ABSTRACT

Different immunotherapy regimens using s.c. recombinant interleukin-2 (rIL-2) were studied in 76 patients with progressive metastatic renal carcinoma, malignant melanoma, colorectal cancer, B-cell lymphoma, or Hodgkin's disease. To assess the immunomodulatory capacity of rIL-2, we measured serum levels of soluble interleukin-2 (sIL-2) receptors, γ-interferon, tumor necrosis factor-α, and various lymphocyte subsets expressing the CD25 Tac IL-2 receptor and the CD56 natural killer (NK) associated antigen. Additionally, we measured serum antibodies specific to rIL-2 in order to evaluate immunogenicity of rIL-2.

In all patients, a significant increase in sIL-2 receptor levels could be observed when comparing values on day 0 and after one treatment course. Patients developing a neutralizing anti-rIL-2 antibody exhibited significantly lower serum sIL-2 receptor levels than patients without antibody. Soluble IL-2 receptors correlated with the percentage of CD25 IL-2 receptor-positive peripheral blood lymphocytes. Both soluble and cell surface IL-2 receptors exhibited a significant increase during rIL-2 therapy but did not correlate with the percentage of CD56-positive peripheral blood lymphocytes. Measurement of treatment-induced second cytokines showed significant increases in γ-interferon serum levels in a proportion of patients tested, although with considerable interindividual variability. No significant increase in mean tumor necrosis factor-α levels was observed during rIL-2 treatment in vivo.

The percentage of CD56-positive NK cells correlated with the clinical outcome of rIL-2 therapy. Thus, partial or complete responders had an increase from a mean of 20% NK cells prior to therapy up to a mean of 40% after the first treatment course. In contrast, patients with progressive disease had a mean of 22 and 24% NK cells before and after treatment, respectively.

INTRODUCTION

The use of recombinant human cytokines has evolved as a novel approach to the treatment of advanced stage human malignancies. Cytokines have shown the ability to mediate partial or complete regression in patients with advanced malignancy (1, 2). rIL-2 was found to be involved in antitumor activity via indirect effects on the immune system, including the activation and expansion of cytotoxic T-lymphocytes and natural killer (NK) cells, and the secretion of secondary cytokines such as IFN-γ and TNF-α (3–6). The immunomodulatory capacity of rIL-2 has been described for the i.v. administration of high-dose cytokine, therapy which was associated with severe adverse effects, including capillary leak-related weight gain, hypotension, malaise, fever, and chills. We recently demonstrated that low-dose s.c. rIL-2 can produce significant tumor regressions in patients with advanced metastatic cancer (2, 7), while drastically reducing treatment-related systemic toxicity.

The biological efficacy and mechanisms of low-dose s.c. rIL-2 in man remain to be fully understood.

The results of these studies are the subject of the present report.

PATIENTS AND METHODS

Patients. Seventy-six patients with progressive metastatic cancer were treated according to different immunotherapy protocols. Forty-eight patients had renal cell cancer, 12 patients had malignant melanoma, 14 patients had colorectal cancer, one patient had a B-cell lymphoma, and one patient had Hodgkin’s disease. Patient sera were obtained prior to, during, and after immunotherapy courses. During therapy, sera were obtained at regular 60-h postinjection intervals. Serum was also obtained at various intervals while patients were not receiving therapy. Patient sera were stored at −70°C prior to testing.

Immunotherapies. Table 1 summarizes the different immunotherapeutic treatment regimens used in this study.

Forty-six patients were treated with a combination of low-dose s.c. rIL-2 (Eurocetus, Amsterdam, The Netherlands). During the first 4 weeks of treatment, rIL-2 was administered every 12 h for 6 consecutive days/week. Doses started at 1.8 million IU/m²/day in week 1 and were escalated by 100% of the previous dose during weeks 2 through 4. Starting at week 5, maintenance therapy was administered once weekly at a dose of 14.4 million IU/m² (7).

Ten patients received s.c. pulsed rIL-2. This therapy consisted of rIL-2 at a single dose of 4.8 million IU/m² given three times daily on days 1, 15, 29, and 43 and twice daily on days 2, 16, 30, and 44, followed by 4.8 million IU/m² on days 3 through 5, 8 through 12, 17 through 19, and 22 through 26.

Six patients were given a combination of tumor cell vaccine and s.c. rIL-2. Eight weekly injections of 2.5 × 10⁹ lyzed autologous tumor cells admixed with Newcastle disease virus were administered. Starting at week 5, patients received s.c. rIL-2 three times daily at a single dose of 4.8 million IU/m² on days 29 and 50 and twice daily at a single dose of 4.8 million IU/m² on days 30 and 51, followed by 4.8 million IU/m² daily on days 31 through 33, 36 through 40, 43 through 47, 53 through 55, 57 through 61, and 64 through 68.

