Systematic Preclinical Study on the Therapeutic Properties of Recombinant Human Interleukin 2 for the Treatment of Metastatic Disease

James E. Talmadge, Hamblin Phillips, John Schindler, Henry Tribble, and Robin Pennington

Preclinical Screening Laboratory, NCI-Frederick Cancer Research Facility, Frederick, Maryland 21701 [J. E. T., H. P., H. T., R. P.], and Biogen, Inc., Cambridge, Massachusetts 02142 [J. S.]

ABSTRACT

The availability of recombinant human interleukin 2 (rH IL 2) has resulted in its clinical utilization both as a single agent and in combination with lymphokine-activated killer cells. In this report, we discuss the effects of rH IL 2, administered by various routes, on effector cell function, pharmacokinetics and bioavailability, and therapeutic activity. Studies of the pharmacokinetics of in vitro natural killer (NK) cell augmentation by rH IL 2 revealed that a short exposure to high levels of rH IL 2 can augment NK cell activity; however, a prolonged exposure (>12 h) was required to augment NK cell activity at lower doses of rH IL 2. These observations suggested that chronic administration of rH IL 2 might improve immunomodulatory and therapeutic activity. This hypothesis was supported by the results of studies in which we treated experimental and spontaneous metastasis, which revealed that the daily i.p. administration of rH IL 2 resulted in significantly greater therapeutic activity than administration three times/week. The therapeutic protocol for daily i.p. administration had a biphasic dosage optimum, such that low dose therapeutic activity was observed at approximately 1000 units/animal in the treatment of experimental metastases or 10 to 100 units/animal in the treatment of spontaneous metastases. There was a second dosage optimum at ≥100000 units/animal rH IL 2 delivered i.p. on a daily basis. Interferon doses had no significant therapeutic activity. Additional studies revealed that low dose therapeutic activity was not observed in nude mice. In contrast, therapeutic activity was observed in nude mice at high doses of rH IL 2 suggesting that low dose activity was associated with a T-cell-mediated effect, whereas high dose activity may have been meditated by NK or lymphokine-activated killer-like cells. This observation was in agreement with the dose response for T-cell adjuvant activity supporting the hypothesis that low dose therapeutic activity was T-cell associated, because adjuvant activity was observed when rH IL 2 was given daily at approximately 100 units/animal for 3 days, and higher doses had no activity or had a suppressive effect. Because we were concerned about the pharmacological aspects of rH IL 2 treatment, we also examined its therapeutic properties after continuous administration i.p. by osmotic pumps. Under these conditions, therapeutic activity was observed after administration of 600 units/h, whereas lower or higher doses did not have significant therapeutic activity.

INTRODUCTION

rH IL 2, a lymphokine that is produced by helper T-cells and large granular lymphocytes, has numerous immunomodulatory properties that have been observed in vitro and in vivo for rodent models (1-9). Furthermore, our laboratory as well as that of Rosenfels et al. (10-12) have demonstrated that rH IL 2 might improve immunomodulatory and therapeutic activity. This hypothesis was supported by the results of studies in which we treated experimental and spontaneous metastasis, which revealed that the daily i.p. administration of rH IL 2 resulted in significantly greater therapeutic activity than administration three times/week. The therapeutic protocol for daily i.p. administration had a biphasic dosage optimum, such that low dose therapeutic activity was observed at approximately 1000 units/animal in the treatment of experimental metastases, or 10 to 100 units/animal in the treatment of spontaneous metastases. There was a second dosage optimum at ≥100000 units/animal rH IL 2 delivered i.p. on a daily basis. Interferon doses had no significant therapeutic activity. Additional studies revealed that low dose therapeutic activity was not observed in nude mice. In contrast, therapeutic activity was observed in nude mice at high doses of rH IL 2 suggesting that low dose activity was associated with a T-cell-mediated effect, whereas high dose activity may have been mediated by NK or lymphokine-activated killer-like cells. This observation was in agreement with the dose response for T-cell adjuvant activity supporting the hypothesis that low dose therapeutic activity was T-cell associated, because adjuvant activity was observed when rH IL 2 was given daily at approximately 100 units/animal for 3 days, and higher doses had no activity or had a suppressive effect. Because we were concerned about the pharmacological aspects of rH IL 2 treatment, we also examined its therapeutic properties after continuous administration i.p. by osmotic pumps. Under these conditions, therapeutic activity was observed after administration of 600 units/h, whereas lower or higher doses did not have significant therapeutic activity.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male C57BL/6N mice H-2k, male BALB/c-nu/nu mice, male BALB/c-H-2 mice, and male C3H/HeN mammary tumor virus-bearing mice (H-2k), 3 or 4 weeks old, were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility.

Tumors. These studies used the Moloney virus-induced lymphoma YAC-1 (33) of A/5N-H-2a origin and the Moloney virus-induced lymphoma MBL-2 of C57BL/6N origin (31). Adherent cell lines included the metastatic melanoma variant B16-BL6 (34) from the B16 melanoma, which spontaneously arose in a C57BL/6N-H-2 mouse, and the spontaneous lung carcinoma M109 (35), which is syngeneic to BALB/c-H-2 mice. All adherent cell lines were maintained as monolayers in Eagle’s minimum essential medium supplemented with 5% fetal bovine serum, 2-fold-concentrated vitamin solution, glutamine, sodium pyruvate, and nonessential amino acids, termed CMEM. The YAC-1 tumor cell line was grown in RPMI 1640 supplemented with 10% fetal bovine serum and the same medium supplements used with Eagle’s minimum essential medium. All cell lines were free of Mycoplasma and pathogenic murine viruses (31).

