Recombinant Interleukin-2 and Lymphokine-activated Killer Cell Treatment of Advanced Bladder Cancer: Clinical Results and Immunological Effects

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ABSTRACT

The purpose of this study was to evaluate the efficacy of treatment with recombinant interleukin-2 (rIL-2) and lymphokine-activated killer cells in patients with advanced bladder cancer and to study the induced changes in the distribution of leukocyte subsets in blood and tumor.

Nine patients with metastatic transitional cell cancer of the bladder were treated with a continuous infusion of rIL-2 combined with lymphocytes stimulated in vitro with rIL-2. None of the patients responded to the therapy despite substantial changes observed in the immunological cells, both in tumor and blood.

The rIL-2 infusion induced migration of leukocytes to the tumors, which was related to increased expression of the adhesion molecule VLA-1 on both peripheral blood mononuclear cells and the endothelial cells of small tumor vessels. Only T-cells, predominantly expressing IL-2 receptors, and macrophages infiltrated the tumors. Natural killer cells remained few or absent in the tumors, even though the natural killer cells in peripheral blood were activated by the treatment.

This study shows that the present technique of rIL-2 and lymphokine-activated killer cell therapy is able to induce substantial changes in the immune system of patients with metastatic bladder cancer. However, this treatment did not induce tumor regression, which may be due to the advanced stage of disease.

INTRODUCTION

Bladder cancer is known to be associated with impaired immunological reactivity: the spontaneous (1) and IL-2-induced cytotoxicity of PBMC is reduced (2), as is the production of IL-2 (3) and IFN-γ (4). The blastogenesis of PBMC after phytohemagglutinin stimulation is decreased (5), and there is a deficiency in the ability to develop delayed-type hypersensitivity (6).

Several types of treatment have been used to stimulate the immune system in patients with bladder cancer. Clinical responses have been found using intravesical instillation of bacillus Calmette-Guerin and rIL-2 either alone (7, 8) or in combination (9, 10) and by direct injection of rIL-2 into tumors (11).

These treatments have all been used in patients with localized bladder cancer. To our knowledge, the present study is the first report of systemic rIL-2 treatment of patients with advanced bladder cancer. The patients were treated by continuous infusion of high-dose rIL-2 combined with autologous PBMC stimulated in vitro with rIL-2 (LAK cells).

The purpose of this study was to evaluate the efficacy of rIL-2 and LAK cells in patients with advanced bladder cancer and to examine the changes in the distribution of leukocyte subsets in blood and in tumor.

MATERIALS AND METHODS

Patient Population. Nine patients with measurable histologically confirmed metastatic TCC of the bladder entered the study. Two patients had performance status 0 (no symptoms) and 7 performance status 1 (symptoms, but fully functional) according to the Eastern Cooperative Oncology Group. All patients had laboratory evidence of normal bone marrow, renal, hepatic, and heart functions. Ineligible were patients with prior malignancy or brain metastases or patients receiving corticosteroids. Informed consent was obtained from all patients. The patient characteristics are summarized in Table 1.

Treatment. The treatment schedule is given in Fig. 1. Treatment was administered during a 36-day cycle. Recombinant IL-2 (Proleukin; EuroCetus Corp., Amsterdam, The Netherlands) was given continuously at a dose of 3 x 10⁹ Cetus units/m²/day from day 0 to 5 and from day 12 to 16. Major toxicity was controlled by dose reductions. PBMC were harvested by leukapheresis on days 8, 9, 10, and 11 using a continuous-flow cell separator (CS-3000; Baxter Healthcare Corp., Deerfield, IL). Vascular access was obtained with a double-lumen central venous catheter. After 4–5 days of culture and rIL-2 stimulation in vitro, the cells were reinfused to the patients on days 12, 13, and 15. The administration of rIL-2 was suspended during cell infusions.

All patients were evaluated by clinical examination, X-ray of the chest, CT scanning of the abdomen and pelvis, and, if possible, cystoscopy before starting the treatment and on day 36 of each treatment cycle. The response to treatment was evaluated according to the World Health Organization criteria (12). Treatment was discontinued if the disease progressed; if it did not, a second cycle was given.

Each patient was given indomethacin (25 mg every 8 h), acetylsalicylic acid (750 mg every 12 h), and cimetidine (400 mg every 12 h) prophylactically. Blood samples for immunological examination were drawn before and after each rIL-2 infusion period (days 0, 5, 12, and 15), between the two rIL-2 infusion periods (day 8), and at the end of the treatment cycle (day 36) (Fig. 1).

Fourteen patients were planned to enter the study. Toxicity was classified according to the World Health Organization criteria (12). Patients progressing during treatment were offered chemotherapy.