Fifty-six patients were treated with a combination of low-dose s.c. rIL-2 and rIFN-α²b (Essex-Schering, Munich, Germany). Patients received between 14.4 and 18.0 million IU/m²³ of rIL-2 on days 1 and 2, followed by 4.8 million IU/m² daily, 5 days/week, for 6 consecutive weeks; rIFN-α²b was administered at 6.0 million units/m³, three times weekly (2).

Prior to therapy, written informed consent was obtained from all patients. Patients demonstrating stable or regressive disease after the first course of treatment received further therapy.

6312
Soluble IL-2 Receptor Assay. Soluble IL-2 receptor levels were determined using a standard two-step sandwich assay (Immunotech S.A., France), as described previously in detail (8). Briefly, the assay used two antibodies recognizing different epitopes of the IL-2 receptor molecule. The second antibody was conjugated to peroxidase, resulting in a color reaction which was then quantitated in an automated plate reader (Thermo Max; Molecular Devices, Menlo Park, CA). The amount of soluble IL-2 receptor per sample was calculated by plotting the absorbance values against a soluble IL-2 receptor standard curve. The results were expressed in pm. Normal donor values ranged from 25–115 pm (1 pm = 42 pg/ml).

γ-Interferon Assay. γ-Interferon was measured using an EIA (Endogen, Boston, MA) based on the sandwich principle as described previously (9). The assay used two antibodies recognizing different epitopes of the γ-interferon molecule. The amount of bound γ-interferon was detected with a secondary antibody conjugated to peroxidase. Absorbance values were read at 490 nm in an automated plate reader (Thermo Max). The amount of γ-interferon per sample was determined by plotting the absorbance values against a γ-interferon standard curve. The results were expressed in pg/ml, with a detection limit of 50 pg/ml.

Tumor Necrosis Factor Assay. For the quantitative determination of TNF-α serum levels, an EIA (Quantikine; RD6 Systems, Minneapolis, MN) based on the sandwich principle was performed as described previously (10). Briefly, a murine monoclonal antibody specific to human TNF was used, and a second horseradish peroxidase-conjugated anti-human TNF antibody allowed for the quantitation of bound TNF by measuring absorbance values against a standard curve, using an automated plate reader (Thermo Max) at 450 nm.

Phenotypic Analysis. Lymphocyte subsets were defined by antibodies against CD25 (Tac α-chain IL-2 receptor) and CD56 (natural killer cell) surface antigens (11). Percentage of positivity was determined using a fluorescence-activated cell sorter (FACS IV; Becton Dickinson, Mountain View, CA) as described elsewhere (12).

Evaluation of Anti-IL-2 Antibodies. For the quantitation of anti-IL-2 IgG antibodies, an enzyme-linked-immunosorbent assay was used as described previously (13). Specificity of antibodies was confirmed by Western blot analysis using a standard technique described elsewhere (14, 15).

For the detection of neutralizing serum activity against recombinant IL-2, patient sera were heat inactivated (52°C, 30 min) and subsequently tested for inhibition of mouse CTLL using a standard [3H]thymidine proliferation assay. A neutralizing titer >100, defined as fold reduction of IL-2-induced proliferation times the final serum dilution, was considered positive (16).

Statistical Analyses. For assessment of statistical significance, t-test analyses were performed wherever applicable.

RESULTS

Soluble IL-2 Receptor Levels. sIL-2 receptor levels during rIL-2 dose escalation are given in Table 2. In all 14 patients treated with escalating doses of s.c. rIL-2 (1.8 up to 14.4 million IU/m²/day), a significant increase (P < 0.005) in sIL-2 receptor levels was observed. Thus, levels ranged from 87–290 pm (mean, 153 pm) before therapy and 220–1169 pm (mean, 700 pm) after 4 weeks of dose escalation. A significant increase in serum sIL-2 receptors was attained after the first 2 weeks of treatment; thereafter further dose escalation of rIL-2 did not significantly enhance sIL-2 receptor levels. In those patients continuing on a single weekly maintenance dose (10.8 million IU/m²) after dose escalation, a slow decrease in sIL-2 receptor levels occurred, resulting in a mean value of 523 pm.