Agents. rH IL 2 was generously provided by Biogen Research Corporation (Cambridge, MA). IL 2 activity was defined as the reciprocal of the dilution required to sustain half-maximal [3H]thymidine incorporation into 10⁶ C16 cells. The final assignment of units was based on reference IL 2 activity determined with the Biological Response Modifiers Program IL 2 reference reagent, lot ISDP 841. This resulted in approximately 5 × 10⁶ units rH IL 2 activity/mg protein; when this rH IL 2 was analyzed on 10% sodium dodecyl sulfate gels under
reducing conditions and stained with silver, it formed a single band with a molecular weight of 14,400. rIL-2 contained less than 0.03 ng/ml endotoxin contamination/5 x 10^4 units/rIL-2, as determined by *Limulus* amebocyte lysate analysis. Polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose was provided by Dr. Hilton Levy, National Institute of Allergy and Infectious Diseases, Frederick, MD. FK-565 was generously provided by Fujisawa, Japan. All media, salt solutions, and agents were endotoxin negative, as determined with the *Limulus* lysate assay (<0.03 ng/ml).

Augmentation of NK Activity. The ability of rIL-2 to augment NK activity in vitro was determined by incubating spleen cells for 24 h with control media or media containing various doses of rIL-2. In vivo augmentation of NK activity was assessed after injection of various doses of rIL-2, polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose (positive control), or saline (negative control). NK augmentation studies routinely used 3-week-old C3H/HeN mice, because mice of this age provide a very low baseline of NK activity (31). NK cell activity was assessed in a 4-h 1^125^I Cpm release assay using YAC target cells as previously described (31).

Induction of Tumor-specific Cytotoxic T-lymphocytes. Syngeneic C3H/HeN mice were immunized i.d. with 5 x 10^6 irradiated, collagenase-dissociated UV-2237 tumor cells with or without an adjuvant, by injecting 10^6 cells in 0.05 ml of the vaccine into each of 5 discrete sites. Vaccines consisted of tumor cells admixed with HBSS or one of several doses of rIL-2. In several experiments, additional i.p. injections of rIL-2 were administered. Control vaccines consisted of HBSS or rIL-2 alone. Target cells for the cytotoxic T-lymphocyte assays were added to the wells of a flat-bottomed, 96-well plate in 0.1 ml of CMEM-5% fetal bovine serum containing 5000 viable tumor cells. Following an incubation to allow for the attachment of the tumor cells, the cells were radiolabeled by adding 1 µCi 35Se-methionine in a 0.1-ml aliquot of methionine-free CMEM with 5% fetal bovine serum. These cells were incubated for 24 h and washed four times with CMEM. Ten days after immunization, splenic effector cells at effector:target cell ratios of 200:1, 100:1, 50:1, and 25:1 in 0.2 ml were added to the cultures; three replicates were used in each experiment. The cocultures were then incubated at 37°C for 18 h and centrifuged for 3 min; 0.1-ml aliquots were removed to determine the amount of radioactivity released. The percentage of cytolysis was calculated as:

\[
\text{% of cytolysis} = \frac{\text{cpm released in cultures with effector cells} - \text{cpm released spontaneously}}{\text{Total Triton-released cpm} \times 100}
\]

In other studies, we immunized syngeneic C57BL/6N mice with i.p. injections of 10^6 irradiated ascites MBL-2 tumor cells. rIL-2 was administered i.p. one or more times as described in the text. The cytotocicity assay was an 18-h indium release assay using peritoneal lymphocytes (cells that adhered to a plastic surface were removed), and the percentage of cytolysis was calculated with the same formula used in the 35Se-methionine assay (31).

Therapy of Established Metastases. Experimental lung metastases were established in 8-week-old C57BL/6 or BALB/c mice with i.v. injections of 5 x 10^5 in vitro-propagated B16-Bl6 melanoma or M109 carcinoma cells, respectively, in 0.2 ml of calcium-magnesium-free Hanks' balanced salts solution (CMF-HBSS). The schedule for therapy of metastases with rIL-2 varied in each experiment and is described within the text. We continued therapy for 4 weeks and mice that had survived 2 weeks after tumor challenge were killed and necropsied. Determinations of therapeutic efficacy were based on the number of pulmonary metastases.

We also evaluated the therapeutic efficacy of rIL-2 against spontaneous metastases derived from B16-Bl6 melanoma cells. B16-Bl6 melanoma cells (5 x 10^5) were injected in 0.05 ml CMF-HBSS into the posterior footpads of 8-week-old syngeneic mice. When the primary tumor reached a diameter of 0.8 to 1 cm, the tumor-bearing leg was resected at midfemur to include the popliteal lymph node. We initiated therapy 24 h later, using various protocols for a total of 4 weeks. Animals were necropsied 1 week after the last injection, and the number of lung metastases in each group was determined.

Statistical Analyses. The difference between the extent of metastasis (experimental or spontaneous) of control (CMF-HBSS-treated animals) and experimental groups was determined with the nonparametric Mann-Whitney U test. Ten animals were included in each group. The paired Student t test was used for comparisons in the effector cell assays.

