Immunotherapy of Mice with a Large Burden of Disseminated Lymphoma with Low-Dose Interleukin 2

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ABSTRACT

Successful immunotherapy with recombinant interleukin 2 (rIL-2) of mice bearing a large burden of lymphokine-activated killer-resistant disseminated SL2 lymphoma is described. When mice were challenged i.p. with 2 × 10^6 SL2 cells on day 0 and treated with daily i.p. injections of 5,000 units rIL-2 on days 3-7, no therapeutic effect was observed. However after treatment with daily IL-2 injections on day 10-14, 25% of the mice survived. Ten days after this tumor challenge more than 10^8 SL2 cells were present growing as ascitic tumor. On day 10, SL2 cells were also present as solid tumor in the greater omentum and as metastases in lungs and liver. Surviving mice were able to reject a second challenge with SL2 cells given on day 60. A second challenge with P815, another DBA/2 tumor, resulted in death of the mice due to tumor development. This finding is of particular importance as the SL2 cells are resistant to lymphokine-activated killer activity.

Thus local (i.p.) injection of low dose rIL-2 can cause the systemic rejection of advanced and metastasized cancer. Our data indicate that IL-2 can strongly enhance a specific immune reaction against tumor cells.

INTRODUCTION

Recently much attention has been focussed on the use of rIL-2 in immunotherapy of cancer. Human peripheral mononuclear blood cells or murine spleen cells cultured with rIL-2 become cytotoxic against a wide range of tumor cells, but not against normal cells (1, 2). These so-called LAK cells, in combination with injections of rIL-2, can induce tumor regression in vivo, as demonstrated in a number of animal models and clinical trials (3-8). Administration of high doses of rIL-2 only, can also induce tumor regression. This thought is to be mediated through LAK cells generated in vivo (9, 10). rIL-2 also helps adoptively transferred LAK cells to survive and to proliferate in vivo (11). In vitro LAK cell cytotoxicity to tumors seems to correlate with the antitumor effectiveness of treatment with rIL-2 and LAK cells in vivo. This applies to systemic therapy with high dose rIL-2 (12) as well as to local therapy with low dose rIL-2 (13).

However the question remains whether IL-2 therapy can be effective against tumors that are resistant to LAK cell cytotoxicity in vitro. In these cases such tumor cells may only be killed by a specific immune reaction. Low dose rIL-2 preferentially induces specific T-lymphocytes that have no LAK activity (14-16). As these lymphocytes should be able to recognize tumor cells, immunotherapy with low dose IL-2 should be studied in mice bearing immunogenic tumors.

We studied IL-2 immunotherapy of mice bearing SL2 lymphoma, a weakly immunogenic tumor (17-19). It was shown that successful therapy with low dose rIL-2 is possible against metastasized SL2 tumor. Our findings indicate that specific immune reactions mediate this tumor rejection.

MATERIALS AND METHODS

Animals. Inbred DBA/2 mice were obtained from CPB-TNO, Zeist, The Netherlands, and our own breeding colony. Male mice were used at the age of 8-12 weeks.

Tumors. The DBA/2 derived SL2 lymphoma and P815 mastocytoma were used. Both tumors grow i.p. as ascitic tumors and were maintained by weekly i.p. passage. TU5 kidney carcinoma cells (BALB/c derived) were obtained from Dr. A. Mantovani, Milano, Italy. These cells were maintained by in vitro culture.

Interleukin 2. The rIL-2 was a gift from Sanofi, Toulouse, France (batch IL-031-P; specific activity, 18.2 × 10^4 units/mg as determined by [3H]thymidine incorporation in CTLL2 cells). This rIL-2 was produced by mammalian Chinese hamster ovary cells, in which the human IL-2 gene was inserted (20). The IL-2 was diluted to 2 × 10^4 units rIL-2/ml in PBS supplemented with 0.1% BSA (Sigma, St. Louis, MO) and stored at -70°C. It was thawed directly before use. PBS contained per liter: 8.75 g NaCl, 1.53 g NaHPO4, and 0.27 g K2HPO4.

LAK Cells. Spleens from DBA/2 mice were dissected and pressed through a metal sieve. The cell suspension was filtered through glass wool and washed three times in CM before suspension in CM. CM consisted of RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated FCS (Flow Laboratories, Irvine, Scotland), 0.03% glutamin, 0.1% nonessential amino acids (Sigma, St. Louis, MO), 1 μM sodium pyruvate, 5 × 10^-3 M 2-mercaptoethanol, 0.25 mg penicillin/ml, 0.100 mg neomycin/ml, and 0.050 mg streptomycin/ml. LAK cells were generated by incubating 2 × 10^6 spleen cells per milliliter CM containing 500 units rIL-2/ml in 75-cm² tissue culture flasks. The flasks were incubated at 37°C in a 5% CO2 atmosphere for 5 days.

