Potentiation of *in vivo* Antitumor Effects of Recombinant Interleukin-1α by Gelatin Conjugation

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Chemical conjugation of a recombinant human interleukin-1α (IL-1) with gelatin was conducted using a water-soluble carbodiimide in an attempt to augment the indirect effect of IL-1 on *in vivo* tumor cell growth in mice. Chromatographic studies of the IL-1-gelatin conjugate demonstrated that the apparent molecular weight of IL-1 was increased by the gelatin conjugation and about 60% of IL-1 activity was retained in the prepared conjugate. Intraperitoneal (i.p.) injection of the conjugate significantly suppressed the intraperitoneal growth of a subline of Meth A fibrosarcoma cells (RR1 cells), compared with the effect of free IL-1 at the same dose, although the cells *per se* were resistant not only to free IL-1 but also to gelatin-conjugated IL-1. Simple mixing of gelatin with free IL-1 did not augment the *in vivo* antitumor effect as compared with that of free IL-1. Gelatin conjugation improved the *in vivo* stability of IL-1. Prolonged retention of IL-1 activity in the peritoneal cavity as well as the circulation of mice was observed after i.p. injection of the IL-1-gelatin conjugate in comparison with free IL-1 injection, irrespective of the presence of tumor cells. Gelatin conjugation was effective in augmenting the *in vivo* antitumor effects of IL-1 to activate host cells, e.g. macrophages (Mφ). The i.p. injection of the conjugate enhanced Mφ infiltration into the peritoneal cavity of tumor-bearing mice and peritoneal Mφ were strongly activated to inhibit the *in vitro* growth of RR1 cells. Thus, gelatin conjugation was effective in augmenting the indirect effect of IL-1 via host cells, leading to a high suppressive effect on *in vivo* growth of tumor cells.

Key words: Antitumor effect *in vivo* — Gelatin conjugation — Interleukin-1α

Interleukin-1 (IL-1) is a cytokine produced by various cell types including macrophages (Mφ).[1-4] IL-1 has a wide range of biological activities associated with host responses to infectious, immunological, and inflammatory stimuli,[2-5] and it functions effectively as an antitumor cytokine.[5-8] Plausible mechanisms of the antitumor activity have been proposed: the augmentation of cytotoxic activity of monocytes,[9,10] of natural killer (NK) cells,[10-12] and of T lymphocytes,[10-13] the stimulation of antitumor cytokine production (interleukin-2, interferon-γ, and TNF),[10,14-16] and direct cytotoxic action on tumor cells.[17-20]

Recently cDNAs coding for the precursors of two distinct IL-1 species, α and β,[10,21] were successfully expressed in *Escherichia coli*. It is becoming feasible to employ large quantities of pure recombinant IL-1 in clinical trials. However, several problems (e.g. hypotension development, rapid catabolism, and pyrogenicity) remain to be overcome before therapeutic use of IL-1 becomes feasible. One approach would be to reduce the necessary dose of IL-1 by increasing the *in vivo* stability of IL-1. Stabilization of protein drugs has been achieved through chemical modification with various water-soluble polymers.[22-26] We have demonstrated that gelatin functions as a polymer carrier of drugs to increase the therapeutic efficacy.[27-29] Gelatin conjugation of interferon (IFN) not only prolonged the biological life-time but also augmented the antitumor effects mediated by Mφ and NK cells.[29]

In the present study we conducted chemical conjugation of IL-1 with gelatin in an attempt to improve the *in vivo* stability. Therapeutic experiments using tumor-bearing mice were conducted to estimate the antitumor effect of the IL-1-gelatin conjugate. We also examined the antitumor effect of Mφ harvested from tumor-bearing mice given the conjugate injection.

MATERIALS AND METHODS

Mice Specific pathogen-free female (BALB/c×DBA/2)F₁ mice aged 3–5 months were obtained from Japan SLC Inc., Shizuoka. C3H/HeJJcl mice aged 4–6 weeks were purchased from Clea Japan Inc., Osaka.

IL-1 Recombinant human interleukin-1α (IL-1, 2.26× 10⁷ IU/mg protein) was provided by the Dainippon Pharmaceutical Co. Ltd., Osaka. The endotoxin level in

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original preparations was determined by the Limulus test to be 0.32 ng/mg IL-1 (2.26×10^7 IU) or less. The IL-1 activity was evaluated in terms of the proliferation, as ^{3}H-thymidine incorporation, of C3H/HeJ mouse thymocytes in response to phytohemagglutinin P (Difco Laboratories, Detroit, MI) according to the method of Nakano et al.[30]

Culture medium and reagents Culture medium (RPMI-FCS) was prepared by supplementing RPMI-1640 medium (Nissui Seiyaku Co. Ltd., Tokyo) with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, MD), 5 mM L-glutamine, and penicillin (100 IU/ml) and buffered with 5 mM 4-(2-hydroxyethyl)-1-ethanesulfonic acid and $NaHCO_3$ at pH 7.2. Hanks' balanced salt solution (HBSS) and phosphate-buffered saline solution (PBS) were obtained from Nissui Seiyaku Co., Ltd., Tokyo. Gelatin used was of alkaline type, isolated from pig skin (pI 5.1, Nitta Gelatine Co. Ltd., Osaka). The endotoxin level was 0.12 ng/mg gelatin when determined using the Endospecy ES-6 Set (Seikagaku Corp., Tokyo). Other chemical reagents of guaranteed grade were purchased from Wako Pure Chemical Industries, Ltd., Osaka and used as obtained.

