Marked Reduction of Subcutaneous Tumor Growth by Intraperitoneal Administration of Recombinant Human Interleukin 2 with a Cell Accumulator, Proteose-Peptone, in Mice

Seiichi Tanida, Hiroyuki Uchida, Kazuto Taniguchi, and Kikuo Nomoto

ABSTRACT

The growth of 3-methylcholanthrene-induced fibrosarcomas, Meth 1 and Meth A, was strongly suppressed by a combination of recombinant human interleukin 2 (rIL-2) with proteose-peptone (PP) administered i.p. to syngeneic mice. When 1 ml of 10% PP was injected i.p. on Day 6 followed by rIL-2 (50 µg) administered i.p. on Days 7 and 8 after the s.c. inoculation of tumor cells into female BALB/c mice, the tumors regressed. A similar result was also obtained when 12.5 µg of rIL-2 were injected on Days 7, 8, and 9 after s.c. inoculation of Meth 1 cells. The treatment with an anti-asialo-GM1 antibody had no effect on the regression of the Meth 1 tumor induced by the combination. However, the combined treatment with rIL-2 and PP did not suppress the growth of the Meth 1 tumor in adult thymectomized, irradiated, and fetal liver cell-reconstituted BALB/c mice. Therefore, this suggests that the T-cells might be the principal effectors of this antitumor system. The cytolytic activity of splenocytes and peritoneal exudate cells from Meth 1 tumor-bearing mice against Meth 1 cells was significantly augmented by the combined treatment. This peritoneal exudate cell also showed cytolytic activity against other target cells such as Meth A, antigenically distinct from Meth 1, YAC-1, a leukemia cell line sensitive to natural killer cells, and EL-4, a lymphoma cell line resistant to natural killer cells. The cytolytic activity of these effectors was reduced by the treatment with anti-thy1.2 antibody plus complement. The adherent cells in this peritoneal cavity had only a small cytolytic activity on Meth 1 and Meth A targets. The mechanism of antitumor immunity by rIL-2 in combination with PP and the therapeutic availability of this lymphokine are discussed.

INTRODUCTION

IL-2 is a lymphokine that plays an important role in a variety of immune reactions. IL-2 transmits a signal for the proliferation and/or differentiation of T-, B-, NK, and LAK cells via binding with specific receptors on their surfaces (1-4). Therefore, it has been presumed that IL-2 might be therapeutically useful to augment the immune responses of patients suffering from certain immunodeficiencies or malignant neoplasia. Studies in this area have been accelerated by the success of the production of large amounts of highly purified IL-2 by utilizing the recombinant DNA technique.

The use of rIL-2 for the immunotherapy of animals and human tumors has progressed substantially in recent years. However, because of the short in vivo half-life of rIL-2 (5-7), the induction of an adequate antitumor effect using the here-tofore common approaches for anticancer chemotherapy still presents many difficulties. Current trials in adoptive immunotherapy with in vitro activated effector cells by rIL-2 have attracted much attention as a promising effort (8). However, this therapeutic approach is technically complicated, since LAK cells are generated in vitro by the incubation of murine splenocytes or human peripheral blood lymphocytes in a medium containing IL-2.

The biological function of IL-2 in vivo seems to be regulated by endogenous inhibitors (9). Thus, the major functional activity of IL-2 is thought to be potentially limited or localized to the vicinity of its production. In order to establish the principles for a more effective use of rIL-2 to augment antitumor immunity, we attempted to apply rIL-2 to a specific locus in vivo, where a large number of T-lymphocytes expressing IL-2 receptors should have emigrated.

In the following study, we describe the dramatic regression of Meth 1 and Meth A tumors in syngeneic mice administered i.p. with rIL-2 and PP as an irritant for elicitation of T-lymphocytes from the peripheral blood to the peritoneal cavity in tumor-bearing mice.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased from Shizuoka Laboratory Animal Center, Japan, and were used for all experiments at 8 to 9 wk of age.

