Pharmacokinetic Alteration in Rats of Recombinant Interleukin-2 (rIL-2) by Immunocomplexing with a Monoclonal Antibody against rIL-2

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We have investigated the pharmacokinetic alteration in rats of recombinant interleukin-2 (rIL-2) by immunocomplexing with a monoclonal antibody against rIL-2. Serum rIL-2 levels after the intravenous administration of the immune complex at a dose of 100 μg/rat as rIL-2 were significantly higher than those after intravenous administration of rIL-2 alone at the same dose. Pharmacokinetic analysis indicated that the distribution volume of rIL-2 decreased from 74.0 to 10.3 ml/rat, while the elimination rate of rIL-2 was little changed by immunocomplexing with the antibody. On the other hand, serum rIL-2 levels after the subcutaneous administration of the immune complex at a dose of 100 μg/rat as rIL-2 were sustained longer than those after the subcutaneous administration of rIL-2 alone at the same dose, and T max shifted from 0.83 to 3.0 h by immunocomplexing with the antibody. Pharmacokinetic analysis also revealed that the mean-residence-time of rIL-2 increased from 1.98 to 6.52 h, and the area-under-the-curve of rIL-2 decreased slightly, from 834 to 548 ng·h/ml by immunocomplexing with the antibody.

Keywords: recombinant interleukin-2; monoclonal antibody; immune complex; pharmacokinetic alteration

Interleukin-2 (IL-2) plays a critical role in a variety of immune reactions and it has been expected to be therapeutically useful for augmenting the immune responses of patients suffering from certain immunodeficiencies or malignant neoplasms. In fact, it was proven to be effective therapy for advanced cancer. However, exogenous IL-2 has a short half-life, and repeated injections of a high-dose of IL-2 are required, which would cause serious side effects. In a previous paper we demonstrated that the antitumor activity of recombinant IL-2 (rIL-2) was enhanced in Meth-A fibrosarcoma-bearing mice by immunocomplexing with a monoclonal antibody (MoAb) against rIL-2. The present paper describes pharmacokinetic alteration in rats of rIL-2 by immunocomplexing with the antibody. Various pharmacokinetic parameters, including serum clearance, elimination rate, distribution volume and mean residence time of the immune complex are compared with those of unconjugated rIL-2 in rats given drugs both intravenously and subcutaneously.

MATERIALS AND METHODS

rIL-2, Anti-rIL-2 MoAb and Immune Complex: rIL-2 was purified from Escherichia coli. transected with human IL-2-encoding cDNA. A hybridoma secreting anti-rIL-2 antibody was obtained from the splenocytes of rIL-2-immunized mice by a conventional cell-fusion technique using mouse myeloma P3-X63-Ag8-U1 cells and polyethylene glycol. The antibody, MoAb-HRL1-52 (IgG1, κ), was purified from mouse ascites by ABx column chromatography. Immune complex was prepared by mixing rIL-2 and MoAb-HRL1-52 at a molar ratio of 2:1 as described before. ELISA Procedures Two enzyme-linked immunoabsorbent assay (ELISA) systems were adopted for determination of the immune complex in serum. One was an IL-2-ELISA employing microplates coated with affinity-purified anti-IL-2 goat immunoglobulin G (IgG) and horseradish peroxidase (HRP)-labeled anti-IL-2 rabbit Fab' fragment. In this system there was no or little difference in immunoreactivity between unconjugated and immunocomplexed rIL-2, giving similar standard curves at IL-2 levels (data not shown). The other assay has been newly developed for the specific determination of an immune complex, where the complex was sandwiched between anti-IL-2 goat IgG on microplates and HRP-labeled anti-mouse IgG goat antibody with a detection limit of 3.4 ng/ml. Unconjugated forms of rIL-2 and anti-rIL-2 antibody were undetectable in this ELISA system.

Pharmacokinetic Studies: Male Sprague-Dawley rats weighing 170—190 g (6-week-old) were purchased from Japan Charles River Co., Ltd. (Japan) and fed a commercial pellet diet CF-2 (CLEA Inc., Japan) with water ad libitum. One hundred ml portions of rIL-2 alone or its immune complex in phosphate-buffered saline were administered intravenously via the femoral vein or subcutaneously via the back neck at a dose of 100 μg/rat as rIL-2. Blood samples were collected from the tail vein and centrifuged at 3000 rpm for 10 min. Serum specimens were stored in a freezer until ELISA.