RESULTS

Augmentation of NK Cell Activity. We examined the effect of the route of administration of rIL-2 on NK cell augmentation (Fig. 1), as well as the site specificity of NK cell augmentation. The i.p. injection of rIL-2 significantly augmented NK cell activity of peritoneal exudate cells at levels greater than 100 units/animal; however, maximal activation required the injection of 5,000–10,000 units/rIL-2/animal. We also found that i.v. injection of rIL-2 could augment NK cell activity of splenic cells and peritoneal exudate cells. The augmentation of splenic NK cell activity required i.p. or i.v. injection of higher doses of rIL-2; significant activity was observed at doses of 50,000 units/animal and maximal activity was observed at doses of greater than 100,000 units/animal. Although NK cell activity could be augmented only by high doses of rIL-2 when the lymphokine was administered by i.v. or i.p. injection, continuous infusion of rIL-2 by osmotic pumps (Alzert) in 9-week-old mice could augment splenic and peritoneal exudate cells. The augmentation of splenic NK cell activity required i.p. or i.v. injection of higher doses of rIL-2; significant activity was observed at doses of 50,000 units/animal and maximal activity was observed at doses of greater than 100,000 units/animal. Although NK cell activity could be augmented only by high doses of rIL-2 when the lymphokine was administered by i.v. or i.p. injection, continuous infusion of rIL-2 by osmotic pumps (Alzert) in 9-week-old mice could augment splenic and peritoneal exudate cells.
old C3H mice resulted in the augmentation of splenic (Fig. 2) or peritoneal (results not shown) NK cell activity in vivo at lower doses. Thus, Fig. 2 demonstrates that the 7-day osmotic pumps, which released approximately 25,000 units rH IL 2 activity/24-h period or slightly greater than 1000 units/h, augmented splenic NK cell activity, while the i.p. injection of even 10,000 units/animal of rH IL 2 did not augment splenic NK activity. However, lower doses (approximately 100 units/h) were unable to augment splenic or peritoneal NK cell activity. It should be noted that multiple injections of high doses of rH IL 2, continuous infusion of rH IL 2, or prolonged in vitro incubation (3 to 5 days) increases the spectrum of cytotoxic activity of NK cells to include P815 targets in a 4-h 51Cr release assay. However, spleen cells exposed to rH IL 2 in vitro for a short time (24 h) or following a single injection of rH IL 2 do not have cytotoxic activity for P815 targets but only for YAC target cells (Ref. 9; results not shown).

The pharmacokinetics of NK augmentation was examined by incubating spleen cells with rH IL 2 for various periods, washing them three times, and incubating them in normal medium for a total of 24 h. These studies demonstrated that spleen cells had to be continuously incubated with 100 units/ml rH IL 2 for 24 h to maximally augment NK cell activity (Table 1). A brief incubation (10 min to 2 h) in rH IL 2, followed by incubation in normal medium, did not significantly augment NK cell activity. However, incubation of spleen cells for 7–16 h with rH IL 2, followed by incubation in normal medium, significantly augmented NK cell activity but to a lesser extent than was observed after 24 h of incubation with rH IL 2.

In contrast, coincubation of murine spleen cells in vitro with 2000 units/ml rH IL 2 for 2 h augmented NK cell activity (Table 2). Indeed, this resulted in near maximal augmentation of NK cell activity, although at lower effectortarget cell ratios, there was significantly less NK cell augmentation compared to cells incubated with 2000 units/ml rH IL 2 for 24 h. To achieve maximal augmentation, lymphocytes cultured with 200 units/ml rH IL 2 required longer incubations than cells cultured with 2000 units/ml, although significant augmentation was observed after a 2-h coincubation.

Serum Pharmacokinetics of rH IL 2. The serum pharmacokinetics of rH IL 2 following i.v., i.p., or i.m. administration is illustrated in Fig. 3. These log-log plots demonstrate that serum levels of i.v. administration of rH IL 2, display a nearly linear reduction in activity with an ‘a’ to serum half-life of approximately 3 min, as has been reported previously (36–38). In contrast, i.m. administration of 25,000 units rH IL 2 results in a relatively rapid increase in rH IL 2 levels forming a plateau of rH IL 2 activity from 5 min to 8 h after administration and decreasing to background levels within 24 h. The peak level of rH IL 2 activity achieved after i.m. administration of 25,000 units rH IL 2 was approximately 150 units/ml compared to 800 units/ml achieved after i.v. administration of 25,000 units rH IL 2/animal. After i.p. injection, the pattern of serum half-life is similar to that seen after i.m. administration of rH IL 2 with the same broad plateau of activity. Higher peak levels were observed after i.p. administration than i.m. administration of 25,000 units/animal with a peak activity of nearly 500 units/ml.

Immunoadjuvancy of rH IL 2. The immunoadjuvant potential of rH IL 2 was assessed by examining its effect on the in vivo development of splenic or peritoneal exudate cytotoxic T-lym-
**THERAPEUTIC PROPERTIES OF RH IL 2 FOR METASTATIC DISEASE**

**Table 4 Adjuvant activity of RH IL 2 in development of syngeneic cytotoxic T-lymphocytes**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/animal)</th>
<th>Schedule (days)</th>
<th>Vaccine</th>
<th>% of cytotoxicity at effector:target cell ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>0</td>
<td>0-3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HBSS</td>
<td>0</td>
<td>0-3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>200:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>100:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>50:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>25:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>1:1</td>
<td>3</td>
</tr>
</tbody>
</table>

* Significant increase in cytotoxicity compared to animals that received saline alone; *P* ≤ 0.05 (paired Student’s *t* test).