ATXFL Mice. BALB/c mice were thymectomized at 5 wk of age and 2 wk later received 950 rads of whole-body irradiation. They were infused with 2 to 10 x 10^6 syngeneic fetal liver cells within 24 h of irradiation (ATXFL). These ATXFL mice were used in the experiment 4 wk after the reconstitution with syngeneic cells. Additional mice were checked at this time for T-cell depletion by fluorescence-activated cell sorter analysis and by the failure of splenocytes to generate alloreactive T-lymphocytes after in vitro allosensitization. All the experiments conducted in vivo were carried out under the specific-pathogen-free barrier system.

Tumors. Two different lines, Meth 1 (10) and Meth A (11), of the 3-methylcholanthrene-induced fibrosarcoma of BALB/c origin, were used in this study. They were maintained in ascites form in syngeneic BALB/c mice by weekly passages. These tumor cells were suspended in Dulbecco's Ca^2+- and Mg^2+-free PBS and adjusted to a density of 1 x 10^7 cells/ml. The mice were inoculated s.c. with 0.1 ml of the cell suspension in the right flank; the day of inoculation is referred to as Day 0. Tumor growth in vivo was measured as 2 diameters at a right angle to each other. The EL-4 lymphoma, syngeneic to C57BL/6 mice, was maintained in vivo as ascites tumors. YAC-1, a Moloney virus-induced lymphoma of A/Sn mouse origin, was maintained in vitro passage in a conditioned medium. All tumors were free of viral pathogens or Mycoplasma. Both Meth 1 and Meth A tumors are resistant to lysis by natural killer cells in vitro.

rIL-2 Administration. A purified preparation of rIL-2 of human origin was provided by the Central Research Division of Takeda Chemical, Inc., Osaka, Japan (12, 13). The preparation, 1.2 x 10^7 units/mg of protein, calculated on the basis of the Biological Response Modifier Program reference reagent human IL-2 (Jurkat) of the National Cancer Institute (Bethesda, MD), was diluted with 0.15 M NaCl containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) to

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: IL-2, interleukin 2; rIL-2, recombinant human IL-2; PP, proteose-peptone; NK, natural killer; LAK, lymphokine-activated killer; PEC, peritoneal exudate cells; ATXFL, adult thymectomized, irradiated, and reconstituted with fetal liver cells; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; CM, complete culture medium.

284 Unpublished results.
a final concentration of 100 μg/ml. Mice, previously given 1 ml of 10% PP (Difco Co.) i.p. on Day 6, were usually given injections i.p. 2 or 3 times with 0.5 ml of the diluent of rIL-2 on Days 7 and 8, or Days 7, 8, and 9.

Preparation of Splenocyte Suspension. The spleens were removed aseptically and gently teased into suspension in HBSS. The cell suspension, which was passed through cotton gauze, was centrifuged, and the erythrocytes were lysed by treatment with buffered ammonium chloride solution. The cells were washed and adjusted to proper concentration in a complete culture medium. RPMI-1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 30 μg/ml of gentamicin, and 0.2% sodium bicarbonate was used as a CM for cytotoxicity assay.

Preparation of Peritoneal Exudate Cells. PEC were obtained from BALB/c mice by peritoneal lavage of the peritoneal cavity with 10 ml of cold Ca²⁺- and Mg²⁺-free HBSS 3 days after a single i.p. injection of 1 ml of PP (Difco Co.). The PEC were centrifuged at 350 x g at 4°C for 5 min and were resuspended in a CM to a final density of 1 x 10⁶ cells/ml. To obtain adherent cells from PEC as peritoneal macrophage monolayers, 0.1-ml aliquots of PEC suspensions were added to the wells of 96-well flat-bottomed plates (No. 2586; Corning Glass Works, Corning, NY) and incubated for 2 h at 37°C in 5% CO₂. After the incubation, the plates were washed 3 times with warm CM to remove any nonadherent PEC. The resultant populations of cells were greater than 95% macrophages as determined morphologically and by staining for nonspecific esterase. For the preparation of nonadherent PEC, 3-ml aliquots of the PEC suspension (1 x 10⁶ cells/ml) were incubated in wells of 6-well cluster dishes (No. 3406; Coster, Cambridge, MA) at 37°C in a CO₂ incubator. After 1.5-h incubation, the dishes were shaken gently, and the nonadherent cells were harvested. The same procedure was repeated 2 more times for further purification of the nonadherent PEC population. The final yields of nonadherent PEC were usually 25 to 30% of the originally cultured PEC, and it was used in the desired concentration.