Conjugation of IL-1 to gelatin IL-1 was conjugated to gelatin according to the carbodiimide method described previously.[29, 31] Varying amounts of IL-1 were dissolved in 2.5 ml of 0.05 M phosphate buffer solution (pH 4.7) containing 1.25 mg of gelatin. The conjugation was carried out for various periods at 4°C in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl salt (EDCI, 0.05 and 0.5 mg/ml). The resulting IL-1-gelatin conjugate was purified by dialysis against PBS at 4°C for 24 h, sterilized by filtration through a 0.22 μm Millex-GS millipore filter (type: SLGS025OS, Japan Millipore Ltd., Tokyo), and stored at 4°C until use. The endotoxin assay demonstrated that the level of endotoxin in the final conjugate preparation used for *in vivo* experiments was 30.4 pg/190 μg gelatin/0.5 ml PBS, irrespective of the IL-1 dose incorporated into the conjugate.

Change of the molecular size of IL-1-gelatin conjugates with the reaction time was evaluated by measuring the retention time in gel filtration chromatography (GFC, Toyo Soda Co., Ltd., Tokyo) with TSK-gel G6000PW$_{XL}$ and G4000PW$_{XL}$ columns. In addition, the molecular size and molecular weight distribution of the conjugate prepared were investigated by gel exclusion chromatography using a Sepharose 4B column (1.7×47 cm). The flow rate of 0.5 M potassium phosphate buffer (pH 7.0) was 20 ml/h. The IL-1 activity and the total amount of protein in each fraction (2.6 ml) were assayed. The activity was measured by the method described in the previous section. The amount of proteins was determined by measuring the fluorescence intensity at 308 nm on exposure to ultraviolet light of 280 nm.

^{125}I-labeled IL-1, prepared by the chloramine T method,[32] was similarly conjugated to gelatin. The radioactivity of the conjugate was measured to estimate the percentage of IL-1 incorporated into the conjugate. In this paper, the IL-1 activity of the conjugate was expressed on the basis of this percentage of IL-1 incorporated. The activity was calculated simply from the value and the specific activity of original IL-1.

Tumor cells RR1 cells were isolated and cloned in our laboratory as an ascitic nonadherent Meth A cell line (methylcholanthrene-induced fibrosarcoma of BALB/c mice). They are IFN (α, β, γ) and TNF-α-resistant under *in vitro* conditions.[33, 34] Moreover, the *in vitro* growth of RR1 cells was not suppressed by either free IL-1 or IL-1-gelatin conjugate (data not shown). RR1 cells have been maintained by *in vitro* culture and regularly monitored to confirm the absence of mycoplasma.

Measurement of IL-1 activity in peritoneal cavity and blood circulation after i.p. injection of IL-1-gelatin conjugate IL-1-gelatin conjugate (190 μg) containing 1×10^4 IU of IL-1 and 1×10^4 IU of free IL-1 were intraperitoneally injected into normal or RR1-bearing mice, which had received i.p. inoculation of 2×10^6 RR1 cells 8 days before. At various time intervals after injection, blood samples and ascites were collected by bleeding from the orbital plexus and lavage of the peritoneal cavity with 3 ml of HBSS, respectively. The *in vitro* thymocyte proliferation assay of the serum and peritoneal fluid collected was conducted to assess IL-1 activity based on the assumption that the total volume was 2 ml and 3 ml, respectively. Three mice were used for each group and the same experiment was performed independently 3 times.

***In vivo* experiment** RR1 cells maintained *in vitro* were inoculated intraperitoneally to acclimatize them to *in vivo* conditions.[29, 33] The RR1 cells were harvested 7 days later and used for *in vivo* experiments. Mice received i.p. inoculation of 2×10^6 RR1 cells at Day 0 and then were intraperitoneally injected with IL-1-gelatin conjugates and other agents at Days 3, 5, and 7. The agents were 0.5 ml of PBS alone or PBS containing 190 μg of gelatin, free IL-1, and 190 μg of IL-1-gelatin conjugate containing IL-1. The dose of IL-1 injected per mouse was 1×10^3 or 1×10^4 IU. A mixture of 190 μg of gelatin and 1×10^4 IU of free IL-1 was injected as a control.

The suppressive effect on *in vivo* tumor cell growth was estimated by counting the number of RR1 cells in the peritoneal cavity. At the day after the last injection of the agents, peritoneal cells were collected by lavage of the peritoneal cavity with HBSS for counting of viable RR1 cells and host peritoneal exudate cells (PEC) by the trypan blue dye exclusion test. RR1 cells did not form solid tumors in the peritoneal cavity, and were readily distinguishable from host PEC by their large size. The *in*

vivo experiment was conducted twice independently on the same injection schedule. Data were treated statistically using Student's *t* test and differences with a *P* value of less than 0.005 were considered significant.

***In vitro* antitumor activity of peritoneal Mφ from RR1-bearing mice receiving IL-1-gelatin conjugate** RR1-bearing mice were intraperitoneally injected with IL-1-gelatin conjugates or other agents according to the same schedule as described above. Peritoneal cells harvested from these mice at Day 8 were pooled and fractionated by a counter-flow centrifugal elutriator (Beckman, JE-6B rotor) to obtain Mφ populations. These cells were fractionated in the following order: erythrocytes, lymphocytes, Mφ/PMN, and RR1 cells with increase in the flow rate or decrease in the rotation speed of the elutriation apparatus. Various numbers of Mφ in 200 μl of RPMI-FCS were plated into each dish of 96-well multiwell culture plates (Nunc No. 1 67008, Kampstrup, Denmark) and incubated at 37°C in a 5% CO_2-95% air atmosphere for 2 h. After removal of nonadherent cells, the adherent cells were cultured with 1×10^4 RR1 cells in 200 μl of RPMI-FCS. The number of viable RR1 cells was determined by the trypan blue dye exclusion test 24 h later. The inhibitory activity of Mφ was evaluated according to the following formula.

