Enhancement of the Anti-tumor Activity of Recombinant Interleukin-2 (rIL-2) by Immunocomplexing with an F(ab')₂ Fragment of Murine Monoclonal Antibody against rIL-2

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We have investigated the biological properties of an immune complex composed of recombinant interleukin-2 (rIL-2) and an F(ab')₂ fragment of a monoclonal antibody against rIL-2 in mice for the induction of killer cells and anti-tumor activity, as well as the pharmacokinetic properties of the immune complex injected subcutaneously into mice. The immune complex demonstrated sustained serum rIL-2 levels, with a 2.4-times longer "mean-residence-time" than free rIL-2. A more significant portion of rIL-2 was detected in lymph nodes after the subcutaneous injection of the immune complex than with rIL-2 alone. Splenic lymphocytes from mice given the immune complex showed higher killer cell activity against YAC-1 and P815 cells than those from mice given rIL-2 alone. The immune complex also exerted a more significant anti-tumor effect in a dose-dependent manner in Meth-A fibrosarcoma-bearing mice than did rIL-2 alone.

Keywords recombinant interleukin-2; monoclonal antibody; F(ab')₂ fragment immune complex; pharmacokinetics; anti-tumor effect

Interleukin-2 (rIL-2) produced in large amounts by recombinant DNA technology has been used in clinical fields, since the lymphokine plays the important role of inducing natural killer (NK), cytotoxic T (CT) and lymphokine-activated killer (LAK) cell activity with anti-tumor responses.¹⁾ IL-2, however, has a short half-life in circulation when injected intramuscularly or subcutaneously, so a high-dose administration has been required for effective therapy, often accompanied by serious side effects including fever, hypotension and renal dysfunction.²⁻⁵⁾ In order to overcome this short half-life, several delivery systems have been investigated: IL-2 mini-pellet, rIL-2 modified with polyethylene glycol (PEG), and in combination with a protease inhibitor, pepstatin, that inhibited IL-2 metabolism in mouse kidneys to prolong the plasma half-life of IL-2 and to augment LAK cell activity.⁶⁻⁹⁾ We demonstrated in our previous papers that immunocomplexing rIL-2 with a monoclonal antibody (MoAb) against rIL-2 altered its pharmacokinetic behavior and enhanced its anti-tumor activity in tumor-bearing mice.¹⁰⁾ For clinical applications, however, murine immunoglobulin G (IgG) antibodies are highly immunogenic and cause a human anti-mouse antibody (HAMA) response.^{11,12)} In the present paper, an F(ab')₂ fragment of the IgG antibody molecule, which is expected to be less immunogenic than the whole molecule, was prepared, and the pharmacokinetic and biological properties of an immune complex of rIL-2 and the F(ab')₂ antibody were compared with those of free rIL-2 in mice.

MATERIALS AND METHODS

Recombinant IL-2 rIL-2 was extracted from *Escherichia coli* transfected with a plasmid containing human IL-2-encoding cDNA. The purification and biochemical characterization of the rIL-2 preparation has been detailed

elsewhere.^{13,14)}

Anti-rIL-2 Monoclonal Antibody Mice were immunized with rIL-2 in complete Freund's adjuvant injected at multiple subcutaneous sites along the dorsal surface and intramuscularly into both thighs. After repeated immunization, splenocytes were harvested and fused with mouse myeloma P3-X63-Ag8-U1 cells using PEG.¹⁵⁾ Fused cells were selected in a hypoxanthine-aminopterin-thymidine medium, and their culture supernatants were screened for the ability to bind to rIL-2 by an enzyme-linked immunosorbent assay (ELISA). Positive hybridomas were cloned by a limiting dilution method and the hybridoma HRL1-52 (IgG1, κ) was selected. An antibody was purified from the ascites of BALB/c mice which were intraperitoneally injected with HRL1-52 by high-performance liquid chromatography (HPLC) employing an ABx column (J. T. Baker Inc., NJ, U.S.A.).