51Cr-release assay. Cytotoxicity of the LAK cells was tested in a 4-h 51Cr-release assay. Tumor cells (10^4) were labeled with 25 μCi Na2CrO4 (Amersham, UK) in 0.100 ml PBS for 1.5 h. After washing the tumor cells three times in RPMI 1640 with 10% FCS, they were incubated with 0.100 ml of the effector cell suspension in 96-well microtiter plates (μ-bottom) at 37°C in a moist atmosphere containing 5% CO2 for 4 h. Cytotoxicity was measured at varying effector/target ratios.

These plates were centrifuged for 5 min at 1,000 rpm (200 × g) before and after incubation. Supernatants (0.100 ml) were collected and the released 51Cr was measured in a gamma-counter. All experiments were performed in triplicate. The CI was calculated as follows:

\[
CI = \frac{CPM \text{ test} - CPM \text{ spontaneous}}{CPM \text{ maximal} - CPM \text{ spontaneous}} \times 100%
\]

where CPM test is CPM measured in the supernatants of effector cells and target cells. CPM spontaneous is CPM measured in the supernatants of target cells in the absence of effector cells. CPM maximal is CPM measured in the supernatants of target cells lysed with 0.1% sodium dodecyl sulfate.

Immunotherapy Model. DBA/2 mice were injected i.p. with 2 × 10^4 SL2 cells suspended in 1 ml RPMI 1640 on day 0. Mice were injected with rIL-2 diluted in 0.5 ml PBS supplemented with 0.1% BSA according to varying protocols (see "Results"). Control mice were injected with SL2 cells and treated with PBS/BSA. Mice were followed until death. Mice were considered as being cured if they were healthy.

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2The abbreviations used are: rIL-2, recombinant interleukin 2; LAK, lymphokine-activated killer; CM, complete medium; PBS, phosphate buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; CI, cytotoxicity index.
days after tumor challenge. At least five mice were included in each experimental group.

Measurement of the Influence of rIL-2 on SL2 Proliferation in Vitro. SL2 cells (4 x 10⁴/well) were cultured in 96-well tissue culture plates in RPMI 1640 with 10% FCS and varying concentrations of rIL-2. The total volume was 0.200 ml. Four or 20 h later 0.2 μCi [³H]-thymidine/well was added and the culture was continued for 4 h. Thereafter the amount of incorporated [³H]-thymidine was determined using a cell harvester (Titertek) and a liquid scintillation counter.

Statistical Analysis. The Wilcoxon rank sum test (two-tailed) was used to test the significance of differences in survival times of groups of mice. The Student's t test was used to test the significance of differences in proliferation of SL2 cells in different concentrations IL-2.

RESULTS

IL-2 Immunotherapy of Mice Bearing SL2 Lymphoma. DBA/2 mice were challenged with SL2 cells i.p. and we studied whether effective immunotherapy with low dose rIL-2 was possible. The results of a series of experiments in which rIL-2 was injected i.p. according to varying protocols during the first week after tumor inoculation were all negative (data not shown). Positive therapeutic results were obtained when mice were challenged with 2 x 10⁴ SL2 cells i.p. on day 0 and treated with daily i.p. injections of 5,000 units rIL-2 on days 3-7 and 10-14 (Fig. 1). Daily injections with 10,000 units rIL-2 were equally effective (data not shown). Control animals all died within 20 days, whereas treated mice survived significantly longer (P < 0.05). About 25% of the treated animals survived for more than 60 days. These mice were considered as being cured. In subsequent experiments it was shown that the daily injections on day 10-14 with 5,000 units rIL-2 were essential for successful therapy (Table 1). Injections with rIL-2 before day 10 had no significant additive effect (P > 0.05). In order to estimate the effectiveness of this IL-2 therapy, we determined the number of tumor cells present in the ascitic fluid 10 days after i.p. challenge with 2 x 10⁴ SL2 cells. As shown in Fig. 2, more than 10⁴ SL2 cells are present at that time. On day 10 SL2 cells are also present as solid tumor in the greater omentum, SL2 cells have infiltrated the muscles of the abdominal wall and metastases are present in liver and lungs (19). The total tumor burden was estimated to be at least 3 x 10⁴ SL2 cells, i.e., about 0.3 g.

Specific Immunity in Mice Cured of Disseminated SL2. Mice cured of disseminated SL2 by IL-2 immunotherapy were able to reject a second i.p. inoculum with SL2 cells (Table 2). These mice rejected up to 10⁹ SL2 lymphoma cells, but were not able to reject P815 mastocytoma, another DBA/2 derived tumor. Injection of 10⁶ P815 cells i.p. resulted in tumor growth and mice injected with the same tumor challenge died within 3 weeks.