Percent growth inhibition = [(No. of tumor cells cultured without Mφ)–(No. of tumor cells cultured with Mφ)/ (No. of tumor cells cultured without Mφ)] × 100

As the Mφ/PMN ratio determined from smeared-stained preparations varied from 0.8 to 2.2 depending on the therapeutic agents given to mice, the number of Mφ was calculated from the total cell number and the Mφ/PMN ratio.

Measurement of TNF activity in peritoneal cavity after i.p. injection of IL-1-gelatin conjugate Normal mice received i.p. injection of 0.5 ml of PBS alone, PBS containing 190 μg of IL-1-free gelatin, 1×10^4 IU of free IL-1 with or without 190 μg of gelatin, or 190 μg of IL-1-gelatin conjugate containing 1×10^4 IU of IL-1. The mouse ascites were collected by lavage with 3 ml of HBSS of the peritoneal cavity 15 min, 30 min, 1 h, 3 h, 6 h, or 22 h later. TNF activity in each sample was assessed by *in vitro* cytotoxicity assay using L-M cells.[35]

RESULTS

Characterization of IL-1-gelatin conjugate The time course of IL-1 activity during the conjugation is shown in Fig. 1. IL-1 activity of the conjugate decreased up to 10 h to attain a constant level of about 60%, irrespective of the ratio of carbodiimide and IL-1 to gelatin. In addition, GFC studies demonstrated that the elution time of the conjugate decreased with the reaction time up to
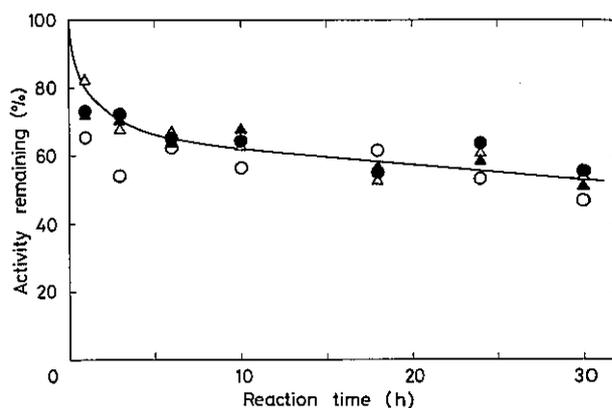

Fig. 1. Time course of conjugation of IL-1 to gelatin. EDCI (1.25 mg) was added to 2.5 ml of phosphate buffer solution containing 1.25 mg of gelatin and 4.42 μg (○), 442 ng (△), or 44.2 ng of IL-1 (▲). EDCI (0.125 mg) was added to 1 ml of the solution containing 1.25 mg of gelatin and 4.42 μg of IL-1 (●). $(2.26 \times 10^7$ IU/mg IL-1)

10 h, and thereafter showed a constant value (data not shown). The solution did not become cloudy during the conjugation.

Fig. 2 shows representative gel filtration profiles of IL-1-gelatin conjugates prepared. Globular proteins of different molecular weights (e.g. bovine thyroglobulin (Mw=670,000), bovine serum albumin (Mw=67,000), bovine pancreatic trypsin (Mw=24,000), and chicken egg lysozyme (Mw=12,500)) were used to calibrate the column. As shown in Fig. 2(A), the mixture of free IL-1 and IL-1-free gelatin was separated into gelatin, devoid of IL-1 activity, in fractions 21 to 32, and IL-1 of molecular weight 15,000 in fractions 34 to 39. On the other hand, the conjugate was eluted in early fractions ranging from 13 to 24, and IL-1 activity was detected in each fraction (Fig. 2(B)). Similar elution behavior of the conjugate was observed, irrespective of the amounts of gelatin, IL-1, and EDCI employed for conjugation. The total amount of proteins recovered was about 80% of that initially added. This value was similar to the percentage of IL-1 incorporated into the conjugate, estimated by using ^{125}I-labeled IL-1.