**Table 3 Adjuvant activity of RH IL 2 and development of syngeneic cytotoxic T-lymphocytes**

C57BL/6 mice were immunized with syngeneic ascites MBL-2 tumor cells (10^9 cells) i.p. in either HBSS or RH IL 2. The adjuvant was administered at time 0 as an admixture and by i.p. injection on days 1, 2, and 3. Ten days after the immunization, effector cell activity was examined in a 24-h radiorelease assay with indium-labeled MBL-2 tumor cells. There were three mice/group.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/animal)</th>
<th>Schedule (days)</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>200:1</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>100:1</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>50:1</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>25:1</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>1:1</td>
</tr>
</tbody>
</table>

* Significant increases compared to sham-treated group; *P* < 0.01 (paired Student’s *t* test).

**Fig. 3. Age- and sex-matched C57BL/6N mice received either an i.v., i.m., or i.p. injection of 0.2 ml of 25,000 units of RH IL 2. Individual mice were bled at various times thereafter, and the serum levels of RH IL 2 activity determined by bioassay. A log-log plot of the serum pharmacokinetics following the i.v., i.p., or i.m. bolus injection of 25,000 units/animal (U/4) of RH IL 2. The i.v. injection has a T½ half-life of 2.94 min and a T0 half-life of 112.73 min while the i.m. injection results in a half-life of 28.0 min and the i.p. injection has a serum half-life of 24.9 min.**

**Table 5**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/animal)</th>
<th>Schedule (days)</th>
<th>Vaccine</th>
<th>% of cytotoxicity at effector:target cell ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>0</td>
<td>0-3</td>
<td>200:1</td>
<td>3</td>
</tr>
<tr>
<td>HBSS</td>
<td>0</td>
<td>0-3</td>
<td>100:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>50:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>25:1</td>
<td>3</td>
</tr>
<tr>
<td>RH IL 2</td>
<td>10,000</td>
<td>0-3</td>
<td>1:1</td>
<td>3</td>
</tr>
</tbody>
</table>

* Significant increase in cytotoxicity compared to animals that received saline alone; *P* ≤ 0.05 (paired Student’s *t* test).

**Immunotherapy with RH IL 2.** Therapy studies with RH IL 2 for MBL-2 (a Moloney virus-induced lymphoma) tumor ascites revealed a dose-dependent prolongation of survival (Table 5). In this study, optimal therapeutic activity was observed at 100 units/animal of RH IL 2 delivered three times/week by i.p. injection. Significantly, less therapeutic activity was observed at higher doses of RH IL 2 (1000 units/animal) and no therapeutic activity was observed at 10,000 units/animal. Similarly, less therapeutic activity was observed at 25-units/animal doses of RH IL 2. Animals with no apparent tumor burden on day 55 received a s.c. MBL-2 tumor challenge. Tumor cells grew progressively and killed control animals, but the animals that were “cured” of their peritoneal ascites by RH IL 2 rejected the tumor challenge and were alive 6 months later.
Table 5 Immunotherapy of MBL-2 tumor ascites with rH IL 2
Syngeneic C57BL/6N mice were given injections of 5 × 10^6 B16-BL6 cells. Twenty-nine days later, when the tumors were 1 cm in diameter, the tumor-bearing leg was resected and therapy was initiated 24 h later. The mice were necropsied 35 days postresection (N = 10).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/animal)</th>
<th>Median survival time (days)</th>
<th>% &quot;cured&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>50 MM</td>
<td>36 (31–43)</td>
<td>0</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>10,000 units/animal</td>
<td>36 (29–43)</td>
<td>0</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>1,000 units/animal</td>
<td>35 (32–55)</td>
<td>0</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100 units/animal</td>
<td>&gt;55 (48–55)</td>
<td>0</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>25 units/animal</td>
<td>39 (36–55)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant prolongation of survival compared to acetic acid control; P < 0.05 (Kruskal-Wallis test).
* Mice alive on day 55 were challenged with a s.c. injection of 100,000 MBL-2 and remained tumor free 6 months later when necropsied. Control animals had a median survival time of 27 (23–31) days.
* Significant increase in "cured" animals compared to acetic acid control cohort; P < 0.05 (Fisher exact test).

Table 6 Treatment of spontaneous metastases with rH IL 2
Syngeneic C57BL/6 mice were given injections in posterior footpad of 5 × 10^4 DIM10 or B16-BL6 cells. Twenty-nine days later, when the tumors were 1 cm in diameter, the tumor-bearing leg was resected and therapy was initiated 24 h later. The mice were necropsied 35 days postresection (N = 10).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/animal)</th>
<th>Metastases [median (range)]</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100,000</td>
<td>150 (19–300)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>10,000</td>
<td>4 (0–73)</td>
<td>0.43</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>1,000</td>
<td>161 (23–300)</td>
<td>0.72</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100</td>
<td>61.5 (0–300)</td>
<td>0.007</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>50</td>
<td>27 (20–200)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mice received HBSS or rH IL 2 by i.p. injection daily (5 times/week) for 4 weeks.
* Difference in the median number of spontaneous metastases (Mann-Whitney U test).