F(ab')₂ of MoAb-HRL1-52 Purified MoAb-HRL1-52 was dialysed against 0.1 M citrate buffer, pH 3.5, and then digested by incubation for 16 h at 37 °C with pepsin (Sigma Chemical Co., MO, U.S.A.) at a ratio of 0.2 mg/0.8 mg. The resulting pepsin digest was purified by HPLC employing an ABx column. The F(ab')₂ preparation thus obtained was confirmed for its purity and antibody activity by polyacrylamide gel electrophoresis and an ELISA, respectively.¹⁶⁾

Immune Complex An immune complex was prepared by mixing rIL-2 and the F(ab')₂ of MoAb-HRL1-52 at a molar ratio of 2 : 1. In brief, 0.2 mg of rIL-2 was incubated at room temperature for 10 min with 0.7 mg of F(ab')₂ in 1.2 ml of phosphate-buffered saline (PBS). Complex formation was estimated by HPLC employing an Asahipak GS-520 column (Asahi Kasei, Japan), PBS as the elution buffer, a flow rate of 1.0 ml/min and detection by absorbance at 280 nm.

Mice Specific pathogen-free female BALB/c mice, 7

weeks old, were purchased from Japan Charles River Co., Ltd. (Japan) and fed a commercial pellet diet, CE-2 (CLEA Inc., Japan), with water *ad libitum*.

Administration and Determination of rIL-2 rIL-2 or its immune complex was dissolved in PBS, and a 100 μ l portion of the solution was subcutaneously injected into the right side of each mouse abdomen at a dose of 10 μ g/mouse as rIL-2. Blood and lymph nodes were taken from the retroorbital sinus and right auxiliary axilla, respectively, at various times after administration. Lymph nodes were homogenized in PBS containing 0.2% bovine serum albumin with a Polytron (Kinematica, Switzerland), and subjected to an ELISA for the determination of rIL-2.

ELISA Procedures rIL-2 concentrations in the serum specimens and extracts of lymph nodes were determined by an ELISA developed by Moriya *et al.*¹⁷⁾ In brief, samples were added to microplates coated with affinity purified anti-IL-2 goat immunoglobulin G, followed by incubation overnight at 4°C. After washing of the plates, bound rIL-2 was detected with horseradish peroxidase-labeled anti-IL-2 rabbit Fab' fragment, followed by an enzyme reaction using 6 mM hydrogen peroxide and 40 mM *o*-phenylenediamine in 0.1 M citrate buffer (pH 5.5) as substrates. In this ELISA, as little as 30 pg/ml of IL-2 was detectable, and the immunoreactivity of rIL-2 complexed with the F(ab')₂ of MoAb-HRL1-52 was found to be equivalent to that of free rIL-2.

In Vitro Proliferation Assay NKC3 cells, a murine NK cell line, were added at a concentration of 4×10^5 cells/well on 96-well micropates containing serial dilutions of rIL-2 alone or the immune complex. The cells were cultured at 37°C for 24 h and then the number of viable cells was measured by a conventional colorimetric method using 3-(4,5-dimethyl-thiazol-3-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., MO, U.S.A.).¹⁸⁾

In Vitro Cytotoxicity Assay NK-sensitive YAC-1 lymphoma cells and LAK-sensitive P815 leukemia cells were cultured at 37°C in RPMI-1640 (Flow Laboratories, UK) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 100 units/ml penicillin, 100 μ g/ml streptomycin sulfate and 0.05 mM 2-mercaptoethanol, and labeled as target cells with Na₂⁵¹CrO₄ (Daiichi Radioisotope Laboratory, Japan) according to the method of Kumagai *et al.*¹⁹⁾ Mouse splenocytes were taken as effector cells one day after the last injection of rIL-2 or its immune complex, and their killer activity was measured by adding them to tumor target cells in round-bottomed 96-well microplates at a cell number ratio of 12.5:1, 25:1, 50:1 or 100:1 in a final volume of 200 μ l. After incubation at 37°C for 4 h, the cultured supernatant (100 μ l) was harvested and its radioactivity measured in a γ -counter. Specific lysis was determined by the formula:

$$\text{lysis (\%)} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total count} - \text{spontaneous release})} \times 100$$

Spontaneous release and total count were determined by incubating target cells without splenocytes and by collecting a 100 μ l portion of the cell suspension from the well, respectively.