Activation of PBMC in Vitro. PBMC were purified and cultured in RPMI-1640 medium containing 1000 Cetus units rIL-2/ml as previously described (13). After 4–5 days of culture, the cells were washed and resuspended in 0.9% sodium chloride solution containing 1000 Cetus units rIL-2/ml and reinfused to the patient over a period of about 90 min.

Prior to termination of the cell culture, samples of the cell suspensions were obtained for detection of contamination by bacteria and Mycoplasma and for in vitro examination of LAK cell cytotoxicity.

Each day during culture, aliquots of cells were frozen (graduated freezing, 90% fetal calf serum, 10% dimethyl sulfoxide, liquid nitrogen) for flow-cytometric analysis. Also, samples of the supernatants from the cultures were frozen at −135°C for analysis of soluble rIL-2 receptors.

Leukocyte Determination. The WBC count and the percentage of...
neutrophils, lymphocytes, eosinophils, and monocytes were measured in whole blood using a Technicon H.1 automated hematology counter (Technicon, Tarrytown, NY). The absolute number of neutrophils, lymphocytes, eosinophils, and monocytes was calculated by multiplying the percentage of each cell type by the WBC count.

PBMC for Immunological Monitoring. PBMC were purified from whole blood mixed with calcium heparinate (5000 IU) by Ficoll-Hypaque density gradient centrifugation (14). The PBMC were washed 3 times and resuspended in RPMI-1640 tissue culture medium supplemented with 10% FCS for use in 51Cr release assays or resuspended in phosphate-buffered saline and 2.5% FCS for immunofluorescence staining. PBMC purified on days 0 and 36 were cultured (3 x 10^6 PBMC/ml, 37°C, humidified 5% CO2 incubator) for 4 days in RPMI-1640 containing 10% FCS and 1000 Cetus units rIL-2/ml and used in 51Cr release assays. To evaluate the maximal inducible LAK cytotoxicity before, during, and after rIL-2 treatment, these cytotoxicities were compared with those of PBMC harvested by leukapheresis on day 8 and stimulated for 4 days with rIL-2.

Assay for LAK Activity. The T24 transitional cell carcinoma cell line (15-17) was used as target cell in the 4-h 51Cr-release assays as the target cell (15-17). T24 cells are insensitive to NK cell-mediated lysis but are sensitive to LAK cells. The LAK cell-induced lysis of the T24 cell line in a 4-h 51Cr release assay is about two-thirds of the lysis of Daudi Burkitt lymphoma cells, which is the classical LAK target cell (2). The 4-h 51Cr release assays were performed as previously described using effector to target cell ratios at 50:1, 25:1, 5:1, and 1:1, respectively (2). In vivo rIL-2 therapy accelerates the development of LAK activity/unit time during in vitro rIL-2 stimulation (18). To measure this priming effect, 51Cr release assays were performed using culture medium containing either 1000 Cetus units rIL-2/ml (4-h stimulation) or no rIL-2.

LAK activity was expressed as lytic units/10^6 effector cells, 1 lytic unit (LU10) being the number of PBMC capable of inducing 10% specific lysis of the target cells. LU10 was calculated by the exponential fit method (19).

Immunofluorescence and Flow Cytometry. The subpopulations of PBMC were examined by dual immunofluorescence experiments and analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with mAbs directly conjugated with FITC, phycoerythrin, or biotin as previously described (2). The panel of mAbs comprised 26 different combinations of the following mAbs: anti-CD3 (Leu4, Pan-T), anti-CD4 (Leu3, T4), anti-CD8 (Leu2, T8), anti-T-cell receptor (TCR-1, a-chain/chain), anti-CD16 (Leu11, NK cells), anti-CD56 (Leu19, NK cells), anti-CD57 (Leu7, predominantly NK cells), anti-CD19 (Leu12, B-cells) anti-CD25 (IL-2R p55, low-affinity IL-2 receptor, a-chain), anti-IL-2R p75 (intermediate-affinity IL-2 receptor, b-chain), anti-CD14 (LeuM3, monocytes), TCR-3-1 (T-cell receptor, y-chain/chain), and anti-VLA-1. Tissue specimens from hyperplastic tonsils, hyperplastic lymph nodes, and native thymus served as positive controls. In vitro rIL-2-activated PBMC were used as positive controls for the staining against VLA-1. Negative controls were performed by using nonsense mAbs of similar isotype as the primary mAbs and by omitting the primary mAb.

Statistics. The relations between cytotoxicity and IL-2R p75+ PBMC fluorescence experiments using mAbs against IL-2R p55 and IL-2R p75 were performed separately using both thawed PBMC from all blood specimens and samples from LAK cell cultures. rIL-2 can interfere with the binding of these antibodies to the IL-2 receptor. To evaluate this effect, IL-2R p75+ PBMC were saturated at acid pH in 10 mM sodium citrate (pH 4.0) for 20 s, followed by washing to remove bound rIL-2 from the receptors. No differences were observed compared with cells that had not been treated at acid pH, indicating that rIL-2 binding did not influence the results of the fluorescence experiments (data not shown).