IL-1 activity in peritoneal cavity and blood circulation after i.p. injection of IL-1-gelatin conjugate The fate of intraperitoneally injected IL-1-gelatin conjugate is shown in Fig. 3. The IL-1 biodistribution was considerably changed by gelatin conjugation. The i.p. injection of the conjugate prolonged the retention period of IL-1 activity in the peritoneal cavity of mice. More than 30% of IL-1 activity was retained 3 h after conjugate injection, while little activity was recovered 3 h after free IL-1 injection. Simple mixing of gelatin and IL-1 did not increase the
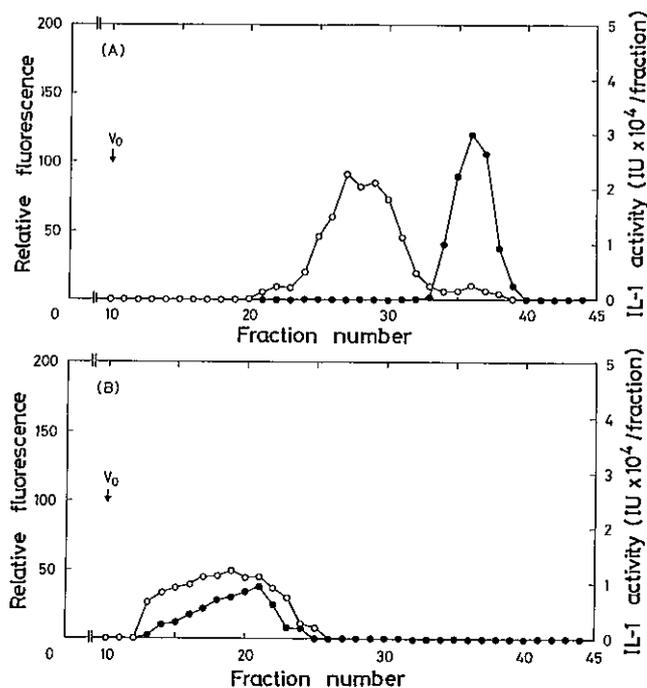
Fig. 2. Gel filtration profiles demonstrating the conjugation of IL-1 to gelatin by EDCI. Chromatographic behavior of (A) a mixture of 1×10^5 IU of free IL-1 and 1.25 mg of gelatin in 2.5 ml of PBS on a Sepharose 4B column and (B) the reaction mixture after 10 h conjugation of 1×10^5 IU of IL-1 and 1.25 mg of gelatin on the same column under the same conditions as in (A). The open symbol represents the fluorescence intensity of proteins and the solid symbol shows IL-1 activity. V_0 represents the exclusion volume.
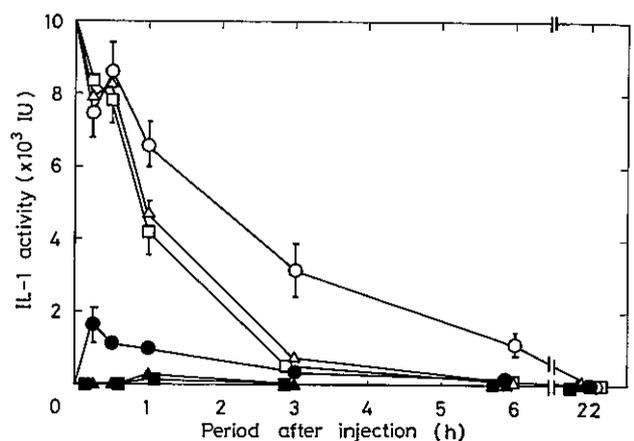

Fig. 3. IL-1 activity in peritoneal cavity (open symbols) and in serum (solid symbols) of normal mice after i.p. injection of free IL-1 or IL-1-gelatin conjugate. Mice were i.p. injected with 190 μg of IL-1-gelatin conjugate containing 1×10^4 IU of IL-1 (O, ●), 1×10^4 IU of free IL-1 (△, ▲), and 190 μg of gelatin plus 1×10^4 IU of free IL-1 (□, ■). Points, means of triplicate experiments; bars, SE.

retention time of IL-1 in the peritoneal cavity. Moreover, gelatin conjugation affected the distribution of IL-1. Up to 6 h after injection, high activity of IL-1 was detected in serum, while it was hardly detected after the injection of free IL-1 with or without gelatin.

The effect of a tumor in the peritoneal cavity on the biodistribution profile of IL-1 was investigated after i.p. injection of free IL-1 and IL-1-gelatin conjugate. The time course of IL-1 in the peritoneal cavity as well as the circulation of tumor-bearing mice was similar to that of normal mice as shown in Fig. 3. The presence of tumor cells in the peritoneal cavity, however somewhat prolonged the retention period of IL-1 in the peritoneal cavity. Thus, IL-1 activity detected in the peritoneal cavity 3 and 6 h after conjugate injection was $5.02 \pm 0.32 \times 10^3$ and $2.68 \pm 0.22 \times 10^3$ IU, respectively. The same trend was observed after i.p. injection of IFN-gelatin and TNF-gelatin conjugates.[29, 31] This could be caused by the difference in physiological status between normal mice and tumor-bearing mice.

Suppressive effect of IL-1-gelatin conjugate on the *in vivo* growth of RR1 cells Fig. 4 shows the effect of i.p. injection of free IL-1 or IL-1-gelatin conjugate on the *in vivo* growth of RR1 cells. The number of viable RR1 cells in the peritoneal cavity of mice was counted after several i.p. injections of the conjugate or other agents. The intraperitoneal growth of RR1 cells was hardly suppressed by treatment with free IL-1 at 1×10^3 or 1×10^4 IU. The i.p. injection of gelatin alone was not effective in inhibiting the *in vivo* growth of RR1 cells and the simultaneous injection of gelatin had no influence on the suppressive effect induced by free IL-1 injection (Exp. 1). In addition, no antitumor effect was observed in the injection of a gelatin preparation which had undergone the EDCI conjugation reaction in the absence of IL-1 (data not shown). On the contrary, the injection of the conjugate led to a marked suppression of the *in vivo* growth of RR1 cells and the suppressive effect was significant compared with that induced by free IL-1 injection ($P < 0.005$).

