Effects of Interleukin 2 Treatment Combined with Local Hyperthermia in Mice Inoculated with Lewis Lung Carcinoma Cells

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ABSTRACT

Recombinant human (rhu) interleukin 2 (IL-2) was evaluated alone and in combination with local hyperthermia (LH) in mice inoculated s.c. with 5 x 10^5 Lewis lung carcinoma cells. Four treatment regimens were begun 6 days postinoculation at a time when the tumor had grown to approximately 8.0 mm in diameter. Treatments were: group 1, saline injected as control; group 2, LH; group 3, rhuIL-2; or group 4, LH combined with rhuIL-2. LH utilized hot water circulation by a Brann Thermonix 1420. The intratumor temperature was maintained at 43 ± 0.2°C for 30 min each on days 6 and 10 and rhuIL-2 was given s.c. at 5 x 10^4 units twice a day for 5 days. Thirty mice in each group were sacrificed 28 days after tumor inoculation. An additional 20 mice in each group were observed for survival time. The size of primary tumor and the number of lung metastases were reduced and the survival time was prolonged in mice treated by either LH or IL-2. However, a greater antitumor effect in Lewis lung carcinoma tumor-bearing mice was observed using IL-2 therapy combined with LH. Tumor growth was associated with increased splenic granulocyte-macrophage progenitor cells and an abnormal L3T4*/Lyt-2* lymphocyte subset ratio (<1.0). Splenic granulocyte-macrophage progenitor cell numbers and the L3T4*/Lyt-2* ratio returned to normal in the group treated with combination therapy, the best responder group. The L3T4*/Lyt-2* ratio did not change in the groups treated with single therapy. These results suggest the efficacy and possible clinical relevance of combined therapy with rhuIL-2 and LH for certain metastatic tumors.

INTRODUCTION

Lymphokines are important in the development of host immunity and may be useful in modifying biological responses in cancer patients or other diseases associated with immune dysfunction (1). IL-2 is one of the earliest lymphokines considered for these types of applications (2, 3). However, a problem with IL-2 therapy is that in order to produce antitumor activity it is used in high doses, which have been associated with toxicity (4-6). It has been reported that the antitumor activity of IL-2 can be enhanced when IL-2 is used in combination with other cytokines and/or monoclonal antibodies and in combination with activated effector cytotoxic lymphocytes (7-13).

There is a biological rationale for the use of hyperthermia in the treatment of cancer (14, 15). It has been theorized that following tumor heating, absorbed necrotic cancer cell by-products provide the antigenic stimulus needed to enhance the host immune system which then leads to destruction of distant metastatic foci (16, 17). Local hyperthermia (LH) alone can induce regression of tumor (18), but better results have been obtained by combining heat with low doses of irradiation (19-22). Activities of a number of immunological cytokines can be modulated by hyperthermia (23-28).

The present study was initiated to evaluate the effects of rhuIL-2, alone and in combination with local hyperthermia, on the growth of tumors in mice inoculated with LLC cells.

MATERIALS AND METHODS

Mice. Female C57BL/6J mice, 6-8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in plastic cages, 5/cage, and provided with water and standard laboratory diet ad libitum.

Tumor. Lewis lung carcinoma is a malignant type of epidermoid carcinoma developed spontaneously in a C57BL/6J mouse. Inoculation of tumor cells into syngeneic hosts results in the consistent appearance of metastatic foci in the lungs. Sterile tumor cell suspensions were prepared by mincing tumor fragments in RPMI 1640 and filtering the crude suspension through a fine sterile screen mesh as described previously (29). Cell viability was determined by trypan blue exclusion. Mice were inoculated s.c. with 5 x 10^6 viable tumor cells in 0.1 ml of culture medium into the right hind limb. Six days postinoculation the tumor averaged 8.0-10.0 mm in diameter. The tumor-bearing mice were randomly divided into four groups with treatment started 6 days postinoculation: group 1, saline injected as control; group 2, LH (43 ± 0.2°C for 30 min, twice a week); group 3, rhuIL-2 (1.8 x 10^4 IU/mg or 3 x 10^6 Cetus units/mg; Cetus Corp., Emeryville, CA); group 4, rhuIL-2 combined with LH.

LH. Mice bearing Lewis lung carcinoma cells in their right hind limb were treated with local hyperthermia on days 6 and 10. Mice were placed on a specially designed wooden table and their tumor-bearing limbs were covered with a rubber tube and warmed with hot water circulated by a Thermonix 1420. Intradatum temperature was monitored by inserting a thermocouple temperature probe (Baily IT-1E) into the tumor. The thermocouple probe was connected to a calibrated Baily BAT-8 thermometer. Twenty-eight days after tumor inoculation, 30 mice in each group were sacrificed and an additional 20 mice in each group were kept to determine the survival time.