Soluble IL-2 Receptors. The development of soluble IL-2 receptors (IL-2R p55) in the supernatant during the culture of PBMC stimulated by IL-2 in vitro was measured in duplicate by ELISA (T Cell Sciences). Two additional IL-2 concentrations were added to the recommended standard curve to achieve more precise measurements. Data were expressed as units/ml, with 1 unit representing the amount of soluble IL-2R p55/ml supernatant in normal IL-2-dependent human T-cell lines after phytomagglutinin stimulation (20, 21). All samples were analyzed on the same 96-well ELISA plate. The intrassay coefficient of variation of the ELISA was 3.5%, and the detection limit was 50 units soluble IL-2R/ml. The supernatant examined contained rIL-2. To evaluate a possible effect of this rIL-2 on the ELISA determinations, 1000 Cetus units rIL-2/ml were added to the samples of a duplicate standard curve. The addition of rIL-2 did not influence the values compared with the standard curve made without rIL-2 (data not shown).

Immunohistological Experiments. In three patients, tumor biopsies were obtained prior to therapy, after cessation of rIL-2 infusion (day 16 or 17) and 3 weeks later (day 36). The biopsies were snap frozen (liquid isopenthane, -80°C). For immunohistological experiments 3- to 4-μm tissue sections were fixed for 5 min in acetone, dried at room temperature, and stained by a three-stage alkaline phosphatase-antialkaline phosphatase technique as described previously (22).

The following mAbs were used: anti-CD2 (T-cells; DAKO, Copenhagen, Denmark), anti-CD3 (DAKO), anti-CD8 (DAKO), anti-CD22 (B-cells; DAKO), anti-CD16 (Beckton Dickinson), anti-CD57 (Beckton Dickinson), anti-CD68 (EMB11, macrophages; DAKO), anti-CD25 (DAKO), transferrin receptor (DAKO), anti-HLA-DR (DAKO), and anti-VLA-1 (T Cell Sciences).

Tissue specimens from hyperplastic tonsils, hyperplastic lymph nodes, and native thymus served as positive controls. In vitro rIL-2-activated PBMC were used as positive controls for the staining against VLA-1. Negative controls were performed by using nonsense mAbs of similar isotype as the primary mAbs and by omitting the primary mAb.

Fig. 1. Treatment schedule for one cycle of rIL-2 and LAK therapy. Leukaphereses (L) was carried out on 4 consecutive days, and the in vitro cultured cells were then infused (R) on 3 separate days. Six blood samples were drawn during each cycle (arrows).

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Table 1 Characteristics of patients in study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Local Site of Disease</th>
<th>Previous Treatment</th>
<th>Administered rIL-2 dose (x10^6 units)</th>
<th>Leukocytes harvested (x10^6/liter)</th>
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</thead>
<tbody>
<tr>
<td>J. N.</td>
<td>58/M</td>
<td>No</td>
<td>Lungs, nodes</td>
<td>Radiotherapy</td>
<td>Cycle 1:37</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cystectomy</td>
<td>Cycle 2:48</td>
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<td>T4B bladder</td>
<td></td>
<td>Cycle 1:37</td>
<td>164</td>
</tr>
<tr>
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<td>61/M</td>
<td>No</td>
<td>T3B bladder</td>
<td></td>
<td>Cycle 1:37</td>
<td>54</td>
</tr>
<tr>
<td>E. D.</td>
<td>72/F</td>
<td>No</td>
<td>T3A bladder</td>
<td></td>
<td>Cycle 1:37</td>
<td>28</td>
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<tr>
<td>R. M.</td>
<td>42/M</td>
<td>No</td>
<td></td>
<td></td>
<td>Cycle 1:37</td>
<td>59</td>
</tr>
<tr>
<td>K. M.</td>
<td>57/M</td>
<td>No</td>
<td></td>
<td></td>
<td>Cycle 1:37</td>
<td>50</td>
</tr>
<tr>
<td>L. R.</td>
<td>55/M</td>
<td>No</td>
<td>T4B bladder (recurrence)</td>
<td></td>
<td>Cycle 1:37</td>
<td>38</td>
</tr>
<tr>
<td>B. L.</td>
<td>43/M</td>
<td>No</td>
<td></td>
<td></td>
<td>Cycle 1:37</td>
<td>51</td>
</tr>
<tr>
<td>K. I.</td>
<td>58/M</td>
<td>No</td>
<td></td>
<td></td>
<td>Cycle 1:37</td>
<td>25</td>
</tr>
</tbody>
</table>
or IL-2R p75⁺ NK cells were evaluated by the nonparametric Spearman test.