In Vivo Anti-tumor Assay Meth-A fibrosarcoma cells

obtained from the National Cancer Research Institute (Japan) were passaged *in vivo* as ascites in BALB/c mice. The tumor cells were transplanted subcutaneously into the left flank of mice, and 7 d afterwards, rIL-2 either alone or complexed with the F(ab')₂ of MoAb-HRL1-52 against rIL-2 was subcutaneously injected on a daily basis for 12 consecutive days.²⁰⁾ Tumor weights were measured 21 d after tumor implantation, and the anti-tumor effect was expressed as a percentage of a control group (untreated mice) (% *T/C* values).

RESULTS

Immunocomplexing of rIL-2 with F(ab')₂ of MoAb-HRL1-52 rIL-2 (1.0 mg/ml) was mixed with the F(ab')₂ at various molar ratios ranging from 8:1 to 2:1, and complex formation was determined by gel-filtration HPLC (Fig. 1). rIL-2 was eluted at 13.6 min (Fig. 1A) and its peak height was reduced with increasing amounts of rIL-2 to F(ab')₂ (Fig. 1B–D). Finally, there was no observation of the rIL-2 peak at a molar ratio of 2:1, with only one peak eluted at 11.0 min (Fig. 1E), indicating that one molecule of the F(ab')₂ stoichiometrically bound to two

Fig. 1. High-Performance Liquid Chromatography Employing a GS-520 Column

Recombinant interleukin-2 (rIL-2) (A) and the immune complexes composed of rIL-2 and the F(ab')₂ of the monoclonal antibody against rIL-2 (MoAb-HRL1-52) at molar ratios of 8:1 (B), 4:1 (C), 2.6:1 (D) and 2:1 (E) were eluted at a flow rate of 1.0 ml/min with phosphate-buffered saline and detected by an absorbance at 280 nm.

Fig. 2. (A) Serum Levels of Recombinant Interleukin-2 (rIL-2) after Subcutaneous Injection into Mice of rIL-2 Either Alone (○) or Complexed with the F(ab')₂ of the Monoclonal Antibody against rIL-2 (MoAb-HRL 1-52) (●) at a Dose of 10 µg/mouse as rIL-2
(B) rIL-2 Content in Auxiliary Lymph Nodes from Mice Given rIL-2 as Described in (A)

TABLE I. Pharmacokinetic Parameters of rIL-2^{a)} Following Subcutaneous Administration of rIL-2 either Alone or Complexed with F(ab')₂ Fragment of MoAb-HRL1-52^{b)} in mice

Parameter ^{c)}	rIL-2 alone	Immune complex
AUC (ng·h/ml)	412	752
MRT (h)	0.938	2.22
VRT (h)	0.424	2.29

a) Recombinant interleukin-2. b) Monoclonal antibody against rIL-2. c) AUC, area-under-the-curve; MRT, mean-residence-time; VRT, variance-of-residence-time.

rIL-2 molecules. The immune complex formed at a molar ratio of 2:1 demonstrated a specific activity of 1.19×10^7 units/mg as rIL-2 in *in vitro* NKC-3 cell-proliferation assay, which was equivalent to that of free rIL-2 (1.12×10^7 units/mg). This result indicated that the F(ab')₂ antibody did not neutralize the biological properties of rIL-2 in the immune complex formation.