Pharmacokinetic Analysis: The pharmacokinetic parameters were determined as follows. Since serum rIL-2 concentration (C s ) versus time (t) after intravenous administration declined monoexponentially, the data were fitted to the following equation by nonlinear least-squares regression,

\[ C_s = C_0 e^{-kt} \]

where C 0 and k are the deduced initial concentration and
elimination rate, respectively. The area under serum concentration–time curve (AUC) was calculated by:

\[ AUC = \frac{C_0}{k} \]

Total clearance (CL) and the distribution volume (Vd), following intravenous administration, was calculated by:

\[ CL = \frac{D}{AUC} \text{ and } V_d = \frac{D}{C_0} \]

where D is a dose administered intravenously.

Pharmacokinetic behavior following subcutaneous administration was evaluated by moment analysis which was model-independent. Mean-residence-time (MRT) in the systemic circulation was calculated by:

\[ MRT = \frac{AUMC}{AUC} \]

where AUMC is the area under the first moment curve and AUC was calculated by the trapezoidal rule. Mean-absorption-time (MAT) was calculated by:

\[ MAT = MRT_a - MRT_v = MRT_v - \frac{1}{k} \]

Biological Activity of rIL-2 in Serum Murine NK cells, NKC3, were added at a concentration of 4 x 10^5 cells/well on 96-well microplates containing serial dilutions of serum specimens. The cells were cultured at 37°C for 24h and then the number of viable cells was measured by a conventional colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

RESULTS

Intravenous Administration Following the intravenous administration of rIL-2 alone and its immune complex at a dose of 100 μg/rat as rIL-2, serum rIL-2 levels were determined by two ELISA systems described in Materials and Methods. Serum levels determined by an rIL-2-ELISA declined monoeXponentially for both formulations: rIL-2 alone and the immune complex; but administration of the immune complex gave significantly higher rIL-2 values than that of rIL-2 alone (Fig.1). Pharmacokinetic parameters are shown in Table I. Total serum clearance (CL) for the immune complex was 7-fold lower than that for rIL-2 alone and it was due to a small distribution volume (Vd) of the immune complex. There was little difference in the elimination rate (k) between both formulations.

To assess the dissociation of rIL-2 molecules from immune complex in circulation, serum specimens were also subjected to the second ELISA system for specific determination of the immune complex, and rIL-2 levels determined by both ELISAs were compared. As shown in Fig. 2, clearance of the immune complex from serum was almost coincident with that determined by an rIL-2-ELISA, suggesting that rIL-2 existed in serum as a complex form following the intravenous injection of the immune complex.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>rIL-2 solution (mean ± S.E.)</th>
<th>Immune complex (mean ± S.E.)</th>
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<tbody>
<tr>
<td>C₀ (ng/ml)</td>
<td>1358 ± 82.8</td>
<td>9716 ± 200</td>
</tr>
<tr>
<td>k (1/h)</td>
<td>3.04 ± 0.28</td>
<td>3.14 ± 0.15</td>
</tr>
<tr>
<td>CL_total (ml/h/rat)</td>
<td>224 ± 18.7</td>
<td>32.3 ± 0.97</td>
</tr>
<tr>
<td>V₀ (ml/rat)</td>
<td>74.0 ± 4.3</td>
<td>10.3 ± 0.22</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. of 3 rats.

Fig. 1. Serum Levels of rIL-2 after Intravenous Administration into Rats of rIL-2 Either Alone (○) or Complexed with the Monoclonal Antibody against rIL-2 (MoAb-HRL1-52) (●) at a Dose of 100 μg/rat as rIL-2

Fig. 2. Relationship between Serum rIL-2 Levels (●) and Serum Immune Complex Levels (○) after Intravenous Administration into Rats at a Dose of 100 μg/rat as rIL-2

Fig. 3. Serum Levels of rIL-2 after Subcutaneous Administration into Rats of rIL-2 Either Alone (○) or Complexed with the Monoclonal Antibody against rIL-2 (MoAb-HRL1-52) (●) at a Dose of 100 μg/rat as rIL-2
complex.

Subcutaneous Administration  Figure 3 shows the time course of serum rIL-2 levels determined by an rIL-2-ELISA following subcutaneous administration of rIL-2 alone and the immune complex. Table II summarizes the pharmacokinetic parameters obtained by moment analysis. In the injection of rIL-2 alone, a maximum serum level ($C_{\text{max}}$) was 358 ng/ml at 50 min and rIL-2 was rapidly cleared from the serum, with little detection at 6 h. The mean residence time ($MRT$) was 1.98 h. Injection of the immune complex, on the other hand, gave a $C_{\text{max}}$ of 45.3 ng/ml at 3 h with a slower disappearance of rIL-2 from the serum. Its $MRT$ value was 3.3-times longer than that of free rIL-2. Furthermore, its $MAT$ value was 3.8 times longer than that of free rIL-2.