Table 7 Therapeutic activity of rH IL 2 in the treatment of M109 lung nodules
Syngeneic BALB/c mice were given i.v. injections of 5 × 10^4 M109 tumor cells, and l.v. therapy was initiated 48 h later (N = 10). Therapy was continued for 4 weeks, and the mice were necropsied 2 weeks after tumor challenge (day 42).

<table>
<thead>
<tr>
<th>Agents</th>
<th>Dose/animal (units/animal)</th>
<th>Schedule</th>
<th>Metastases [median (range)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td></td>
<td>tiw</td>
<td>181 (0–300)</td>
</tr>
<tr>
<td>Poly(I,C)-LC</td>
<td>10 mM</td>
<td>tiw</td>
<td>23 (0–81)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>10,000 units</td>
<td>tiw</td>
<td>50 (0–300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100 units</td>
<td>tiw</td>
<td>61 (0–300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>10 units</td>
<td>tiw</td>
<td>111 (0–300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100 units</td>
<td>S</td>
<td>39 (0–300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>1,000 units</td>
<td>L</td>
<td>24 (0–300)</td>
</tr>
</tbody>
</table>

* Significant reduction in experimental metastases (Mann-Whitney U test).
* rH IL 2 was administered either three times/week (tiw) or daily (qd).
* Poly(I,C)-LC, polyinosinic-polycytidylic acid complexed with polyl-lysine and carboxymethylcellulose.

When we treated experimental and spontaneous metastases with rH IL 2, we discovered that the optimal therapeutic protocol for rH IL 2 depends on the dose and therapeutic schedule (Table 6). The greatest reduction in metastases was observed when the results of daily administration of 50 units (median, 27 metastases) and 100,000 units (median, 4 metastases) was compared to the saline control (median, 150 metastases). In contrast, intermediate or similar doses administered less frequently (results not shown) had less therapeutic activity.

The low dose therapeutic optimum for treating experimental M109 or B16-BL6 [results not shown] metastases was higher [100 to 1000 units/animal (Table 7)] than the optimum low dose against spontaneous metastasis [Table 6]. The greatest reduction in the median number of metastases (determined at necropsy) was observed in animals that received 100 or 1000 units rH IL 2 on a daily basis. Although significant therapeutic activity was demonstrated by the administration of 10,000, 1,000, or 100 units rH IL 2 three times/week, this reduction in metastasis was significantly less than when rH IL 2 was administered at 100 units/animal every day. The lowest level of therapeutic activity was observed with doses of 10 and 10,000 units rH IL 2 three times/week.

We examined the therapeutic activity of rH IL 2 as a continuous infusion by placing 14-day osmotic pumps in the peritoneal cavity (Table 8). Significant therapeutic activity (P = 0.008) was observed with the infusion of approximately 14,000 units/24 h (approximately 600 units/h). This therapeutic activity was significantly greater (P < 0.05) than that observed with a log higher dose (P = 0.03) or a log lower dose (P = 0.04) of rH IL 2. At the same time, we treated animals with no tumor burden to determine the effect of rH IL 2 on the host's histopathology. Significant increases in organ weight were observed in animals receiving 6000 units/h rH IL 2 (Table 9). Similarly, there was an increase in the spleen, lung, and liver weights of mice receiving lower levels of rH IL 2, although the greatest increase was observed at 6000 units/h. Thus, chronically infused rH IL 2 has therapeutic activity at doses that are less than the maximum tolerated dose as determined by organ weights. Table 9 also reveals that splenomegaly and pulmonary leukoplasia could be reversed after rH IL 2 infusion was discontinued (14-day osmotic pumps were utilized). Histological studies revealed that animals receiving the highest dose of rH IL 2 had marked evidence of pulmonary lymphoplasia and hepatic lymphoplasia (Fig. 4). One characteristic of the leukocyte hyperplasia was the perivascular cuffing of leukocytes. This suggests that one mechanism of the leukocyte hyperplasia was sequestering of peripheral blood leukocytes to these organs.

The nature of the effector cells responsible for the therapeutic activity of rH IL 2 was examined by the administration of rH IL 2 to nude mice by daily i.p. injections (Table 10). These studies revealed no therapeutic activity in nude mice at low doses of rH IL 2 but significant therapeutic activity in animals receiving 10,000 or 100,000 units/animal of rH IL 2. In contrast, in the same experiment using the same tumor cell inoculum with 7-week-old syngeneic mice both significant low dose (100 units/animal) and high doses (100,000 units/animal), therapeutic activity was observed. These results suggest that low dose therapeutic activity may be T-cell mediated, whereas the high dose therapeutic activity may be mediated by NK and/or LAK-like cells precipitated on \textit{ex vivo} observations.

**DISCUSSION**

rH IL 2 is a potent immunomodulatory agent that affects many host immune effector cells. The cloning of the gene for
THERAPEUTIC PROPERTIES OF RH IL 2 FOR METASTATIC DISEASE

Fourteen-day osmotic pumps were implanted into the peritoneal cavities of mice, and 5 or 21 days later their lungs, livers, and spleens were removed and weighed (N = 5).