Mϕ activation in RR1-bearing mice receiving IL-1-gelatin conjugate Fig. 5 shows the *in vitro* antitumor effect of Mϕ populations harvested from the peritoneal cavity of RR1-bearing mice. Mice received i.p. injection of IL-1-gelatin conjugate or other agents according to the same schedule as in the therapeutic experiments. Mϕ obtained from mice given the injection of PBS and gelatin did not inhibit the *in vitro* growth of RR1 cells. However, the i.p. injection of the conjugate was effective in activating Mϕ *in vivo* and the harvested Mϕ strongly inhibited the *in vitro* growth of RR1 cells. Although the
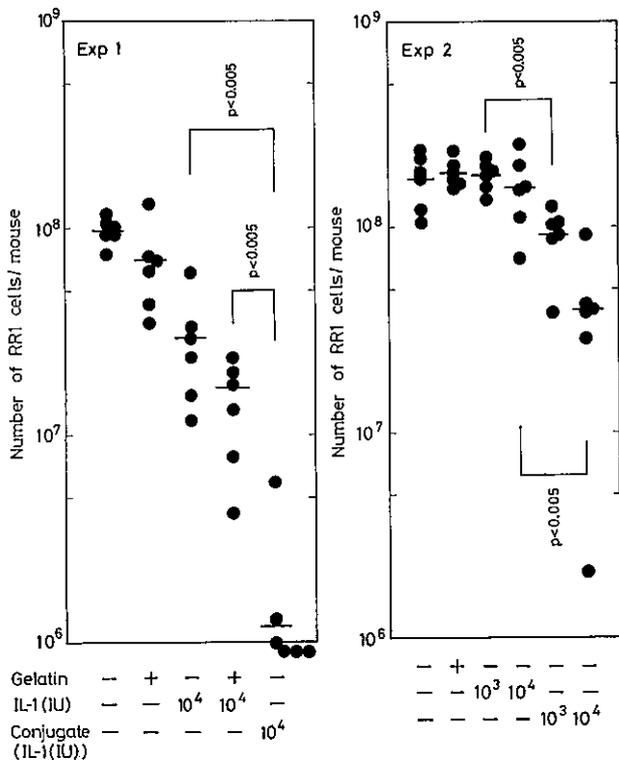
Fig. 4. *In vivo* growth of RR1 cells in the peritoneal cavity of mice given i.p. injection of free IL-1 or IL-1-gelatin conjugate. RR1 cells (2×10^6) were inoculated i.p. at Day 0 and the recipient mice received three i.p. injections at Days 3, 5, and 7 of the IL-1-gelatin conjugate and other agents. Viable RR1 cells were counted at Day 8. Points, number of cells in each mouse; bars, mean.
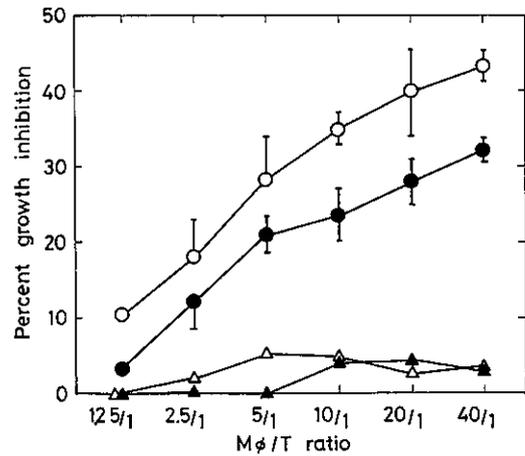

Fig. 5. *In vitro* antitumor activity of Mϕ populations obtained from RR1-bearing mice given i.p. injection of free IL-1 or IL-1-gelatin conjugate. Mice were i.p. injected with PBS alone (▲) or that containing 190 μg of IL-1-gelatin conjugate containing 1×10^4 IU of IL-1 (○), 1×10^4 IU of IL-1 (●), and 190 μg of gelatin (△) according to the schedule in the experiment of Fig. 4. Mϕ populations were cultured from 4 to 6 mice each and pooled. Varying numbers of Mϕ were cultured with 1×10^4 RR1 cells and the viable RR1 cells were counted 24 h later. Points, means of triplicate cultures; bars, SE. T; RR1 cells, Mϕ; macrophages.

free IL-1 injection at the same dose activated Mϕ, the antitumor effect was significantly lower than that induced by the conjugate injection.

The number of RR1, PEC or Mϕ and their cell ratio in PEC in the peritoneal cavity of RR1-bearing mice given the conjugate injection are shown in Table I. Treatment with the conjugate or free IL-1 was conducted at Days 3, 5, and 7, and the number of cells in the peritoneal cavity of 5 mice was counted at Day 8. Treatment with IL-1 in any form increased the number of PEC and Mϕ although the extent of the increase was higher for free IL-1 than the conjugate. However, when the numbers of PEC and RR1 cells were compared, the number of PEC was smaller in control and free IL-1-injected mice and larger in the conjugate-treated mice than that of RR1 cells. The ratio of the PEC to RR1 cells was beyond 1 after treatment of the conjugate, in contrast with other agents. Moreover, the largest Mϕ/RR1 ratio was obtained after conjugate injection. The free IL-1 injection led to PEC infiltration into the peritoneal cavity but the PEC/RR1

and Mϕ/RR1 ratios were lower than those in the conjugate-treated mice. In addition, the number of Mϕ was hardly increased by gelatin injection.

TNF activity in peritoneal cavity after i.p. injection of IL-1-gelatin conjugate The ascites were collected from mice given i.p. injection of IL-1-gelatin conjugate or free IL-1 to assess their TNF activity. As shown in Fig. 6, injection of the conjugate was effective in inducing a high level of TNF in the peritoneal cavity in comparison with that of gelatin with or without free IL-1. TNF was not detectable in the circulation after injection of IL-1 in any form (data not shown).

