-2 may activate specific antitumor reactive cells as well as nonspecific (LAK-like) lymphocytes. Furthermore, long-term incubation of TIL in IL-2 may stimulate the overgrowth of nonlytic clones, thus contributing to the decline in vitro of lytic activity as well as to the cell surface phenotype changes observed in our cultures as cell number had increased. Although TIL from renal cell cancers are not tumor-specific cytotoxic T-lymphocytes, the cytotoxic potential of TIL may not be the important function associated with possible in vivo effects. Other aspects of
cell function may also be relevant, such as proliferative potential to specific antigen and the production of other lymphokines. In animal models, TIL have far greater therapeutic effects than do LAK cells and these in vitro effects do not necessarily correlate with in vivo cytotoxicity against tumor cells. These cellular functional correlates of in vitro therapeutic effects are under study.

In this paper we have shown that lymphoid cells infiltrating human renal cancer could be isolated from single cell suspensions of tumors when cultured in IL-2 containing medium in a relatively routine fashion. Furthermore, the total number of cells required for human adoptive immunotherapy protocols can be reached by the in vitro expansion of TIL but often not for PBL. An average size renal cell tumor will yield approximately 2 x 10^6–4 x 10^6 cells containing 1 to 2 x 10^6 lymphocytes (median of 55% lymphocytes). Three to 4 passages in vitro (approximately 18–22 days in culture) will suffice to bring up the total number of lymphoid cells to 2–3 x 10^11 cells. The reason for the variable total growth of TIL from individual patients may be due to intrinsic biological differences among tumors. It may be related to the degree of antigenicity and/or lymphoid infiltrate in the tumor, the need for additional growth factors in the medium, or production of suppressor factors by individuals.

Cell surface marker analysis of TIL and PBL reported by various groups have revealed inconsistent results. No attempts were made to follow these cultures by repeat phenotyping with time in culture. Moy et al. (15) and Colatta et al. (27), studying lung and ovarian cancers, respectively, found no phenotypic differences in Leu 2*, Leu 3*, or NK cells between tumor and blood lymphocytes. Itoh et al. (24), however, observed higher Leu 2* (61%) and lower Leu 3* (18%) and NK cell markers versus PBL. An average size renal cell tumor will yield approximately 2 x 10^6–4 x 10^6 cells containing 1 to 2 x 10^6 lymphocytes (median of 55% lymphocytes). Three to 4 passages in vitro will suffice to bring up the total number of lymphoid cells to 2–3 x 10^11 cells. The reason for the variable total growth of TIL from individual patients may be due to intrinsic biological differences among tumors. It may be related to the degree of antigenicity and/or lymphoid infiltrate in the tumor, the need for additional growth factors in the medium, or production of suppressor factors by individuals.

Our data demonstrate significant changes in the expressions of surface markers of TIL and PBL with time in culture. Changes were not consistent in all patients. A general pattern, however, of increasing Leu 3* and Leu 4* cells with decrease in Leu 2* and HLA-DR in the TIL population was seen. We have noticed only minor differences in the percentage of Leu 2*, Leu 3*, and Leu 4* cells between PBL and TIL cultured in vitro for 2 weeks under the same conditions. IL-2 receptor and HLA-DR antigens however were higher in the TIL group, possibly related to the far better expansion capabilities of TIL versus PBL.

The ability to obtain expanded cytotoxic TIL from cryopreserved tumor suspensions in approximately two-thirds of the preparations is of practical importance. This opens the possibility of harvesting TIL from routine surgical resections of kidney tumors, storing the suspensions in liquid nitrogen with the possibility of thawing, activating with IL-2, expanding, and reinfusing the patient with his own cells once distant metastases appear.

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Interleukin 2 Expanded Tumor-infiltrating Lymphocytes in Human Renal Cell Cancer: Isolation, Characterization, and Antitumor Activity

Arie Belldegrun, Linda M. Muul and Steven A. Rosenberg


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