Measurement of Tumor Volume. The primary tumor was measured with calipers. The two perpendicular diameters and the mean diameter were used to calculate tumor volume. Volume was calculated as

\[ V = 0.4 (ab^2) \]

where \( a \) and \( b \) are two perpendicular diameters, and \( b \) is the smaller one (30).

Determination of Lung Metastases. During autopsy, the lungs were stained by inflation with a 15% solution of India ink and fixed in Fekete's solution (31). With this technique, the metastatic nodules become prominent and are easily counted using a magnifying lens (×8).

Source of Cells. Cells obtained from femoral bone marrows and spleens of individual mice were made into single cell suspensions by filtering through a fine sterile screen mesh. Nucleated cells were counted using a Coulter Counter (Model ZM; Coulter Electronics, Hialeah, FL).

Colony Formation by Granulocyte-Macrophage Progenitor Cells. Mouse bone marrow and spleen cells were plated, respectively, at 5 x 10^4 cells/ml and 10^6 cells/ml in 35-mm standard tissue culture dishes in 1 ml of 0.3% agar (Difco) culture medium containing McCoy's 5A medium supplemented with additional essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate (Gibco Lab...
oratories); 10% heat-inactivated fetal calf serum; and 10% (v/v) test CM (32, 33). Culture dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO2 at lowered (5%) O2 tension and scored after 5 days for colonies (>40 cells/aggregate) and clusters (5–40 cells/aggregate).

Monoclonal Antibodies. Anti-L3T4 and anti-Lyt-2 were purchased from Becton-Dickinson (Mountain View, CA).

Flow Cytometry. Mouse spleen cells were analyzed by indirect immunofluorescence, using an EPICS flow cytometry system (Coulter Electronics). One × 106 cells were incubated with anti-L3T4 (4 μg phycoerythrin-conjugate) for background fluorescence determination, mouse IgG2a + b (5 μg fluorescein). The antibodies were obtained from Becton-Dickinson and incubation was done at 4°C for 30 min. After cells were washed 3 times, they were diluted to 1.0 ml with phosphate-buffered saline for analysis. Cells were kept at 4°C until they were analyzed.

Statistics. Results are expressed as mean ± SE. Statistical significance was determined by use of Student’s t test (two tailed) or log rank test.

RESULTS

Primary Tumor and Lung Metastases. LLC tumor-bearing mice were routinely monitored for tumor growth twice a week and the final tumor volume was measured on day 28-post tumor cell inoculation. Table 1 shows the effects on tumor growth of LH, rhuIL-2, or LH combined with rhuIL-2. The mean primary tumor volume in mice treated with LH or rhuIL-2 is about one-third of that in the control mice. The primary volume was reduced even further in mice treated with LH combined with rhuIL-2. The number of lung metastases is significantly less in the combination therapy arm (LH- and IL-2-treated groups) compared to controls or to either therapy arm itself. The effects of either therapy alone reduced mainly the numbers of small metastases, while the numbers of both small and large metastases were reduced by combination therapy. No metastases were noted in 2 of 30 mice in the combined therapy group and no lung nodules over 4 mm were found in this group.

Survival Time. Table 2 shows the survival times for the 4 groups of mice averaging the results of 20 mice/group from a total of 2 complete experiments. Mice were examined daily. More than one-half of the saline-treated control mice had died by day 36, while at this time all mice in the treatment groups were alive. By day 45 all untreated mice had died, whereas, respectively, 80, 100, and 100% of the mice treated with LH, rhuIL-2, and LH plus rhuIL-2 survived. In all cases treatment increased survival time significantly when compared to controls but mice treated with combination therapy had the longest survival times.

Hematological Parameters. Analysis of data for absolute numbers of CFU-GM per femur and per spleen (Table 3) demonstrated that the greatest effects of LLC inoculation were apparent in the spleens which manifested large increases in progenitor cell numbers. These increases were significantly decreased by treatment with rhuIL-2 or the combination of rhuIL-2 with LH. Treatment with LH alone had no significant effect on numbers of bone marrow or spleen CFU-GM and the effects of combination therapy were not greater than that of rhuIL-2 alone. Analysis of data for L3T4+ and Lyt-2+ spleen lymphocyte subsets demonstrated a decrease in the percentage of L3T4+ cells in mice bearing LLC cells which resulted in a decreased L3T4*/Lyt-2+ ratio (Table 4; average results of 3 experiments). Only the tumor-bearing mice receiving combination therapy had their percentage of L3T4+ cells and their L3T4*/Lyt-2+ ratios restored to normal levels.