Serum specimens taken at 3, 6 and 10 h were also applied to an NKc3 cell-proliferation assay. The biological activity of each specimen corresponded well with the ELISA value, showing that rIL-2 molecules existed in a biologically-active form in serum. Free rIL-2 showed a specific activity of $1.29 \times 10^4$ units/mg and the rIL-2 complexed with antibody also possessed a specific activity of $1.17 \times 10^4$ units/mg as rIL-2. These results indicated that the MoAb-HRL1-52 did not neutralize the biological properties of rIL-2 in immune complex formation.

**DISCUSSION**

Alterations of pharmacokinetic behavior can be adopted to increase the efficacy and decrease the toxicity of a drug. As for IL-2 therapies, prolongation of the serum half-life and reduction of distribution into unfavorable organ sites are the major concerns. We have attempted in the present study to alter the pharmacokinetic behavior of rIL-2 by immunocomplexing with a monoclonal antibody against rIL-2, since IgG molecules possess a long serum half-life and small distribution volume as intrinsic properties.\(^{15,16}\) When the molar ratio of rIL-2 and the MoAb is 2:1, only one peak corresponding to the immune complex was detected without free rIL-2 by gel filtration analysis.\(^{9}\) Therefore, the immune complex was stoichiometrically composed of one monoclonal antibody molecule (150 kDa, IgG,) and two rIL-2 (15 kDa) molecules, giving a molecular weight of 180 kDa. When intravenously administered as a free form into rats, rIL-2 was found to be cleared from the serum in a rapid and monophasic manner. The distribution volume of 74 ml/rat was approximately 7-fold larger than the total serum volume in a rat. When administered as an immune complex, however, rIL-2 gave a significantly decreased distribution volume of 10 ml/rat equivalent to the total serum volume, and higher serum rIL-2 levels were observed, although the elimination rate was unchanged. It is clarified that rIL-2 does not exist in serum as a free form but as a complexed form, because rIL-2 levels declined with time in a similar manner, as shown in Fig. 2. An apparent increase of the molecular weight seemed to result in a smaller distribution volume by preventing the diffusion of rIL-2 from the blood compartment.

With respect to sustaining serum rIL-2 levels, the immune complex was more favorable in subcutaneous injection into rats than free rIL-2. The immune complex gradually appeared in the blood stream and disappeared slowly from the blood, while free rIL-2 showed a maximum serum level within one hour and was rapidly cleared from circulation. Ballard reported that macromolecules with a molecular weight of more than 30 kDa appeared through the lymph system in the blood stream after subcutaneous injection.\(^{17}\) The present immune complex of rIL-2 and the monoclonal antibody also seemed to be slowly delivered to blood through the lymph system following subcutaneous injection, because our previous paper demonstrated significant rIL-2 levels in lymph nodes.\(^{9}\) The accumulation of rIL-2 complexed with the antibody in lymph nodes could be favorable to stimulation of lymph tissues, and in fact contributed to an enhanced anti-tumor activity in Meth-A fibrosarcoma-bearing mice.\(^{9}\) As for $AUC$ values, the immune complex gave a slightly lower value than free rIL-2. Sustained serum levels could be effective for tumor therapy.

Several researchers have already reported that the covalent conjugation of IL-2 with macromolecular compounds provided prolongation of the serum half-life and enhancement of its anti-tumor effect.\(^{18,19}\) However, these chemical conjugations were often accompanied by impairment of their biological activities and difficulty in the stable production of a homogeneous preparation. The immune complex described here can be easily prepared by mixing rIL-2 and the monoclonal antibody at a molar ratio of 2:1 with only a trace of free forms of the two constituent proteins and without any loss of IL-2 biological activity.

Rosenblum et al. showed an immune complex of human leukocyte interferon (IFN) and anti-IFN monoclonal antibody gave an approximately 3-times longer plasma half-life with only a slight decrease in distribution volume after intravenous administration into rats.\(^{20}\) In the present study, however, the immune complex of rIL-2 and the monoclonal antibody gave a significantly decreased distribution volume with little effect on the serum half-life. The pharmacokinetic behavior of an immune complex might depend on a combination of a cytokine and its antibody, and therefore on the clearance pathway of the cytokine itself. In this study, competitive exchange of rIL-2 between the MoAb:rIL-2 immune complex and rIL-2 clearance receptor in the kidney seemed to result in little change in the elimination rate.

In conclusion, we developed rIL-2 complexed with an anti-rIL-2 monoclonal antibody in order to alter its
pharmacokinetic behavior. This alteration could improve the therapeutic index of rIL-2 for anti-tumor therapy.

REFERENCES