Table 9 Organ weights of mice receiving RH IL 2 by osmotic pump

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
<th>Day 5</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td></td>
<td>170 ± 40 a</td>
<td>980 ± 190</td>
<td>70 ± 10</td>
<td>200 ± 10</td>
<td>1140 ± 110</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>50 mm</td>
<td>190 ± 30</td>
<td>900 ± 120</td>
<td>90 ± 10</td>
<td>210 ± 20</td>
<td>1170 ± 80</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>6 units/h</td>
<td>210 ± 10</td>
<td>1070 ± 60</td>
<td>120 ± 50</td>
<td>210 ± 20</td>
<td>990 ± 80</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>60 units/h</td>
<td>170 ± 30</td>
<td>1002 ± 80</td>
<td>130 ± 20</td>
<td>190 ± 20</td>
<td>1130 ± 60</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>600 units/h</td>
<td>230 ± 70</td>
<td>900 ± 150</td>
<td>130 ± 70</td>
<td>220 ± 20</td>
<td>1110 ± 90</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>6000 units/h</td>
<td>230 ± 30</td>
<td>1110 ± 100</td>
<td>150 ± 30</td>
<td>210 ± 20</td>
<td>1120 ± 50</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>6000 units/h</td>
<td>220 ± 40</td>
<td>1340 ± 230</td>
<td>230 ± 70</td>
<td>210 ± 15</td>
<td>1110 ± 60</td>
</tr>
</tbody>
</table>

\* Mean ± SD.
\* Significant difference in average organ weight compared to mice with pumps containing HBSS (P < 0.05 by the paired Student t test).

Table 10 Treatment of experimental B16-BL6 metastases with rH IL 2 in nude mice

Nude or C57BL/6N mice were given i.v. injections of 5 x 10^6 B16-BL6 tumor cells; i.p. therapy was continued for 4 weeks, and the mice were necropsied 1 week after tumor challenge (N = 10).

<table>
<thead>
<tr>
<th>BRM</th>
<th>Dose/animal</th>
<th>Schedule</th>
<th>Metastasis [Median (range)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td></td>
<td>tiw</td>
<td></td>
</tr>
<tr>
<td>Poly(l,C)-LC a</td>
<td>10 µg</td>
<td>Daily</td>
<td>&gt;300 (52-&gt;300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>25 units</td>
<td>Daily</td>
<td>175 (0-&gt;300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100 units</td>
<td>Daily</td>
<td>&gt;300(10-&gt;300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>1,000 units</td>
<td>Daily</td>
<td>&gt;300(30-&gt;300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>10,000 units</td>
<td>Daily</td>
<td>38.5* (0-&gt;300)</td>
</tr>
<tr>
<td>rH IL 2</td>
<td>100,000 units</td>
<td>Daily</td>
<td>19.5* (3-&gt;300)</td>
</tr>
</tbody>
</table>

a Poly(l,C)-LC, polynonsinic-polycytidylic acid complexed with poly-l-lysine and carboxymethylcellulose.

Fig. 4. Mice received an i.p. implant of a 7-day osmotic pump which released approximately 6000 units/h of rH IL 2. Five days later the mice were necropsied, the organs were weighed, and the tissues were fixed in formalin for processing. Pulmonary histopathology (top) from a mouse receiving a continuous infusion of rH IL 2 for 5 days at 6000 units/h. Note the lymphocytic hyperplasia and the lymphocytic perivascular cuffing. × 200. The hepatic histopathology (bottom) is also shown from a mouse receiving a continuous infusion of rH IL 2 for 5 days at 6000 units/h. The lymphoid hyperplasia was generalized as well as focal. × 400. Perivascular cuffing of lymphocytes was also noted (not shown).

Human IL 2 has made available large amounts of highly purified rH IL 2 for in vitro and in vivo studies (39-41). In vitro studies with rH IL 2 have revealed that it can augment a mixed lymphocyte tumor response, mixed lymphocyte response, macrophage tumoricidal activity, and NK cell activity (1-9). In addition, it supports the proliferation of unstimulated large granular lymphocytes as well as primed or antigen-stimulated T-lymphocytes (42-49). This in vitro augmentation and proliferation have occurred at moderate to low concentrations of rH IL 2. In contrast, the activation or augmentation of NK cell activity in vivo or the induction of proliferation of peripheral blood or organ leukocytes in vivo requires high doses of rH IL 2. The present studies support the observation that high doses of IL 2 are required for NK cell augmentation. Interestingly, lower doses can be sufficient to augment NK activity in the lung and liver, but higher doses are needed to augment splenic and peripheral blood NK cell activity (9). Similarly, high doses of rH IL 2 appear necessary to generate LAK cells in the spleens of mice (6-8, 38). In contrast, the adjuvant activity of rH IL 2 for the induction of specific cytotoxic T-lymphocytes occurs at low levels of IL 2 (approximately 25 to 1000 units/animal). This dosage requirement is related to the number of doses administered, such that higher doses are active following a single administration whereas lower doses are active with multiple treatments. Indeed, multiple high doses of rH IL 2 do not act as an adjuvant and can suppress the development of cytotoxic T-lymphocytes that have been induced with a near optimal tumor vaccine.