The effect of dose and schedule on serum levels of soluble IL-2 receptors is summarized in Table 3. During pulsed rIL-2 treatment (n = 10; pulses of 14.4 million IU/m² every 2 weeks, followed by 4.8 million IU/m² daily, for 5 days/week, for 4 consecutive weeks), mean sIL-2 receptor levels increased from 78 pm (range, 40–113 pm) on day 0 to a mean of 580 pm (range, 146–974 pm) after one treatment course. Within 3 weeks after cessation of rIL-2, sIL-2 receptor levels decreased to a mean of 122 pm (range, 54–185 pm).

When pulsed rIL-2 (n = 6; pulses of 14.4 million IU/m² every 4 weeks, followed by 4.8 million IU/m² daily, for 5 days/week, for 6 consecutive weeks) was administered in conjunction with tumor cell vaccines, we observed an increase in sIL-2 receptor levels up to a mean of 839 pm in the second week of rIL-2 treatment. The vaccination itself did not significantly alter mean sIL-2 receptor levels (mean of 80 pm 4 weeks after vaccination). Three weeks after therapy was discontinued, mean sIL-2 receptor levels decreased to 106 pm (Table 3).

Serum levels of sIL-2 receptor did not correlate with clinical response or tumor type and were unaffected by patients undergoing prior chemotherapy (data not shown).

Soluble and Cell Surface (CD25) IL-2 Receptors as Correlated with Natural Killer Cell (CD56) Phenotype. Fig. 1 gives sIL-2 receptor levels and percentage of CD25- and CD56-positive peripheral blood lymphocytes in 46 patients receiving s.c. rIL-2/rIFN-α2b combination therapy (pulsed rIL-2 in conjunction with 6 weeks of rIFN-α2b at 6 million units/m² thrice weekly). A highly significant increase (P < 0.005) in sIL-2 receptor levels occurred, from a mean level of 121 pm (range, 32–465 pm) at baseline to a mean level of 153 pm (mean ± SD) at the end of 2 weeks of rIFN-α2b combination therapy.

Table 1 Summary of immunotherapeutic treatment regimens

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Wk of treatment</th>
<th>No. of evaluated patients</th>
<th>Projected dose of rIL-2 (million IU/m²/week)</th>
<th>No. of high-dose rIL-2 pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escalated rIL-2</td>
<td>4</td>
<td>14</td>
<td>162</td>
<td>*</td>
</tr>
<tr>
<td>Pulsed rIL-2</td>
<td>6</td>
<td>10</td>
<td>172</td>
<td>4</td>
</tr>
<tr>
<td>rIL-2 plus vaccine</td>
<td>10</td>
<td>6</td>
<td>172</td>
<td>2</td>
</tr>
<tr>
<td>rIL-2 plus IFN-α2b</td>
<td>6</td>
<td>46</td>
<td>162–172</td>
<td>1–2</td>
</tr>
</tbody>
</table>

* A weekly dose escalation was performed, up to a dose of 14.4 million IU/m²/day.

Table 2 Soluble IL-2 receptors during rIL-2 dose escalation in vivo

<table>
<thead>
<tr>
<th>Wk of therapy</th>
<th>No. of patients evaluable</th>
<th>Dose of rIL-2 (million IU/m²/day)</th>
<th>sIL-2 receptor (pm)</th>
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</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>14</td>
<td>0</td>
<td>153 ± 66*</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>3.6</td>
<td>678 ± 172</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>14.4</td>
<td>700 ± 286</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>10.8</td>
<td>523 ± 120</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>10.8</td>
<td>427</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Table 3 Effect of rIL-2 dose and schedule on serum levels of soluble IL-2 receptors

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Pretreatment</th>
<th>Wk 3</th>
<th>End of first treatment cycle</th>
<th>3 wk off therapy</th>
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</thead>
<tbody>
<tr>
<td>Escalated rIL-2</td>
<td>153 ± 66*</td>
<td>ND*</td>
<td>700 ± 286</td>
<td>ND</td>
</tr>
<tr>
<td>Pulsed rIL-2</td>
<td>78 ± 40</td>
<td>602 ± 290</td>
<td>580 ± 311</td>
<td>122 ± 48</td>
</tr>
<tr>
<td>rIL-2 plus vaccine</td>
<td>98 ± 17</td>
<td>839 ± 407</td>
<td>671 ± 495</td>
<td>106 ± 31</td>
</tr>
<tr>
<td>rIL-2 plus IFN-α2b</td>
<td>121 ± 92</td>
<td>797 ± 359</td>
<td>541 ± 207</td>
<td>234 ± 96</td>
</tr>
</tbody>
</table>

* Mean ± SD.