RESULTS

Clinical Results

No patients responded to treatment (response rate, 0%; 95% confidence limits, 0–33%). Eight patients had PD and one patient stable disease at the evaluation on day 36 of the first treatment cycle. Three patients developed metastatic lesions in new sites (bones, muscles, and liver). Tumor progression occurred in known lesions in the remaining patients. The patient with stable disease after the first cycle, developed PD with new liver metastasis during the second cycle. All patients with PD were offered chemotherapy. However, institution of chemotherapy was not possible in two patients because of rapidly progressing disease.

All patients died within 2–14 months (median survival time, 10 months) calculated from the first day of treatment.

Two patients did not receive their in vitro cultured LAK cells. One developed symptoms of an intracranial hygroma during leukapheresis. The other developed a Staphylococcus aureus sepsis originating from the central venous catheter used for leukapheresis, which also infected the corresponding LAK cell cultures.

The toxicity consisted of fatigue (9 of 9 patients), fever (9 of 9 patients), nausea (6 of 9 patients), vomiting (3 of 9 patients), erythema or rash (8 of 9 patients), pruritus (6 of 9 patients), diarrhea (4 of 9 patients), and dyspnea (2 of 9 patients). Five patients developed hypotension which was treated by reduction of rIL-2 administration and infusion of albumin. None of the patients needed vasopressor treatment. Infusion of LAK cells was associated with chills (4 of 7 patients), fever (5 of 7 patients), hypotension (2 of 7 patients), and hypertension (1 of 7 patients). In 7 of 9 patients, rIL-2 infusion was reduced because of the adverse effects. The mean reduction of the scheduled rIL-2 dose was 25% (SD, ±20%). In one patient, CT scanning before treatment revealed a small shadow consistent with a hygroma in the cerebral parietal lobe. During leukapheresis (day 10), the patient became somnolent, and a repeat CT scan showed compression of the ventricular system and a 3-cm increase in the diameter of the hygroma. LAK cell infusion and further rIL-2 infusion were cancelled, and the patient recovered after evacuation of the hygroma. Except for this patient, no life-threatening toxicity occurred, and none of the patients required treatment in the intensive care unit. Adverse reactions disappeared following the completion of rIL-2 treatment, and all patients were discharged home for follow-up.

Immunological Results

WBC and Numbers of Neutrophils, Lymphocytes, Eosinophils, and Monocytes (Table 2). The WBC count doubled from day 0 to 8. The leukaphereses and LAK cell infusions did not induce significant changes in the WBC count. Absolute as well as relative lymphopenia developed from day 0 to 5, which changed to a rebound lymphocytosis on day 8. The neutrophil count remained constant, until it increased substantially on day 12. The eosinophils showed a two-wave pattern with an initial increase on day 8 followed by a more pronounced increase on day 15.

Lymphocyte Subset Distribution. The number of all lymphocyte subsets increased during the rebound lymphocytosis (day 8), and this increase was most pronounced for the CD56⁺ NK cells (Table 3). Even though the absolute number of TCR-γ-1⁺ T-cells which can mediate both non-MHC (23) and MHC-restricted cytolysis (24) and the CD56⁺CD3⁻ T-cells which act in a MHC-unrestricted manner (23) increased, the percentage of these cells showed only minor fluctuations. VLA-1 was predominantly expressed on CD8⁺ cells.

rIL-2 therapy induced a substantial increase in the number of PBMC expressing IL-2R p75 and IL-2R p55 (Table 4). Like the number, the fluorescence intensity of IL-2R p75⁺ lymphocytes increased significantly. The majority of the IL-2R p75⁺ cells coexpressed the CD56 marker and the number of these double-positive cells increased by up to a factor 20. If only CD3⁻CD56⁺ PBMC were included in the analyses, up to 93% of the CD56⁺ cells became IL-2R p75⁺ during therapy. Also, some patients developed hypotension which was treated by reduction of rIL-2 administration and infusion of albumin. None of the patients needed vasopressor treatment. Infusion of LAK cells was associated with chills (4 of 7 patients), fever (5 of 7 patients), hypotension (2 of 7 patients), and hypertension (1 of 7 patients). In 7 of 9 patients, rIL-2 infusion was reduced because of the adverse effects. The mean reduction of the scheduled rIL-2 dose was 25% (SD, ±20%). In one patient, CT scanning before treatment revealed a small shadow consistent with a hygroma in the cerebral parietal lobe. During leukapheresis (day 10), the patient became somnolent, and a repeat CT scan showed compression of the ventricular system and a 3-cm increase in the diameter of the hygroma. LAK cell infusion and further rIL-2 infusion were cancelled, and the patient recovered after evacuation of the hygroma. Except for this patient, no life-threatening toxicity occurred, and none of the patients required treatment in the intensive care unit. Adverse reactions disappeared following the completion of rIL-2 treatment, and all patients were discharged home for follow-up.