DISCUSSION

Hyperthermia may have either a direct suppressive effect on tumor cell growth or it may act indirectly via enhancement of host immune function (34). It has been demonstrated by others that IL-2 is capable of inducing desired changes in immune response and of producing antitumor activity (4–6). Our present experiments demonstrated the most potent antitumor effect in LLC tumor-bearing mice when combination therapy with rhuIL-2 and LH was applied. The size of primary tumors and the numbers of lung metastases were reduced and the survival time was prolonged in mice treated either by rhuIL-2 or LH. However, best antitumor effects were observed using both rhuIL-2 and LH. The mechanism(s) underlying these effects are not known, but they may reflect immune associated effects as discussed below.

Mice inoculated with LLC tumor cells had increased numbers of splenic CFU-GM and a decreased proportion of spleen cells with the L3T4+ phenotype with the ratio of L3T4+/Lyt-2+ (<1.0) being reversed from normal. These abnormalities were corrected by combination therapy. Increased numbers of splenic CFU-GM, which may be associated with immune suppressor cell activities, may reflect a reactive process involving enhanced cytokine production by T-cells or other accessory cells, an effect counteracted by the treatment used. L3T4 is considered to the murine analogue of the human Leu-3/T4 (CD4) molecule and expression of this antigen correlates primarily with major histocompatibility complex class II antigen reactivity (35). Our

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of mice</th>
<th>Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>20</td>
<td>34.5 ± 1.27</td>
</tr>
<tr>
<td>2</td>
<td>LH</td>
<td>20</td>
<td>46.9 ± 0.75</td>
</tr>
<tr>
<td>3</td>
<td>rhuIL-2</td>
<td>20</td>
<td>52.1 ± 1.16</td>
</tr>
<tr>
<td>4</td>
<td>LH + rhuIL-2</td>
<td>20</td>
<td>61.5 ± 1.35</td>
</tr>
</tbody>
</table>

* Data represent two separate experiments with the mean ± SE results for a total of 20 mice in each group. The comparison of survival of different groups was analyzed by Kaplan-Meier and log rank test.
experiments, while establishing an association between T-cells and tumor growth and metastases in animals, do not allow us to determine that there was a cause-effect relationship. However, it is intriguing to speculate that the antitumor activity of IL-2 combined with LH was at least in part mediated through T-lymphocytes, alone or in combination with other treatment modalities, as mean ± SEM per femur and spleen for 1 representative of 2 experiments. Numbers in parentheses, percentage change from saline control with significant levels given. NS, not significant (P > 0.05). The values for the non-tumor-bearing untreated control mice are given for reference.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonies/organ</th>
<th>Colonies + clusters/organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Bone marrow</td>
<td>28,435 ± 385</td>
<td>73,465 ± 5,295</td>
</tr>
<tr>
<td>Saline controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>36,340 ± 5,825 (+28)</td>
<td>NS</td>
</tr>
<tr>
<td>rhuIL-2</td>
<td>22,960 ± 280 (-19)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>rhuIL-2 + LH</td>
<td>20,690 ± 218 (-21)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Non-tumor-bearing controls</td>
<td>20,480 ± 255</td>
<td>45,120 ± 3,217</td>
</tr>
<tr>
<td>B. Spleen</td>
<td>15,796 ± 5,751</td>
<td>26,098 ± 9,585</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>22,250 ± 3,745 (+41)</td>
<td>NS</td>
</tr>
<tr>
<td>rhuIL-2</td>
<td>3,292 ± 1,608 (-79)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>rhuIL-2 + LH</td>
<td>4,895 ± 1,510 (-69)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Non-tumor-bearing controls</td>
<td>1,044 ± 185</td>
<td>2,196 ± 274</td>
</tr>
</tbody>
</table>

* Three mice per group were sacrificed on day 28. Bone marrow and spleen cells were plated, respectively, at 5 x 10⁴ and 10⁶ cells/ml and the results are expressed as mean ± SEM per femur and spleen for 1 representative of 2 experiments. Numbers in parentheses, percentage change from saline control with significant levels given. NS, not significant (P > 0.05). The values for the non-tumor-bearing untreated control mice are given for reference.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of positive cells</th>
<th>Ratio</th>
<th>Statistical Analysis of % of L3T4-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L3T4⁺</td>
<td>L3T4⁺/L3T4⁺</td>
<td>Groups</td>
</tr>
<tr>
<td>2 vz. 3</td>
<td>&gt;0.05, not significant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 vz. 4</td>
<td>&lt;0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 vz. 5</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 vz. 2</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 vz. 5</td>
<td>&gt;0.05, not significant</td>
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</tbody>
</table>

* The results are expressed as mean cell percentage ± SEM from the combined average of three experiments for a total of 9 mice/group.

**REFERENCES**


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