ND, not done.

Table 3 Effect of rIL-2 dose and schedule on serum levels of soluble IL-2 receptors

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<td>234 ± 96</td>
</tr>
</tbody>
</table>

* Mean ± SD.

ND, not done.

Value given in parentheses, sIL-2 level after 4 weeks of vaccination, prior to rIL-2.

Value refers to second week of rIL-2 administration.

6313
Fig. 1. Dose and response dependence *in vivo* of soluble and cell surface IL-2 receptors as compared against natural killer cell phenotype. Soluble serum IL-2 receptors, CD25 cell surface IL-2 receptors, and CD56-positive natural killer cells were measured at regular intervals pretreatment, during (week 4) treatment and at the termination (week 8) of therapy. Soluble IL-2 receptors in patient sera were measured with a standard EIA; lymphocyte subsets were phenotyped by immunofluorescence analysis using monoclonal antibodies against CD25 and CD56. Results are shown as mean values in treatment responders (A, n = 10), stable disease patients (B, n = 20), and progressive disease patients (C, n = 16).

pm) on day 0 to a mean of 541 pm (range, 218–961 pm) after 7 weeks of therapy. Mean sIL-2 receptor values remained at an elevated level (mean, 234 pm) 4 weeks after completion of the first treatment course, prior to the second course of therapy.

Mean serum levels of sIL-2 receptors correlated with the expansion of CD25-positive peripheral blood lymphocytes (r = 0.938), which were 21% positive cells pretreatment (range, 5–51%) and 27% (range, 9–46%) after 7 weeks of therapy.

Emergence of soluble and cell surface (CD25) IL-2 receptors did not coincide with the rIL-2-induced expansion of natural killer (CD56-positive) cells. However, there was a significant correlation between clinical response and CD56 positivity; thus, clinical responders exhibited a mean increase from 20% (range, 7–37%) before treatment up to 40% (range, 20–56%) after 7 weeks of therapy (P < 0.001, day 0 versus week 7). In contrast, in patients with stable disease during therapy, CD56 positivity increased from a mean of 23% on day 0 (range, 2–49%) to a mean of 30% (range, 10–57%) after one treatment course (P > 0.05); patients with progressive disease had a mean of 22% (range, 7–56%) and 24% (range, 12–44%) CD56-positive peripheral blood lymphocytes, before and after therapy (P > 0.05), respectively. Significance levels were calculated at P = 0.002 when comparing CD56 positivity in responding versus nonresponding patients.

Effect of Neutralizing Serum Activity against rIL-2 on Soluble IL-2 Receptor Levels. Among patients receiving rIL-2/rIFN-α2b combination therapy, four developed a neutralizing anti-rIL-2 antibody during the second treatment course. These patients exhibited significantly lower (P < 0.005) sIL-2 receptor levels when compared with antibody-negative patients during the second treatment course. Thus, mean sIL-2 receptor levels in antibody-positive patients were 174 pm before onset of the second course, 268 pm after 3 weeks of therapy, and 214 pm after 7 weeks of therapy. In contrast, antibody-negative patients exhibited mean sIL-2 receptor levels of 245 pm prior to the second course of therapy, 745 pm after 3 weeks of treatment, and 516 pm at termination of the second course (Fig. 2). In these patients, no significant differences in mean sIL-2 receptor levels were observed during the first course of therapy.

γ-Interferon Serum Levels. γ-Interferon serum levels in patients receiving rIL-2-based immunotherapy are given in Table 4. Marked increases in serum concentrations of IFN-γ were observed in a proportion of patients, although with considerable interindividual variability.

Serum levels of IFN-γ did not correlate with the dose of rIL-2 administered, with concentrations of soluble and cell surface IL-2 receptors, or with peripheral blood NK cells. Increases in mean IFN-γ levels, when comparing pre- and posttreatment values, were insignificant in patients undergoing pulsed rIL-2 or rIL-2 plus vaccine treatment. In contrast, rIL-2/rIFN-α2b
combination therapy resulted in a significant enhancement (P < 0.001) of IFN-γ levels, from a mean of 141 pg/ml (range, 50-656 pg/ml) before treatment to a mean of 401 pg/ml (range, 55-1,968 pg/ml) after therapy. However, clinical response was not associated with elevated interferon-γ serum levels.

TNF-α Serum Levels. There was no significant increase in mean serum TNF-α levels, when comparing values pretreatment and after rIL-2 therapy. Mean levels before and after therapy were <10 pg/ml (data not shown).