**Table 2 Changes in leukocyte counts and leukocyte subsets in blood during rIL-2 treatment**

<table>
<thead>
<tr>
<th>Treatment day</th>
<th>0</th>
<th>5</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10⁹/liter)</td>
<td>80 (±33)</td>
<td>84 (±28)</td>
<td>149 (±24)</td>
<td>139 (±21)</td>
<td>169 (±49)</td>
<td>101 (±26)</td>
</tr>
<tr>
<td>Neutrophils (10⁹/liter)</td>
<td>62 (±4)</td>
<td>69 (±4)</td>
<td>69 (±6)</td>
<td>104 (±4)</td>
<td>106 (±8)</td>
<td>104 (±18)</td>
</tr>
<tr>
<td>Lymphocytes (10⁹/liter)</td>
<td>10 (±4)</td>
<td>6 (±4)</td>
<td>20 (±2)</td>
<td>12 (±1)</td>
<td>26 (±6)</td>
<td>26 (±6)</td>
</tr>
<tr>
<td>Eosinophils (10⁹/liter)</td>
<td>2 (±4)</td>
<td>4 (±2)</td>
<td>11 (±1)</td>
<td>14 (±5)</td>
<td>29 (±4)</td>
<td>29 (±4)</td>
</tr>
<tr>
<td>Monocytes (10⁹/liter)</td>
<td>5 (±2)</td>
<td>6 (±2)</td>
<td>9 (±6)</td>
<td>6 (±5)</td>
<td>7 (±5)</td>
<td>5 (±5)</td>
</tr>
</tbody>
</table>

* Mean value; number in parentheses, SD.

**Table 3 Changes in lymphocyte subsets in blood during rIL-2 and LAK therapy**

<table>
<thead>
<tr>
<th>Treatment day</th>
<th>0</th>
<th>5</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16⁺</td>
<td>(±13)</td>
<td>(±14)</td>
<td>(±14)</td>
<td>(±16)</td>
<td>(±16)</td>
<td>(±50)</td>
</tr>
<tr>
<td>CD56⁺</td>
<td>(±15)</td>
<td>(±24)</td>
<td>(±98)</td>
<td>(±50)</td>
<td>(±39)</td>
<td>(±62)</td>
</tr>
<tr>
<td>CD56⁺CD3⁺</td>
<td>(±24)</td>
<td>(±3)</td>
<td>(±18)</td>
<td>(±3)</td>
<td>(±4)</td>
<td>(±9)</td>
</tr>
<tr>
<td>CD57⁺</td>
<td>(±16)</td>
<td>(±13)</td>
<td>(±24)</td>
<td>(±14)</td>
<td>(±15)</td>
<td>(±33)</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>(±22)</td>
<td>(±15)</td>
<td>(±115)</td>
<td>(±56)</td>
<td>(±45)</td>
<td>(±65)</td>
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<tr>
<td>CD8⁺</td>
<td>(±11)</td>
<td>(±9)</td>
<td>(±183)</td>
<td>(±15)</td>
<td>(±20)</td>
<td>(±54)</td>
</tr>
<tr>
<td>TCR-γ-1⁺</td>
<td>(±3)</td>
<td>(±2)</td>
<td>(±17)</td>
<td>(±2)</td>
<td>(±2)</td>
<td>(±5)</td>
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<tr>
<td>VLA-1⁺</td>
<td>(±1)</td>
<td>(±38)</td>
<td>(±2)</td>
<td>(±4)</td>
<td>(±7)</td>
<td>(±7)</td>
</tr>
</tbody>
</table>

* Mean lymphocytes (10⁹/liter); numbers in parentheses, SD.

**Table 4 Changes in the number of PBMC expressing IL-2 receptors (p75 and p55) in patients with stable disease after the first cycle of chemotherapy**

<table>
<thead>
<tr>
<th>Treatment day</th>
<th>0</th>
<th>5</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2R p55⁺ (10⁹/liter)</td>
<td>(±2)</td>
<td>(±3)</td>
<td>(±97)</td>
<td>(±22)</td>
<td>(±10)</td>
<td>(±26)</td>
</tr>
<tr>
<td>IL-2R p75⁺ (10⁹/liter)</td>
<td>(±8)</td>
<td>(±15)</td>
<td>(±100)</td>
<td>(±34)</td>
<td>(±31)</td>
<td>(±60)</td>
</tr>
<tr>
<td>IL-2R p75⁺ and CD56⁺ (10⁹/liter)</td>
<td>(±6)</td>
<td>(±12)</td>
<td>(±82)</td>
<td>(±26)</td>
<td>(±20)</td>
<td>(±50)</td>
</tr>
<tr>
<td>IL-2R p75⁺ (%)</td>
<td>45 (±20)</td>
<td>58 (±18)</td>
<td>78 (±12)</td>
<td>75 (±12)</td>
<td>66 (±71)</td>
<td>71 (±12)</td>
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</tbody>
</table>

* Mean values ± SD (in parentheses) of positive cells in the total PBMC population.