**In Vivo Treatment with Anti-asialo-GM1 Antibody.** To reduce the NK activity in vivo, anti-asialo-GM1 antibody obtained from Wako Chemical Co., Osaka, Japan, was diluted 10-fold with PBS, and 0.3 ml of the diluted were injected i.v. on Days 5 and 10 (14, 15).

**In Vitro Cytotoxicity Test.** PEC and splenocytes prepared from tumor-bearing mice on Day 9 were used as the effectors. Meth 1, Meth A, EL-4 thymoma cell line derived from C57BL/6 mice resistant to NK cells, and YAC-1, a leukemic cell line sensitive to NK cells, were used as the targets. The cytotoxicity of splenocytes and PEC was determined by the method of Brunner et al. (15) with some modification (10). Briefly, 10⁶ tumor cells in 0.5 ml of the fresh medium were labeled with 100 μCi of Na²⁸CrO₄ (Japan Atomic Energy Research Institute, Tokyo, Japan) at 37°C for 2 h and washed 3 times with the medium. The ⁵¹Cr-labeled target cells (1 x 10⁶) and varying numbers of effector cells were mixed in 0.2 ml of fresh medium in 96-hole round-bottomed microplates. The plates were incubated at 37°C for 4 h against EL-4 and YAC-1 targets and 18 h against Meth 1 and Meth A targets in a humidified atmosphere of 5% CO₂ in air. Then, the microplates were centrifuged for 5 min, and the amount of ⁵¹Cr released in 0.1 ml of supernatant was measured. To determine the maximal amount of releasable radioactivity, the labeled cells were destroyed by 10% Triton X-100 and centrifuged, and the radioactivity of the supernatant was measured. The minimal release was the radioactivity released from the target cells incubated in the culture medium without effector cells. The percentage of ⁵¹Cr release was calculated from the counts of triplicate wells by the equation

\[
\% \text{ of } ^{51}\text{Cr release} = \frac{\text{experimental release} - \text{minimal release}}{\text{maximal release} - \text{minimal release}} \times 100
\]

**In Vitro Treatment of PEC with Antibody and Complement.** The effectors were adjusted to a concentration of 10⁴ cells/ml, treated with anti-thy-1.2 antibody (× 500) (Cedarlane Laboratories, Ltd., Ontario, Canada) for 45 min at 4°C and anti-asialo-GM1 serum (× 50) (Wako Pure Chemical Industries, Osaka, Japan) for 45 min at 4°C, and further incubated with complement at a 1/10 dilution (Low-Tox-M rabbit complement; Cedarian Laboratories, Ltd.) for an additional 45 min at 37°C to deplete the T-cell and NK cell activity in vitro, respectively. The cells were washed 3 times in HBSS and resuspended in CM for further study.