The NK cell augmentation studies suggested that the optimal route of administration requires i.p. or i.m. rather than i.v. injection of rH IL 2. This hypothesis is supported by pharmacokinetic studies, because both i.p. and i.m. administration result in the longest serum half-life. Our findings concerning the tα and tβ serum half-lives of rH IL 2 after i.v. administration and the half-life after i.p. administration are in agreement with previous reports (36-38). The result with the serum half-life following i.m. administration of rH IL 2 are, however, novel.

The T-cell, adjuvant-like activity of rH IL 2 is observed at moderate to low concentrations of rH IL 2. In contrast, the activation or augmentation of NK cell activity in vivo or the induction of proliferation of peripheral blood or organ leukocytes in vivo requires high doses of rH IL 2. The present studies support the observation that high doses of IL 2 are required for NK cell augmentation. Interestingly, lower doses can be sufficient to augment NK activity in the lung and liver, but higher doses are needed to augment splenic and peripheral blood NK cell activity (9). Similarly, high doses of rH IL 2 appear necessary to generate LAK cells in the spleens of mice (6-8, 38). In contrast, the adjuvant activity of rH IL 2 for the induction of specific cytotoxic T-lymphocytes occurs at low levels of IL 2 (approximately 25 to 1000 units/animal). This dosage requirement is related to the number of doses administered, such that higher doses are active following a single administration whereas lower doses are active with multiple treatments. Indeed, multiple high doses of rH IL 2 do not act as an adjuvant and can suppress the development of cytotoxic T-lymphocytes that have been induced with a near optimal tumor vaccine.

The NK cell augmentation studies suggested that the optimal route of administration requires i.p. or i.m. rather than i.v. injection of rH IL 2. This hypothesis is supported by pharmacokinetic studies, because both i.p. and i.m. administration result in the longest serum half-life. Our findings concerning the tα and tβ serum half-lives of rH IL 2 after i.v. administration and the half-life after i.p. administration are in agreement with previous reports (36-38). The result with the serum half-life following i.m. administration of rH IL 2 are, however, novel.

The T-cell, adjuvant-like activity of rH IL 2 is observed at low doses and the failure of cytotoxic T-effector cells to develop at high doses suggests that the adjuvant activity of rH IL 2 at
low doses may be mediated via helper cells, whereas high doses may support or induce suppressor cell activity. Previous reports have indicated that rH IL 2 can induce and/or support the growth of suppressor cells (50-52), which provides support for the concept of such a biphasic response. Studies to examine this possibility are ongoing. Alternatively, high dose rH IL 2 may induce a suppressor factor such as prostaglandins, which depress or prevent the development of cytotoxic-T effector cells.

One of the intriguing aspects of the therapeutic activity of rH IL 2 is its biphasic dose response. We suggest that low dose therapeutic activity may be associated with T-cells, because the dosage optimum parallels its adjuvant activity for T-effector cell activity. This low dose therapeutic optimal has been observed in three tumor models, B16-BL6 (>10 experiments), M109, and MBL-2 (three experiments) and is thus a highly reproducible observation. This hypothesis is supported by the absence of low dose therapeutic activity in nude mice which was observed in concomitant therapeutic protocols using normal syngeneic mice. High dose therapeutic activity is present in nude mice at the same doses required to activate NK cells or LAK-like effector cells reported by others in normal mice.

The therapeutic activity of rH IL 2 appears to be highly dependent on the schedule of administration. Thus, daily administration by i.p. injection produces greater therapeutic activity than is achieved when rH IL 2 is administered two or three times a week. The pharmacokinetics of rH IL 2 administration, as measured by serum levels, seems to indicate that i.p. and i.m. routes are the most effective routes of administration. Because the i.p. route of administration appeared to have therapeutic activity associated with the pharmacokinetics of the agent, we also undertook studies in which we continuously administered rH IL 2 via i.p. osmotic pumps. We noted that a low daily dose of rH IL 2 had significant therapeutic activity. Interestingly, in several studies when higher or lower doses of rH IL 2 were administered by osmotic pumps, both had significantly less therapeutic activity compared to the therapeutic optimum. Presumably, if we had been able to administer a log higher dose via the osmotic pumps, we might have seen a high dose therapeutic activity (the solubility of rH IL 2 was a rate-limiting factor). The lymphoid hyperplasia as well as therapeutic activity that was observed with continuous administration of higher doses of rH IL 2 suggests that ex vivo LAK cell induction, cultivation, infusion, and technology may be unnecessary (53).