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the number of IL-2R p55+ PBMC increased, and these cells predominantly coexpressed p55 with CD4 (data not shown).

LAK Cell Activity. In vivo stimulation with rIL-2 did not induce LAK cytotoxicity (Fig. 2A). When rIL-2 was added to the assay medium, substantial cytotoxicity was measured in the majority of the PBMC samples drawn during rIL-2 treatment (Fig. 2B). The correlation between the percentage of IL-2R p75+ or IL-2R p75*CD56+ PBMC and the LAK cytotoxicity was low (0.35 < r < 0.49) but statistically significant (0.001 < P < 0.033). PBMC with low rIL-2-induced cytotoxicity were not restricted to particular patients. Surprisingly, the cytotoxicity of PBMC drawn on days 8, 12, and 15 and stimulated by rIL-2 during the 51Cr release assay was similar to that of the PBMC reinfused to patients after 4 days of rIL-2 stimulation (Fig. 2C).

The cytotoxicity of PBMC obtained on days 0, 8, and 36 and stimulated for 4 days in vitro by rIL-2 is shown in Table 5. It is apparent that the maximal inducible LAK cytotoxicity increased during rIL-2 therapy in vivo and that this effect persisted, at a reduced level, even 3 weeks after cessation of rIL-2 administration (day 36).

In Vitro IL-2-stimulated PBMC. The cell products of leukapheresis contained mainly lymphocytes (81 ± 4%, mean ± SD) but also a few monocytes (8 ± 4%) and granulocytes (2 ± 1.5%). During culture, almost all monocytes and granulocytes vanished.

The number of cells expressing IL-2R p75, IL-2R p55, VLA-1, and CD56 showed a remarkable course during culture. Prior to culture, the cells expressed similar amounts of IL-2R p75 as observed in blood. During the first day of culture, the IL-2R p75 decreased to between 1 and 4% (data not shown) and remained constant for the remainder of the culture period. The number of IL-2R p55+ PBMC either remained high or increased during the first 2 days of culture but then decreased for the remaining period (Fig. 3A). It is apparent from Table 6 that shedding of IL-2R p55 from cell surfaces may have contributed to the reduction in CD25+ cells.

The VLA-1+ PBMC count showed a substantial increase during in vitro culture (Fig. 3B) in contrast to the PBMC stimulated by rIL-2 in vivo. However, VLA-1 was predominantly expressed on CD8+ cells in both cases. To examine

<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Patient</th>
<th>R. J.</th>
<th>E. D.</th>
<th>S. P.</th>
<th>Control 1</th>
<th>Control 2</th>
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<tbody>
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<td>1020</td>
<td>1660</td>
<td>2550</td>
<td>3000</td>
<td>450</td>
<td>600</td>
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<td>24</td>
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<td>22</td>
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Table 6: Soluble IL-2R p55 in PBMC cultures

The concentration of soluble IL-2R p55 (units/ml) was determined in samples of supernatants during 4 days culture of PBMC obtained by leukapheresis. Data from 3 representative patients are shown. Supernatants of PBMC from 2 healthy untreated subjects and cultured under identical conditions were included as controls.

Fig. 3. The development of IL-2R p55* (CD25*) cells (A), VLA-1* cells (B), and CD56* (C) cells during 4 days of culture of leukapheresis products from 7 different patients. The number of VLA-1* cells in control PBMC from 4 different buffy coats are included in B (----, mean values).
whether VLA-1 expression only developed during in vitro culture or priming by rIL-2 in vivo contributed to the increased VLA-1 expression, PBMC of untreated healthy subjects were stimulated under similar conditions as those of the patients. Fig. 3B shows that PBMC from 4 buffy coats developed fewer VLA-1+ cells than patient PBMC, suggesting that VLA-1 expression is influenced by the rIL-2 therapy in vivo.

The increased number of VLA-1+ cells was associated with an increase in the intensity of the immunofluorescence staining, suggesting an increased number of VLA-1 molecules/cell (Fig. 4).