**Statistical Analysis.** Student’s t test was used to determine the statistical significance.

**RESULTS**

**Inhibition of Growth of Meth 1 Tumor by rIL-2 in Syngeneic Mice.** When there was no treatment administered to Meth 1-bearing mice, they died within a month after the tumor inoculation. The tumor growth was slightly suppressed in Meth 1-bearing BALB/c mice given injections i.p. twice with a high dose (50 μg) of rIL-2 on Days 7 and 8 (Fig. 1), but these mice died from the progression of this tumor after 1 mo. When 1 ml of 10% PP was injected i.p. on Day 6 followed by i.p. administration of rIL-2, the tumor regressed completely (Fig. 1). The tumor regression induced by the combined treatment with rIL-2 and PP seems to depend on the route of rIL-2 administration, because the growth of the Meth 1 tumor was scarcely affected when the lymphokine was administered i.v. (data not shown). This observation correlates with the rapid loss of bioavailability of rIL-2 in serum (5–7, 9). PP alone could not suppress tumor growth in this system (Fig. 1C). In addition, the mock injection of a rIL-2-free solution (0.15 m NaCl containing 0.1% bovine serum albumin) did not affect tumor growth in this combined treatment. The marked regression of the Meth 1 tumor in syngeneic mice was observed when a lower dose (12.5 μg) of rIL-2 was injected 3 times on Days 7, 8, and 9 in this combination with PP (P < 0.001 compared with untreated control) (Fig. 2A). The 3-time administration of rIL-2 caused a significant inhibition of the tumor growth even at ½ dose (3.75 μg) or even ¼ dose (1.25 μg) of the lymphokine (P < 0.05 compared with Fig. 2A, untreated control).

**Inhibition of Growth of Meth A Tumor by rIL-2 in Syngeneic Mice.** The Meth A tumor is antigenically distinct from the Meth 1 tumor. Further examination demonstrated that combined treatment with rIL-2 and PP caused a marked regression in the Meth A tumor, although rIL-2 alone or PP alone showed little or no effect on the tumor growth (data not shown).

**In Vivo Analysis of Inhibition of Growth of Meth 1 Tumor by Treatment with rIL-2 and PP.** PP is an irritant that elicits...
MURINE TUMOR REGRESSION BY A COMBINATION OF IL-2 AND IRRITANT

Fig. 2. Regression of Meth 1 tumor in syngeneic mice treated with lower doses of rIL-2 in this combination. A, untreated control; B, i.p. administration of 1 ml of 10% PP alone on Day 6 ( ); C, combined administration of rIL-2 (1.25 μg, 3 times) on Days 7, 8, and 9 with PP on Day 6; D, combined administration of rIL-2 (3.75 μg, 3 times) with PP; E, combined administration of rIL-2 (12.5 μg, 3 times) with PP; F, i.p. administration of rIL-2 (12.5 μg, 3 times) alone on Days 7, 8, and 9. Bars, SD.

Fig. 3. Growth of Meth 1 tumor in syngeneic ATXFL mice given rIL-2 and PP. The treatment schedule was the same as described in the legend of Fig. 1. rIL-2 at 50 μg was injected twice. A, untreated control; B, combined administration of rIL-2 and PP. Bars, SD.

Inflammatory cells from the peripheral blood to the peritoneal cavity in mice. Numerous kinds of cells, such as macrophages, polymorphonucleocytes, and lymphocytes, were observed when rIL-2 was administered.

IL-2 binds to the specific receptor on the surface of lymphocytes and induces proliferation and expansion of these cells. rIL-2 combined with PP did not suppress the growth of the Meth 1 tumor in syngeneic ATXFL mice (Fig. 3). Similar results were observed in BALB/c-nu/nu mice (data not shown). This indicates that T-lymphocytes are mostly essential for the antitumor effect of rIL-2 in this treatment. On the other hand, NK cells may not be involved in the tumor regression in this combined treatment, because the growth of the Meth 1 tumor was also suppressed in mice treated with i.v. injection of antiasialo-GM1 antibody (Fig. 4). This antibody is known to reduce the NK activity in vivo (14, 15) and, in the preliminary experiment, fresh spleen cells from these mice had no cytolytic activity against NK-sensitive YAC-1 targets. The same result was obtained in mice treated with an i.p. injection of this antibody (data not shown).