Pharmacokinetics of rIL-2 Following the subcutaneous administration of 10 µg/mouse of rIL-2, rIL-2 levels in serum and lymph nodes were determined by ELISA (Fig. 2, Table I). The injection of rIL-2 complexed with the F(ab')₂ antibody resulted in a longer "mean-residence-time" (MRT) and "variance-of-residence-time" (VRT) than injections of rIL-2 alone. The immune complex showed MRT and VRT values of 2.2 and 2.3 h, respectively, giving a serum level of 1.1 ng/ml of rIL-2 at 10 h after administration, while rIL-2 alone gave 2.4- and 5.4-times shorter MRT and VRT values, respectively, and the serum level became undetectable level at 6 h after administration. As for the "area-under-the-curve" (AUC), the immune complex demonstrated at 1.8-times larger value than free rIL-2. The immune complex showed an AUC value of 752 ng·h/ml, while rIL-2 alone gave the value of 412 ng·h/ml. rIL-2 contents in auxiliary lymph nodes from mice after the subcutaneous injection of rIL-2 alone reached the highest value of 0.7 ng/node of rIL-2 at 30 min and rapidly decreased to an almost undetectable level at 3 h. On the other hand, after the subcutaneous injection of the immune complex of rIL-2 and the F(ab')₂ fragment, mice showed a much higher rIL-2 concentration

in the lymph nodes, with a maximum level of 3.7 ng/node at one hour and a significant level of 1.0 ng/node even at 6 h. These results suggested that immunocomplexing rIL-2 with the F(ab')₂ fragment of MoAb-HRL1-52 contributed to its easy accumulation in lymph nodes and subsequent sustained release into the blood stream.

Killer Cell Activity Splenocytes were taken as effectors from mice given rIL-2 either alone or complexed with the F(ab')₂ fragment once a day for 1, 3 and 10 consecutive days, and their cytotoxic activity was measured by an *in vitro* ⁵¹Cr-release assay using YAC-1 and P815 cells as the targets (Fig. 3). At an effector/target ratio of 100:1, splenocytes from mice given the immune complex revealed significant killer activity against YAC-1 cells, even after a single administration, and the activity reached a maximum level by 3 consecutive doses. On the other hand, those from mice given rIL-2 alone showed gradually increasing cytotoxicity against YAC-1 cells at each effector/target ratio of 12.5:1, 25:1, 50:1 and 100:1 for 1 to 10 consecutive doses, yet the activity after 10 doses was a little less than the maximum level after 3 doses of the immune complex. As for cytotoxic activity against P815 cells, the subcutaneous administration of the immune complex also significantly augmented the killer activity of splenocytes in a dose-dependent manner for more than 3 doses, while that of free rIL-2 showed little augmentation even after multiple doses for 7 d.

Anti-tumor Activity Three doses of the immune complex (1, 5 and 10 µg/d/mouse as rIL-2) were subcutaneously injected into Meth-A fibrosarcoma-bearing mice ($n=5$) for 12 consecutive days starting on the 7th day after tumor implantation. As shown in Table II, the immune complex of rIL-2 and the F(ab')₂ fragment significantly inhibited tumor growth in a dose-dependent manner; the mean weights of tumor masses from mice receiving daily doses of 1, 5 and 10 µg as rIL-2 were 54%, 48% and 25% of that of untreated mice ($n=10$), respectively, while the mean weight of tumor masses from mice ($n=5$) receiving 10 µg of rIL-2 alone daily was 50% of that of untreated mice ($n=10$). rIL-2 complexed with the F(ab')₂ of MoAb-HRL1-52 demonstrated a significantly enhanced anti-tumor activity in Meth-A fibro-

Fig. 3. Killer Cell Activity Against YAC-1 (A, B) and P815 (C, D) Cells of Splenocytes from Mice Given Recombinant Interleukin-2 (rIL-2) Either Alone (A, C) or Complexed with F(ab')₂ Fragment of the Monoclonal Antibody against rIL-2 (MoAb-HRL1-52) (B, D)

■, E/T = 12.5; ▨, E/T = 25; ▩, E/T = 50; ▤, E/T = 100.