The percentage of CD56+ cells was similar in all cultures at the time of leukapheresis. Fig. 3C shows that the number of CD56+ cells changed very differently during culture. Irrespective of the proportion of CD56+ NK cells, the percentage of CD16+ NK cells decreased rapidly during culture (day 4, 7% ± 2%), while the percentage of CD57+ NK cells remained almost constant. The percentage of CD4+, CD8+, TCR-β1+, B-cells, and CD56+CD3+ double-positive cells did not change significantly.

To evaluate whether the in vitro cultured cells were stimulated optimally by cultivation, 4-h 51Cr release assays were performed using either medium containing rIL-2 or rIL-2-free medium. No significant increase in cytotoxicity was observed by the addition of rIL-2, indicating that sufficient rIL-2 was present during the culture to induce optimal cytotoxic activity (data not shown).

Immunohistological Analyses. Prior to rIL-2 treatment, very few lymphocytes and macrophages were present in the tumor tissue and the HLA-DR expression on tumor cells was low or absent (data not shown). During therapy the tumors became infiltrated by macrophages (Fig. 5A) and T-cells, many of which expressed the IL-2R p55 (Fig. 5B). The expression of IL-2 receptors in tumor biopsies became even higher than that observed in hyperplastic lymph nodes, which were used as positive controls in these studies. Also, a distinct membrane expression of HLA-DR developed on the tumor cells during therapy (Fig. 5C). Before rIL-2 therapy, VLA-1 was scarcely expressed, being located only on the endothelial cells and in the basement membrane of the vessels (data not shown). Fig. 5D demonstrates that this VLA-1 expression increased substantially during rIL-2 therapy and was associated with an infiltration of VLA-1+ lymphoid cells. Also, the intercellular collagen became moderately VLA-1 positive. Three weeks after cessation of rIL-2 treatment (day 36), the VLA-1 expression decreased toward pretreatment values. Cells expressing CD2, CD4, CD8, and transferrin receptors increased moderately during treatment, while B-cells (CD22+ cells) and NK cells expressing the CD16 and CD57 markers remained few or absent (data not shown).

DISCUSSION

In the present study we treated 9 patients with metastatic TCC and rIL-2 and LAK cells without achieving any clinical response. Our initial plan was to treat 14 patients. Patient accrual was terminated ahead of schedule because each of our 9 first patients had rapidly progressive disease during the first or second cycle which prevented institution of chemotherapy in two cases owing to their poor general condition.

A longer observation period before the institution of chemotherapy might have been advantageous since delayed responses have previously been observed in studies with rIL-2-based immunotherapy (25). However, since all our patients had a clear PD when they went off the protocol, it was not possible to postpone the planned chemotherapy because of the deteriorating general condition of these patients.

We have treated patients with metastatic renal cell carcinoma with rIL-2 alone achieving a response rate of 20%. In the present study, rIL-2 was administered in comparable doses to those used in the renal cancer study and the number of in vitro stimulated PBMC reinjected to each patient was similar to that used in studies reporting clinical responses in malignant melanoma, renal cell carcinoma, and colorectal cancer (26). The rIL-2-stimulated PBMC achieved considerable cytotoxicity in vitro, and similar changes in the distribution of cells occurred both in blood and tumor during therapy, which is in agreement with the findings of others (27, 28). Accordingly, the therapeutic regimen used in our study was as intensive as that used in trials in which clinical responses were achieved.

The lack of effect of rIL-2 and LAK therapy in the present study suggests the existence of different immunological properties in metastatic and localized TCC, since clinical responses have been observed after immunotherapy of localized TCC (7–11). This is supported by previous reports of a reduced ability of PBMC from patients with advanced TCC to develop cytotoxicity after in vitro stimulation with rIL-2 and of reduced IL-2 production after mitogen stimulation in comparison with patients with noninvasive TCC or healthy controls (3, 4). These reports taken together with our results suggest that the IL-2 mechanisms are impaired, in general, in patients with metastatic TCC and that immunotherapy may be more efficient in localized bladder cancer than in metastatic disease.

To our knowledge the expression of the VLA-1 cell adhesion molecule and the relationship between the number of IL-2R p55+ cells and soluble IL-2R in LAK populations during rIL-2 therapy have not been reported previously. VLA-1 is a member of the integrin family of cell adhesion receptors. It is expressed by a variety of cells, but in the peripheral blood they are restricted to long-term activated T-cells (29, 30). VLA-1 functions as a receptor for collagen IV and may also react with laminin, both of which are components of the endothelial lining and the basement membrane (31, 32). Therefore, VLA-1 expression on lymphoid cells may be important for their migration out of vessels (30–32) and may contribute to the increased infiltration of VLA-1+ lymphoid cells observed in the tumors. Treatment with rIL-2 also increased the VLA-1 expression on the endothelial lining of vessels. This may further contribute to...
the cellular infiltration of tumors because endothelial cell surface structures, i.e., VLA-4, which like VLA-1 is an integrin, are involved in the lymphocyte-homing mechanism (33). The low number of VLA-1+ cells found in peripheral blood compared to that of in vitro stimulated cells may be the result of these cells leaving the peripheral blood, suggesting that VLA-1 increases cell migration.