Augmentation of Cytolytic Activity by Combined Treatment with rIL-2 and PP. The cytolytic activity of splenocytes and PEC prepared from tumor-bearing mice on Day 9 was examined. The activity of both effectors against Meth 1 cells was significantly augmented by the combined treatment of rIL-2 and PP, compared with the treatment of PP alone (Fig. 5A). The splenocytes from mice given rIL-2 i.v. showed a slight activity in the combined treatment (Fig. 5B). This result suggests that effector cells proliferated and expanded in the peritoneal cavity, and then migrated into the peripheral. To clarify the population of effector cells in this combination, the cytolytic activity of PEC against other murine tumor cell lines was examined (Table 1). PEC prepared from Meth 1-bearing mice treated with rIL-2 and PP apparently augmented the cytolytic activity against other murine tumor cell lines such as Meth A, a methylcholanthrene-induced fibrosarcoma cell line derived from a BALB/c mouse and antigenically distinct from Meth 1 cells (16); YAC-1, a leukemic cell line sensitive to NK cells; and EL-4, a lymphoma cell line derived from C57BL/6 and resistant to NK cells (Table 1). The effector cells in the PEC also showed...
MURINE TUMOR REGRESSION BY A COMBINATION OF IL-2 AND IRRITANT

Fig. 4. Effect of anti-asialo-GM1 antibody on the regression of Meth 1 tumor induced by combined administration of rIL-2 and PP. The treatment schedule was the same as described in the legend of Fig. 1. A, untreated control; B, administration of PP alone (solid arrow); C, combined administration of rIL-2 (dashed arrow) with PP (solid arrow); D, C plus i.v. administration of anti-asialo-GM1 antibody on Days 6 and 12 (upper solid arrows). Bars, SD.

Days after tumor inoculation

![Graph showing tumor size (mm sq) over days after tumor inoculation for different treatment conditions.]

Table 1 Cytolytic activity of PEC from Meth 1-bearing mice treated with IL-2 and PP

<table>
<thead>
<tr>
<th>Effectors*</th>
<th>Meth 1b</th>
<th>Meth 2b</th>
<th>EL-4c</th>
<th>YAC-1d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated</td>
<td>26.1 ± 4.0d</td>
<td>24.2 ± 1.2</td>
<td>44.0 ± 3.3</td>
<td>65.0 ± 5.3</td>
</tr>
<tr>
<td>Anti-thy 1 + complement</td>
<td>1.5 ± 1.0d</td>
<td>2.0 ± 2.5d</td>
<td>0.5 ± 0.5d</td>
<td>1.0 ± 0.4d</td>
</tr>
<tr>
<td>Anti-asialo-GM1 + complement</td>
<td>23.0 ± 1.5d</td>
<td>22.0 ± 2.5d</td>
<td>43.0 ± 2.3d</td>
<td>45.0 ± 1.2d</td>
</tr>
<tr>
<td>Adherent</td>
<td>5.0 ± 2.0d</td>
<td>4.5 ± 1.3d</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>25.0 ± 4.0d</td>
<td>22.0 ± 2.2d</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Effectors are nontreated, treated with anti-thy 1.2 antibody plus complement, treated with anti-asialo-GM1 antibody plus complement, adherent cells of PEC, and nonadherent cells of PEC.

DISCUSSION

IL-2 administered i.p. simultaneously with PP, as an irritant, markedly reduced the s.c. growth of fibrosarcomas, Meth 1 and Meth A, in syngeneic BALB/c mice. Significant cytolytic activity against Meth 1 and Meth A targets by peritoneal exudate cells was demonstrated after the combined treatment (Table 1). This might explain the tumor reduction. As no apparent anti-

and in normal BALB/c mice with the same treatment (data not shown).

PEC was separated into two fractions, both adherent and nonadherent cells, and the majority of the former was thought to be macrophage. To assess the cytolytic activity of peritoneal macrophages, the chromium release assay was done against the same targets. Adherent cells had little cytolytic activity against Meth 1 and Meth A targets, while nonadherent cells had a similar activity to that of whole PEC (Table 1). So, the effector that functions in this mechanism was supposed to be in a nonadherent fraction which mainly consisted of lymphocytes, but not macrophages. Therefore, the macrophage was not the main effector which directly lysed the tumor.