DISCUSSION

Chromatographic studies demonstrated that the carbodiimide method employed here was effective in preparing IL-1-gelatin conjugate without much loss of IL-1 activity. About 80% of the IL-1 amount added initially was conjugated to gelatin and the percentage of activity remaining in the resulting conjugate was about 60%. It is likely that the conjugate has the micro-gel structure of gelatin associated with IL-1 with a wide distribution in molecular weight ranging from 600,000 to 5,000,000. It has been demonstrated that the increase in the apparent molecular weight of proteins through polymer conjuga-

Table I. Numbers of RR1, PEC, and Mϕ of RR1-bearing Mice Receiving i.p. Injection of Free IL-1 and IL-1-gelatin Conjugate[a]

Treatment	Cell number ($\times 10^7$)			Cell number ratio		
	RR1	PEC	Mϕ	PEC/RR1	Mϕ/RR1	Mϕ/PEC
PBS	13.7 ± 1.2[b]	4.51 ± 0.6	2.03 ± 0.2	0.33	0.15	0.45
Free IL-1 (1×10^4 IU/mouse)	15.8 ± 2.4	11.4 ± 1.1	6.95 ± 0.9	0.72	0.44	0.61
IL-1(1×10^4 IU)-gelatin conjugate (190 μg/mouse)	4.42 ± 1.2	5.82 ± 0.3	3.96 ± 0.7	1.32	0.90	0.68
Gelatin (190 μg/mouse)	12.2 ± 3.5	6.20 ± 0.7	3.10 ± 0.4	0.51	0.25	0.50

a) Mice were given an i.p. inoculation of 2×10^6 RR1 cells at Day 0 and then intraperitoneally injected with IL-1-gelatin conjugates and other agents at Days 3, 5, and 7. On the day after the last injection, the numbers of viable RR1 cells, peritoneal exudate cells (PEC), and Mϕ were determined as described in "Materials and Methods."
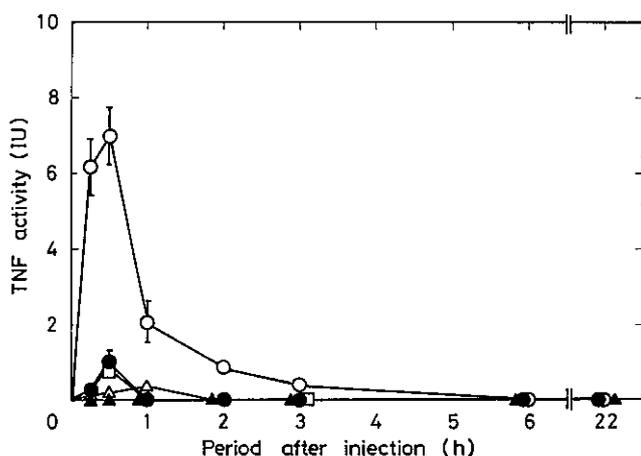b) Means $\pm$ SE.

Fig. 6. TNF activity present in the peritoneal cavity of normal mice after the injection of free IL-1 or IL-1-gelatin conjugate. Mice received a single i.p. injection of PBS alone (▲) or that containing 190 μg of IL-1-gelatin conjugate containing 1×10^4 IU of IL-1 (○), 1×10^4 IU of free IL-1 (●), 190 μg of gelatin (△), and 190 μg of gelatin plus 1×10^4 IU of free IL-1 (□). Points, means of triplicate experiments; bars, SE.

tion can prolong their half-life in the body.[22-26] In the present study, the molecular mass of IL-1 was increased by the gelatin conjugation and consequently, prolonged retention of IL-1 in the peritoneal cavity and the circulation of mice was observed after i.p. injection, compared with IL-1 in free form (Fig. 3). We have investigated the distribution after i.p. injection of TNF-gelatin conjugates with similar molecular size to that of the IL-1-gelatin conjugate. There was a prolonged retention of TNF activity in the peritoneal cavity and the blood circulation after i.p. injection.[31] Large IL-1-gelatin conjugates may be transferred from the peritoneal cavity to the circulation more slowly than free IL-1, leading to the retention of IL-1 activity in the circulation for a longer period. No

serious side effect due to a high level of IL-1 activity in the circulation was apparent in the present study (data not shown).

The present study demonstrates that gelatin conjugation is effective in enhancing the antitumor effects of IL-1 *in vivo*. A higher suppressive effect of the IL-1-gelatin conjugate was observed on the growth of RR1 cells in the peritoneal cavity of mice in comparison with the same dose of IL-1 in free form (Fig. 4). However, simple mixing of gelatin and free IL-1 did not increase the *in vivo* suppressive effect over that induced by the injection of free IL-1 alone, indicating that gelatin conjugation is required for the enhancement of the antitumor effect. This result may be explained in terms of difference in properties between gelatin-conjugated IL-1 and unconjugated IL-1. First of all, gelatin conjugation prolonged the retention period of IL-1 activity in the peritoneal cavity and in the circulation of mice, irrespective of the presence of tumor cells. Secondly, the conjugate augmented the antitumor effect of IL-1 mediated by host cells (e.g. Mϕ) in tumor-bearing mice. Intraperitoneal injection of the conjugate was effective in activating *in vitro* growth inhibition of RR1 cells as compared to free IL-1 (Fig. 5). We have demonstrated the increased association of muramyldipeptide (MDP) and IFN with Mϕ following gelatin conjugation because of the intrinsic opsonic ability of gelatin.[27-29] Thus, gelatin conjugation may enhance the association of IL-1 with Mϕ, leading to efficient antitumor activation of Mϕ. In addition, the injection of the conjugate enhanced infiltration of Mϕ into the peritoneal cavity. It may be concluded that the augmentation of the indirect action of IL-1 by the conjugation led to the suppression of the *in vivo* growth of RR1 cells.