**DISCUSSION**

In the present study, we studied the immunomodulatory effects in cancer patients of low-dose s.c. rIL-2 treatment (2, 7). Using various immunotherapeutic treatment regimens, we could demonstrate the immunoenhancing effects of rIL-2, as assessed by the increase in soluble and cell surface (CD25) IL-2 receptors, CD56-positive natural killer cells, and serum levels of γ-interferon in vivo.

The biological relevance of sIL-2 receptors in humans remains to be defined. In the patients described herein, soluble IL-2 receptors were increased significantly during rIL-2 therapy at doses, 5- to 10-fold lower than reported previously. During long-term low-dose s.c. rIL-2 treatment in vivo, serum levels of sIL-2 receptors correlated with CD25 cell surface antigen expression on peripheral blood lymphocytes.

During systemic administration of rIL-2 in humans, elevated sIL-2 receptor levels have been observed (17). In addition, it has been reported that elevated sIL-2 receptor levels may be associated with disease, such as adult T-cell leukemia, hairy cell leukemia, or various immunodeficiency syndromes (18-21).

The quantitative correlation stated for soluble and cell surface IL-2 receptors is explained by its membrane-bound expression and subsequent shedding without the transmembrane domain of the CD25 molecule (8).

As reported herein, immunogenicity of recombinant IL-2 resulted in the development of neutralizing antibodies against rIL-2 in a total of four patients. In these patients, significantly lower mean sIL-2 receptor levels were observed upon the emergence of neutralizing serum activity specific to rIL-2. Thus, the development of neutralizing antibodies against rIL-2 diminished rIL-2-induced biological activation, but it did not abrogate clinical response. Further investigations are needed to define the clinical relevance of anti-rIL-2 serum antibodies.

In our studies, patients receiving s.c. low-dose rIL-2 exhibited detectable levels of IFN-γ, a finding which was consistent with previous reports demonstrating that rIL-2 induced IFN-γ production in lymphocytes, both in vitro (22, 23) and in vivo (24, 25). As described, IFN-γ levels varied considerably and showed no direct dose dependence; thus, a proportion of patients had serum levels below detection limit during therapy.

In the patients reported, we consistently observed normal TNF-α serum levels, which were measured at pretreatment values during long-term rIL-2 treatment. This was reflected by reduced systemic toxicity due to the drastic reduction of s.c. doses of rIL-2 performed in this study. It has been hypothesized that secondary cytokines, primarily TNF-α, account for a major proportion of therapy-related adverse effects, e.g., fever, chills, malaise, in patients receiving high-dose i.v. rIL-2. In contrast, in some studies, the absence of elevated TNF-α levels during systemic rIL-2 administration has been explained as the result of rapid polymerization of TNF by serum proteases (6, 26).

As shown in the present study, the use of low-dose rIL-2 could effectively expand and activate various subsets of peripheral blood lymphocytes. Previously, this was demonstrated in patients with advanced cancer receiving high-dose rIL-2 by i.v. bolus or constant infusion (3, 27). However, while significant increases were observed in CD25- and CD56-positive cells, these did not correlate with the clinical outcome of patients (27, 28).

In the present study, however, we could demonstrate clinical response associated with significant rIL-2-induced increases in the number of CD56-positive peripheral blood lymphocytes. Conversely, patients with progressive disease showed lower levels of NK (CD56) positivity, regardless of the systemic administration of s.c. rIL-2.

In summary, we could demonstrate the immunomodulatory potency of low-dose rIL-2 regimens at doses approximately 5- to 10-fold lower than reported previously. While the clinical safety and therapeutic efficacy of low-dose rIL-2 has been established (2, 7), further functional characterization of rIL-2-induced cellular and humoral mediators will be needed to improve our current understanding of the biology of this molecule.

**REFERENCES**


**Table 4 γ-Interferon serum levels during systemic rIL-2 in vivo**

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Pretreatment</th>
<th>End of first treatment cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsed rIL-2 (n = 10)</td>
<td>337 ± 265*</td>
<td>447 ± 282</td>
</tr>
<tr>
<td>rIL-2 plus vaccine (n = 6)</td>
<td>171 ± 148</td>
<td>1080 ± 764</td>
</tr>
<tr>
<td>rIL-2 plus IFN-α2b (n = 46)</td>
<td>141 ± 143</td>
<td>401 ± 352*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

* Comparing pre- and posttreatment values, statistical significance was calculated at P < 0.001.


Biological Monitoring of Low-Dose Interleukin 2 in Humans: Soluble Interleukin 2 Receptors, Cytokines, and Cell Surface Phenotypes

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