This augmented cytolytic activity was markedly reduced by the treatment with anti-thy 1.2 antibody plus complement. However, when treated with anti-asialo-GM1 antibody plus complement, the cytolytic activity was not reduced against the Meth 1, Meth A, and EL-4 targets, and only moderately reduced against the YAC-1 target. These data suggest that cells which express thy 1 antigen are the effectors primarily responsible for this cytolytic activity in vitro and function as the main effector in Meth 1-bearing mice to regress the tumor in vivo. However, NK may not play a role in the regression of the Meth 1 tumor, since there was no reduction in cytolytic activity against the Meth 1 target by treatment with the anti-asialo-GM1 antibody.

These results suggest that the combined treatment with rIL-2 and PP might be able to augment effector functions of thy1-expressed nonspecific killer cells. We are now performing the neutralization (Winn) test to determine whether the effectors at the later stage after the combined treatment contain tumor-specific T-lymphocytes. The preliminary results show that specific cytotoxic T-cells, which may contribute to the regression of tumors, were detected in mice which received the combined treatment with IL-2 and PP.5

5 Manuscript in preparation.
tumor effect by the combined treatment was observed in T-cell-free ATXFL mice, the T-cell may be the main effector in this antitumor mechanism. rIL-2 alone neither reduced tumor growth appreciably nor augmented the cytolytic activity of splenocytes and peritoneal cells against the tumor cells (data not shown). The possibility of direct synergism of rIL-2 with PP to augment cytotoxic T-lymphocytes can be ruled out, since PP has little ability to augment the activity of allospecific killer cells in in vitro culture containing IL-2 or IL-2-induced cytotoxic activity (data not shown). Antigen binding to the specific T-cell receptor induces the expression of IL-2 receptors on the T-lymphocytes with IL-2 receptors expressed on their surface that provide the ability to respond to exogenous IL-2 (18–20). rIL-2 administered subsequently to the peritoneal cavity is expected to induce proliferation and expansion of the functional specific cytotoxic T-lymphocytes, and the majority of T-cells accumulated in the peritoneal cavity after PP administration should be efficiently activated and expand by exposure to rIL-2. These T-lymphocytes might migrate to the tumor-growing site where effectors might demonstrate their activity against the tumor. Since rIL-2 administered i.v. caused neither tumor regression nor augmentation of the cytotoxic activity of splenocytes against tumor cells, i.p. treatment must play an important role in the tumor regression.

The function of nonspecific killer cells such as LAK cells and NK cells should also be induced or be augmented by a combined treatment with rIL-2 and PP. The results depicted in Fig. 5 showed that the regression of Meth 1 and Meth A tumors in this combination may not depend on the effector function of NK cells. The combined treatment raised broad reactive killer cells, which are nonadherent and express thy1.2 (Table 1). These effector cells are referred to as LAK cells derived from T-lineaged cell precursors but not from NK cells. These results may reflect the in vivo results. Macrophages are known to play important roles for the activation and proliferation of T-lymphocytes (21, 22). Carbon treatment to block the function of phagocytes did not suppress the regression of the Meth 1 tumor induced by the combined rIL-2 and PP treatment (data not shown), but the low killing activity of the peritoneal adherent cells from mice which received the combined treatment indicates the small possibility of the participation of phagocytes including macrophages. Yet, the possibility that macrophages contribute in some way to the regression of the tumor growth in our system cannot be completely ruled out.

Recombinant DNA technology has opened the way to the availability of large amounts of homogeneous IL-2 as a therapeutically agent (23–25). The current experiments have used human rIL-2 in mice, since human IL-2 can be substituted for murine IL-2 to induce T-cell immunity in mice (26). In fact, no functional differences have been found in vitro between native and recombinant IL-2 molecules (12, 25). Therefore, it is likely that the principles developed for the use of human rIL-2 in mice will be applicable for the use of this lymphokine in human beings.

REFERENCES

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