It is likely that the *in vivo* antitumor effect of IL-1-gelatin conjugate is attributable to the indirect action of IL-1 via host cells, since RR1 cells are resistant to free IL-1 and also to the gelatin conjugate under *in vitro* conditions. We have reported on the indirect antitumor effect of IFN-gelatin conjugate via host cells.[29] The

indirect effect of IFN showed a good correlation with the number and the activation state of host PEC, especially $M\phi$, infiltrating the tumor site.[33, 34] In the case of IL-1, the increase in antitumor effect of $M\phi$ as well as their number was also achieved by gelatin conjugation. The ratio of the number of host PEC infiltrating into the peritoneal cavity of RR1 recipients to that of RR1 cells was increased by gelatin conjugation (Table I). Among the PEC, $M\phi$ populations were increased by the conjugate injection. Injection of the conjugate activated peritoneal $M\phi$ more strongly to inhibit the in vitro growth of RR1 cells than did injection of free IL-1 (Fig. 5). This indicates that $M\phi$ play an important role in exerting the in vivo indirect effect of IL-1 after the injection of IL-1-gelatin conjugate. On the other hand, the injection of free IL-1 was also effective in increasing the $M\phi$ number. However, the antitumor activity of $M\phi$ infiltrating into the peritoneal cavity was low in comparison with that in the case of conjugate injection and the number of $M\phi$ was lower than that RR1 cells. This is consistent with the fact that free IL-1 injection was not effective in suppressing the in vivo growth of RR1 cells.

It is possible that injection of the IL-1-gelatin conjugate was effective in inducing some cytokines. It has been reported that IL-1 induces TNF production.[29] Therefore, the induction of TNF by IL-1 injection was investigated. Injection of the IL-1-gelatin conjugate was effective in inducing a high level of TNF in the peritoneum in comparison with that after injection of gelatin with or without free IL-1 (Fig. 6). Thus, the conjugate may function effectively to augment the production of cytokines and exert a synergistic effect with them, leading to the induction of high in vivo antitumor effect.

It may be concluded that the IL-1-gelatin conjugate is a promising antitumor agent effective in enhancing the in vivo antitumor effect of IL-1 in comparison with free IL-1. Gelatin conjugation prolongs the retention of IL-1 in the body and allows it to associate effectively with $M\phi$, leading to potentiation of the antitumor effect of $M\phi$ and possibly other effector cells. This procedure of gelatin conjugation is applicable to other antitumor agents, as we have reported,[27-29, 31] and further applications will be examined.

REFERENCES

1) Gery, I., Gershon, R. and Waksman, B. H. Potentiation of the T-lymphocyte response to mitogens. J. Exp. Res., 136, 128–142 (1972).

2) Mizel, S. B. The interleukins: regulation of lymphocyte differentiation, proliferation and funtional activation. In "Biological Response in Cancer," ed. E. Mihichi, p. 89–119 (1982). Plenum Press, New York.

3) Oppenheim, J. J. and Gery, I. Interleukin 1 is more than an interleukin. Immunol. Today, 3, 113–119 (1982).

4) Dinarello, C. A. Interleukin 1. Rev. Infect. Dis., 6, 51–95 (1984).

5) Oppenheim, J. J., Onozaki, K., Benezur, M. and Matsushima, K. Biological activities of interleukin 1 with potential antitumor effects. In "Recent Advances in Chemotherapy. Anticancer Section 1," ed. J. Ishigami, pp. 72–95 (1985). University of Tokyo Press, Tokyo.

6) Izumi, Y., Tsuchida, T., Okuno, K., Fujiwara, H. and Hamaoka, T. Enhanced induction of tumor-specific Lyt-1^+2^- T cell-mediated protective immunity by in vivo administration of interleukin 1. Jpn. J. Cancer Res., 76, 863–870 (1985).

7) Nakamura, S., Nakata, K., Kashimoto, S., Yoshida, H. and Yamada, M. Antitumor effect of recombinant human interleukin 1 alpha against murine syngeneic tumors. Jpn. J. Cancer Res., 77, 767–773 (1986).

8) Nakata, K., Kishimoto, S., Yoshida, H., Oku, T. and Nakamura, S. Augumented antitumor effect of recombinant human interleukin-1α by indomethacin. Cancer Res., 48, 584–588 (1988).

9) Onozaki, K., Matsushima, K., Kleinerman, E. S., Saito, T. and Oppenheim, J. J. Role of interleukin 1 (IL1) in promoting human monocyte-mediated tumor cytotoxicity. J. Immunol., 135, 314–320 (1985).

10) Dinarello, C. A. Studies on the biological properties of purified and recombinant human interleukin-1. Methods Find. Exp. Clin. Pharmacol., 8, 57–61 (1986).

11) Dempsey, R. A., Dinarello, C. A., Mair, J. W., Rosenwasser, L. J., Allegretta, M., Brown, T. E. and Darkinson, D. R. The differential effects of human leukocytic pyrogen/lymphocyte-activating factor, T cell growth factor, and interferons on human natural killer activity. J. Immunol., 129, 2504–2510 (1982).

12) Yamashita, U. and Shirakawa, F. Restoration of impaired T cell functions in tumor-bearing mice by the administration of interleukin 1. Jpn. J. Cancer Res., 78, 270–278 (1987).