Although clinical therapeutic activity and toxicity have been noted when rH IL 2 was administered with an aggressive dosage and schedule, most notably in combination with LAK cells or tumor-infiltrating lymphocytes (27-29), the preclinical observation of therapeutic activity at low doses suggests that rH IL 2 might be therapeutically effective using a less toxic protocol. The hypothesis that moderate, nontoxic doses of rH IL 2 have therapeutic activity when administered by continuous infusion has been studied to some extent in the clinic. Clinical studies headed by Dr. William West at Biologic Therapeutics, Inc., have shown therapeutic activity when rH IL 2 was administered by continuous infusion in combination with LAK cells (54). Indeed, in a preliminary report, these investigators described responses in 9 of 16 evaluable melanoma patients. Studies by Dr. Paul Sandel at the University of Wisconsin have shown that continuous infusion of rH IL 2 initially produces leukopenia and later results in lymphoid hyperplasia in the peripheral blood and increased LAK cell activity ex vivo (55). The Biological Response Modifiers Program has developed such a protocol and has noted biological activity following continuous infusions of 100,000 units/m²/h of IL 2 (R. Steis and J. Clark, personal communication). R.H IL 2 has been administered clinically, using a wide variety of doses and routes. However, most of these studies have involved a short duration of administration and by the i.v. route. Both of these parameters may not result in therapeutic or immunomodulatory activity since immunotherapy, at least in rodents, requires chronic administration for significant responses. Thus, the observation of immunomodulation and therapeutic responses following the infusion of 3 × 10⁶ units of rH IL 2/day/m² once daily (55) or 3 × 10⁷ units of rH IL-2 per 24 h/m² once a week (56, 57) is very encouraging. These are better tolerated protocols that use lower cumulative dose than those used to date in the aggressive therapeutic protocols where approximately 100,000 units/kg is administered three times a day for 4 days. This results in a daily dose of approximately 20 million units/day/patient. While the pharmacokinetics of IL 2 administered by continuous infusion results in increased toxicity on a equidose basis compared to an i.v. bolus, the impression has been that significantly less toxicity and greater immunomodulation is observed in patients receiving continuous infusions of rH IL 2 at approximately 3 × 10⁶ units/day once a day or a weekly 24-h infusion of 30 × 10⁷ units/day as compared to high dose i.v. administration three times a day. It remains to be determined in the clinic whether or not LAK cells are required in combination with IL 2, whether i.v., i.p., or continuous infusion will have the greatest therapeutic activity with the least toxicity, and what dose will result in the greatest therapeutic activity.

REFERENCES

mediated by the systemic administration of high-dose recombinant interleu-

14. Donohue, J. H., Rosenstien, M., Chang, A. E., Lotze, M. T., Robb, R. J.,
and Rosenberg, S. A. The systemic administration of purified interleukin 2
enhances the ability of sensitized murine lymphocytes to cure a disseminated

15. Shu, S., and Rosenberg, S. A. Adoptive immunotherapy of newly induced

and human tumors in mice with lymphokines and interleukin-2-propagated

17. Kedar, E., Herberman, R. B., Gorelik, E., Sredni, B., Bonnard, G. D.,
and Navarro, N. Antitumor reactivity in vitro and in vivo of mouse and human
lymphoid cells cultured with T cell growth factor. In: A. Fefer and A.
Goldstein (eds.), The Potential Role of T Cells in Cancer Therapy, pp. 173–

18. Greenberg, P. D., and M. A. Cheever. Effector mechanisms operative in
adoptive therapy of tumor-bearing animals: implications for the use of

19. Mule, J. J., Shu, S., and Rosenberg, S. A. The anti-tumor efficacy of
lymphokine-activated killer cells and recombinant interleukin 2 in vivo. J.

20. Lafreniere, R. R., and Rosenberg, S. A. Successful immunotherapy of murine
experimental hepatic metastases with lymphokine-activated killer cells and

21. Donohue, J. H., Rosenstien, M., Chang, A. E., Lotze, M. T., Robb, R. J.,
and Rosenberg, S. A. The systemic administration of purified interleukin 2
enhances the ability of sensitized murine lymphocytes to cure a disseminated

killer cell-mediated established pulmonary metastases by the intrave-
nous adoptive transfer of syngeneic lymphocytes activated in vitro by inter-

23. Salup, R. R., and Willrout, R. H. Adjuvant immunotherapy of established
murine renal cancer by interleukin 2-stimulated cytotoxic lymphocytes.

24. Kern, P., Toy, J., and Dietrich, M. Preliminary clinical observations with
recombinant interleukin-2 in patients with AIDS or LS. Blut, 50: 1–6,
1985.

25. Lotze, M. T., Robb, R. J., Sharrow, S. O., Frana, L. W., and Rosenberg, S.
A. Systemic administration of interleukin-2 in humans. J. Biol. Response

26. Lane, H. C., Siegel, J. P., Rock, A. H., Masur, H., Gelman, E. P., Quinnan,
G. V., and Fauci, A. S. Use of interleukin-2 in patients with acquired

27. Pizza, G., Severini, G., Mennini, D., De Vinci, C., and Corrado, F. Tumor
regression after intralesional injection of interleukin 2 (IL-2) in bladder

28. Siegel, J. P., Lane, H. C., Stock, N. I., Quinnan, G. V., Jr., and Fauci, A. S.
Pharmacokinetics of lymphocyte-derived and recombinant DNA-derived inter-

29. Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E.,
Etinghhausen, S. E., Matory, Y. L., Skibber, J. M., Shiloni, E., Vetto, J. T.,
Seiger, C. A., Simpson, C., and Reichert, C. M. Observations on the systemic
administration of autologous lymphokine-activated killer cells and recombi-
nant interleukin-2 to patients with metastatic cancer. N. Engl. J. Med., 313:

30. Matory, Y. L., Chang, A. E., Lipford, E. H., Ill, Brazel, R., Hyatt, C. L.,
McDonald, H. D., and Rosenberg, S. A. Toxicity of recombinant human
interleukin-2 in rats following intravenous infusion. J. Biol. Response Modif.,
Systematic Preclinical Study on the Therapeutic Properties of Recombinant Human Interleukin 2 for the Treatment of Metastatic Disease


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/21/5725

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/47/21/5725. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.