Except for the number of IL-2R p55+ T-cells and the number of IL-2R p75+ NK cells, we observed similar changes in blood leukocytes and lymphocyte subsets during in vitro and in vivo rIL-2 treatment as previously described (26, 28, 34).

The observed reduction of T-cells expressing the IL-2R p55 chain during in vitro cultivation could reflect a declining or low T-cell activation in the cell populations rein infused to the patients. However, it is more likely that it is a sign of intensive rIL-2 activation. The IL-2R p55 and IL-2R p75 chains form an IL-2 receptor with high affinity for IL-2. The IL-2R p55 chain is released to form soluble IL-2R p55 when IL-2 binds to the high-affinity receptor (20, 35–37). Thus, the rIL-2 which is present during culture most likely activates the T-cells through the IL-2 receptor, resulting in the observed increased concentration of soluble IL-2R p55 in the supernatants and the corresponding reduced number of IL-2R p55+ cells.

The present immunohistological studies of tumor biopsies showed substantial changes both in the lymphoid cells and the cancer cells. The induction of intensive HLA-DR expression...
on the tumor cells, which probably is mediated by tumor necrosis factor-α, IFN-α, and IFN-γ produced by the tumor-infiltrating macrophages (38–42), should increase the possibility of antineoplastic reactions by activated specific T-cells. The tumors became infiltrated by IL-2R p55+ T-cells, which may represent activated T-cells. However, it is possible that this cell activation reflects an ability of the cells to migrate and does not necessarily include activation for cytotoxicity, which might explain the lack of clinical response. A correlation between HLA-DR expression on tumor cells and clinical response to rIL-2 therapy has been reported in patients with renal cell carcinoma and malignant melanoma (43). Also, a substantial infiltration of macrophages and T-cells was found in responders. Our findings are similar to those reported by others in responding patients, but since we did not observe a clinical effect of rIL-2, it is possible that metastatic bladder cancer cells are able to inhibit the immune mechanisms, i.e., by secreting immune inhibitory substances or by hiding surface structures used by T-cells to recognize foreign cells.

Detailed examination of the IL-2R p75 expression during rIL-2 and LAK cell therapy has not been presented before. LAK cytotoxicity in PBMC is mainly induced by the interaction between rIL-2 and IL-2R p75 receptors on cells with NK antigenic phenotype. During this process, the IL-2R p75 is internalized and development of LAK cytotoxicity is initiated (44, 45). Our findings of about 10% of IL-2R p75* PBMC in untreated PBMC is in accordance with other reports (46, 47). The discrepancy between the relatively high number of IL-2R p75* cells in peripheral blood during rIL-2 treatment and the low count in the in vitro cultures may be caused by differences in rIL-2 concentration. The high in vitro concentrations may result in an almost immediate internalization of IL-2R p75 chains. The low rIL-2 concentration in vivo [i.e., approximately 30 Cetus units/ml serum (18)] that is reduced further by the interruptions in rIL-2 infusion may not be able to induce IL-2R p75 internalization at the same rate as the chain is expressed. The increased in vivo development of IL-2R p75 receptors/cell may also contribute to the persistent expression of IL-2R p75 in vivo because higher rIL-2 concentrations are required to saturate the receptors. This indicates that the phenotypically NK cells are only partially activated during in vivo therapy, which is in agreement with the results of the 51Cr release assays showing that additional rIL-2 was necessary to achieve optimal LAK cytotoxicity. These findings suggest that a more intensive rIL-2 treatment should be used. On the other hand, rIL-2 therapy did not influence the number of cells with NK phenotype in the tumors and scintigraphic studies using 111In-labeled tumor-infiltrating T-cells or LAK cells have shown that only the T-cells home significantly to tumors (48, 49). Since NK cells have been shown not to infiltrate tumors in responding patients (43), it is not likely that they mediate the antineoplastic actions of rIL-2. Therefore, measurements of NK and LAK cell activation may be of minor relevance in evaluation of the intensity of rIL-2 and LAK therapy.

In conclusion, our study suggests that the present modality of rIL-2 and LAK cell treatment is not able to induce clinical responses in patients with metastatic TCC, despite substantial action on the leukocytes, both in tumor and in peripheral blood. Since clinical responses have been observed with immunotherapy of localized bladder cancer, this form of therapy may be more efficient in limited and localized, rather than metastatic, disease.


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