13) Farrar, W. L., Mizel, S. B. and Farrar, J. J. Participation of lymphocyte activating factor (interleukin 1) in the induction of cytotoxic T cell responses. J. Immunol., 124, 1371–1377 (1980).

14) Smith, K. A., Lachman, L. B., Oppenheim, J. J. and Favata, M. F. The functional relationship of the interleukins. J. Exp. Med., 151, 1551–1556 (1980).

15) Kasahara, T., Mukaida, N., Hatake, K., Motoyoshi, K., Kawai, T. and Nakano, K. Interleukin 1 (IL1)-dependent lymphokine production by human leukemic T cell line HSB.2 subclones. J. Immunol., 134, 1682–1689 (1985).

16) Philip, R. and Epstein, L. B. Tumour necrosis factor as

immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin 1. *Nature,* **323**, 86–89 (1986).

17) Onozaki, K., Matsushima, K., Aggarwal, B. B. and Oppenheim, J. J. Human interleukin 1 is a cytocidal factor for several tumor cell lines. *J. Immunol.,* **135**, 3962–3968 (1985).

18) Lachman, L. B., Dinarello, C. A., Llansa, N. D. and Fidler, I. J. Natural and recombinant human interleukin-1β is cytotoxic for human melanoma cells. *J. Immunol.,* **136**, 3098–3102 (1986).

19) Lovett, D., Kozan, B., Hadam, M., Resch, K. and Gemsa, D. Macrophage cytotoxicity: interleukin 1 as a mediator of tumor cytostasis. *J. Immunol.,* **136**, 340–347 (1986).

20) Gaffney, E. V. and Tsui, S. C. Lymphocyte-activating and growth-inhibitory activities for several sources of native and recombinant interleukin 1. *Cancer Res.,* **46**, 3834–3837 (1986).

21) Krakauer, T. Human interleukin 1. *CRC Crit. Rev. Immunol.,* **6**, 213–244 (1986).

22) Pyataki, P. S., Abuchowski, A. and Davis, F. F. Preparation of a polyethylene glycol: superoxide dismutase adduct, and examination of its blood circulation life and anti-inflammatory activity. *Res. Commun. Chem. Pathol. Pharmacol.,* **29**, 113–127 (1980).

23) Wileman, T., Foster, R. L. and Elliot, P. N. C. Soluble asparaginase-dextran conjugates show increased circulatory resistance and lowered antigen reactivity. *J. Pharm. Pharmacol.,* **38**, 264–271 (1986).

24) Hershfield, M. S., Buckley, R. H., Greenberg, M. L., Melton, A. L., Schiff, R., Hatem, C., Kurtzberg, J., Markert, M. L., Kobayashi, A. L. and Abuchowski, A. Treatment of adenosine deaminase deficiency with poly-ethylene glycol-modified adenosine deaminase. *N. Eng. J. Med.,* **316**, 589–594 (1987).

25) Katre, N. V., Knauf, M. J. and Laird, W. J. Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. USA,* **84**, 1487–1492 (1987).

26) Morikawa, K., Okada, F., Hosokawa, M. and Kobayashi, H. Enhancement of therapeutic effects of recombinant interleukin 2 on a transplantable rat fibrosarcoma by the use of a sustained release vehicle, pluronic gel. *Cancer Res.,* **47**, 37–41 (1987).

27) Tabata, Y. and Ikada, Y. Macrophage activation of antitumor function by muramyl dipeptide-protein conjugates. *J. Pharm. Pharmacol.,* **42**, 13–19 (1990).

28) Tabata, Y. and Ikada, Y. Augmentation of antitumor activity of macrophage through chemical modification with gelatin. *In* "Polymers and Biomaterials, Vol. 3," ed. T.-F. Xi and H. Li, pp. 317–325 (1991). Elsevier, Amsterdam.

29) Tabata, Y., Uno, K., Yamaoka, T., Ikada, Y. and Muramatsu, S. Effects of recombinant α-interferon-gelatin conjugate on *in vivo* murine tumor cell growth. *Cancer Res.,* **51**, 5532–5538 (1991).

30) Nakano, K., Okugawa, K., Hayashi, H., Abe, S., Sohmura, Y. and Tsuboi, T. Establishment of dye-uptake method (A375 assay) for quantitative measurement of IL1: correlation with LAF assay. *Dev. Biol. Stand.,* **69**, 93–119 (1988).

31) Tabata, Y., Uno, K., Ikada, Y. and Muramatsu, S. Suppressive effect of recombinant TNF-gelatin conjugate on tumour growth *in vivo. J. Pharm. Pharmacol.,* **45**, 303–308 (1993).

32) Greenwood, F. C. and Hunter, W. M. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.,* **89**, 114–123 (1963).

33) Uno, K., Shimizu, S., Ido, M., Naito, K., Inaba, K., Oku, T., Kishida, T. and Muramatsu, S. Direct and indirect effects of interferon on *in vivo* murine tumor cell growth. *Cancer Res.,* **45**, 1320–1327 (1985).

34) Uno, K., Shimizu, S., Inaba, K., Kitaura, M., Nakahira, K., Kato, T., Yamaguchi, Y. and Muramatsu, S. Effect of recombinant interferon-αA/D on *in vivo* murine tumor cell growth. *Cancer Res.,* **48**, 2366–2371 (1988).

35) Nakano, K., Abe, S. and Sohmura, Y. Recombinant human tumor necrosis factor-I. Cytotoxic activity *in vitro. Int. J. Immunopharmacol.,* **8**, 347–355 (1986).