Table 1 Immunotherapy of SL2-bearing mice with varying doses rIL-2

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Days 3-7</th>
<th>Days 10-14</th>
<th>MST ± SD</th>
<th>Surviving mice⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000⁶</td>
<td>5000</td>
<td>18.1 ± 2.6</td>
<td>4/15*</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>5000</td>
<td>18.7 ± 2.3</td>
<td>6/15*</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>100</td>
<td>17.1 ± 1.5</td>
<td>0/15</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>5000</td>
<td>18.5 ± 2.5</td>
<td>4/15*</td>
<td></td>
</tr>
</tbody>
</table>

* SL2 cells (2 x 10⁴) were injected i.p. on day 0. Mice were injected i.p. with PBS/BSA, 100 or 5000 units rIL-2 on days 3-7 and days 10-14.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Cytoxicity index (mean ± SD)₇, E:T ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁶ SL2</td>
<td>51.9 ± 0.8, 100:1</td>
</tr>
<tr>
<td>10⁶ P815</td>
<td>55.4 ± 2.5, 20:1</td>
</tr>
<tr>
<td>SL2</td>
<td>3.5 ± 3.3, 4:1</td>
</tr>
</tbody>
</table>

* LAK cells were generated by culturing DBA/2 spleen cells 5 days with 500 units rIL-2/ml CM.

* Cytoxicity was measured in a 4-h ⁵¹Cr release assay at effector cell:target cell ratio (E:T ratio) 100:1, 20:1, and 4:1. Targets were P815, Tu5, and SL2 cells. The cytoxicity index of fresh spleen cells against the tumors was always below 5.

* Results are shown from a representative experiment.

Specific Immunity in Mice Cured of Disseminated SL2. Mice cured of disseminated SL2 by IL-2 immunotherapy were able to reject a second i.p. inoculum with SL2 cells (Table 2). These mice rejected up to 10⁹ SL2 lymphoma cells, but were not able to reject P815 mastocytoma, another DBA/2 derived tumor. Injection of 10⁷ P815 cells i.p. resulted in tumor growth and death of all mice.

LAK-cell Cytotoxicity. Murine cells with LAK activity generated by culturing spleen cells for 5 days with 500 units rIL-2 per ml clearly lysed P815 and Tu5 tumor cells (Table 3). These cells did not show cytotoxicity against SL2 lymphoma cells. In addition LAK activity against SL2 cells was measured in a series of experiments in which the culture time with rIL-2 varied from 1-11 days and the rIL-2 concentration from 5 to 1000 units per milliliter. LAK activity against SL2 cells was always absent (data not shown).

Influence of rIL-2 on the In Vitro Growth of SL2 Cells. SL2 cells were cultured in vitro in varying concentrations rIL-2.
These control experiments demonstrated that rIL-2 had no significant direct influence on the in vitro growth of SL2 cells ($P > 0.05$, Table 4).

**DISCUSSION**

We demonstrated that local immunotherapy with low dose rIL-2 can cure mice bearing a large burden of disseminated SL2 lymphoma. This is of particular importance as these tumor cells are resistant to IL-2 activity. In our model it appeared essential for successful immunotherapy that daily i.p. injections with 5000 units rIL-2 were given late after tumor challenge. As it takes about a week before a specific immune response reaches its effector phase, these data suggest that the administered rIL-2 reinforces the effector phase of a specific antitumor immune reaction. This was confirmed by the finding that mice cured from SL2 tumor after IL-2 therapy show specific immunity against SL2. In DBA/2 mice SL2 is immunogenic, for it is possible to immunize mice with irradiated SL2 cells. T-lymphocytes isolated from these immunized mice can transfer tumor resistance to naive mice (17, 18). However in SL2 bearing mice no effective T-cell population seems to be generated (21).

The generation of these effector cells is apparently enhanced by the addition of rIL-2. It has also been shown in several other models that exogenously administered IL-2 can augment specific immune reactions in vitro after the transfer of immune lymphocytes (22-24) or during immunization (25, 26). It was demonstrated that the injection of low dose IL-2 during immunization stimulated the induction and function of cytotoxic T-cells (25, 26). Furthermore data have been published indicating that effective immunotherapy with low dose IL-2 is preferentially mediated by T-lymphocytes whereas therapy with high dose IL-2 is mediated by natural killer cells (16). Additionally it has been shown that IL-2 therapy can be most effective when given late after tumor challenge (9, 27). However, these groups studied high dose IL-2 therapy of mice bearing LAK-sensitive tumors. Interestingly it has been reported that early IL-2 treatment of mice bearing weakly immunogenic tumors is mediated by both ASGM-1- and Lyt2-positive cells, whereas late treatment is mediated by Lyt2-positive cells, indicating a change in effector cells (28).

It is unlikely that in vivo-generated LAK activity is important during tumor rejection in our model as LAK cells do not lyse SL2 cells in vitro. However it cannot be excluded that LAK cells are involved as it has been shown that LAK cells are capable of antibody-mediated cellular cytotoxicity against otherwise LAK-resistant tumor cells (29, 30).

It can be concluded that immunotherapy with relatively low dose IL-2 can be highly effective in mice bearing a large tumor burden. In our model it is estimated that at the onset of IL-2 therapy $3 \times 10^6$ tumor cells are present, i.e., approximately 0.3 g tumor. As the mean weight of male DBA/2 mice is 24 g, these mice bear a tumor load that accounts for more than 1% of their total body weight. In humans this would be a tumor of about 1 kg.

Most important is that the i.p. administration of relatively low dose rIL-2 results in an effective systemic antitumor reaction, as SL2 lymphoma cells metastasize rapidly. This finding is very promising for future development of better protocols for effective IL-2 therapy of human cancer.

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**REFERENCES**


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