TABLE II. Anti-tumor Effect of rIL-2^{a)} Either Alone or Complexed with F(ab')₂ Fragment of MoAb-HRL1-52^{a)} on Tumor Growth in Meth-A Fibrosarcoma-Bearing Mice

Treatment	Dose (μ g/mouse as rIL-2) ^{b)}		Tumor weight ^{c)} (mg)	Tumor growth T/C (%)	Weight ^{d)} gain (g)
	Total	Daily			
Untreated control	—	—	4622 $\pm$ 629	100	3.6
Vehicle	0	0	4031 $\pm$ 461	87	3.4
rIL-2 alone	120	10	2292 $\pm$ 494	50	2.2
Immune complex	12	1	2479 $\pm$ 816	54	2.9
Immune complex	60	5	2210 $\pm$ 837	48	2.0
Immune complex	120	10	1166 $\pm$ 479	25	0.9

a) Described in Table I. b) BALB/c mice were subcutaneously administered rIL-2 or its immune complex for 12 consecutive days starting on the 7th day after tumor implantation. Each group consisted of 5 mice except the untreated control consisting of 10 mice. Values were mean $\pm$ S.D. c) Tumor weights of treated mice were measured 21 d after tumor implantation and compared with that of the untreated control. d) The values represent body weight increases through 7 to 21 d after tumor implantation.

sarcoma-bearing mice. With respect to weight gain, the immunocomplex treatment caused a lower weight increase in a dose-dependent manner than the untreated control (Table II). But, the extent of weight gain was comparable to that with the rIL-2 solution treatment (Table II).

DISCUSSION

We investigated the effects of immunocomplexing rIL-2 with the F(ab')₂ fragment of anti-rIL-2 MoAb-HRL1-52 on its pharmacokinetics, the induction of killer cell activity and tumor growth inhibition in mice. We had already reported the prolongation of serum half-life and the enhancement of anti-tumor activity of rIL-2 by immunocomplexing with the antibody whole molecule, but the administration of the immune complex was accompanied by the problem of raising immune responses against mouse IgG.¹⁰⁾ On the other hand, it has been reported that the use of F(ab')₂ fragments instead of whole IgG molecules results in a reduced immunogenicity in primates and in patients.^{21,22)} We have presented here that the immune complex of rIL-2 and the F(ab')₂ antibody showed a strong induction of killer cells and anti-tumor effect in mice equal to that of rIL-2 and the IgG antibody.

The present immune complex of rIL-2 and F(ab')₂ demonstrated prolonged serum levels, with a 2.4-times longer *MRT* value after its subcutaneous administration, and produced a higher content of rIL-2 in lymph nodes. The accumulation of the immune complex in lymph nodes probably contributed to the systemic stimulation of lymph

tissues, resulting in effective anti-tumor treatment. Ballard reported that macromolecules with a molecular weight of more than 30 kDa appeared *via* the lymph system in the blood stream after subcutaneous injection.²³⁾ The molecular weight of this immunocomplex is calculated to be 130 kDa, including the 2 molecules of rIL-2, 15 kDa, and one molecule of F(ab')₂, 100 kDa. Therefore, we speculate that the complex given subcutaneously appeared in blood *via* lymph system and accumulated in lymph nodes.

NK and LAK cells were both reported to play a critical role in mediating anti-tumor responses, and their activities were assessed respectively by cytotoxicities against NK-sensitive YAC-1 cells and LAK-sensitive P815 cells of splenocytes from mice given rIL-2 consecutively.²⁴⁻²⁶⁾ The immune complex of rIL-2 and the F(ab')₂ augmented NK cell activity more rapidly and significantly than free rIL-2. For the induction of LAK cells, the immune complex also played a significant role, while free rIL-2 did little.

The anti-tumor activity of the immune complex of rIL-2 and the F(ab')₂ antibody was also estimated using Meth-A fibrosarcoma-bearing mice which are sensitive to IL-2 immunotherapy, and the complex was found to be more potent than free rIL-2.²⁰⁾ The immune complex seemed to induce higher anti-tumor effects in this animal model through the effective activation of NK and LAK cells.

Thus, the immune complex of rIL-2 and the F(ab')₂ demonstrated similar pharmacokinetic and biological properties in mice to those of rIL-2 and the whole IgG molecule. Recent progress in recombinant DNA techniques, however, has made it possible to construct a humanized antibody which is much less immunogenic to human beings,²⁷⁾ and, therefore such an immune complex composed of some cytokine and its humanized antibody could provide a therapeutically useful drug delivery system to enhance biological activity by altering its pharmacokinetic parameters.

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