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Acta Medica Okayama

Volume 47, *Issue* 2 1993 *Article* 2

APRIL 1993

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Abstract

A sustained release system for interleukin-2 (IL-2), and IL-2 mini-pellet (IL-2 mp), was developed by fusing IL-2 into a needle shaped collagen. Serum concentration of IL-2 after a single subcutaneous injection of the IL-2 mp into C57BL/6 mice remained elevated longer than after an injection of aqueous IL-2. IL-2 in the serum became undetectable by 6h after a subcutaneous injection of 1 x 10(6) unit of IL-2 in phosphate-buffered saline (PBS). In contrast, after a single subcutaneous injection of IL-2 mp containing the same amount of IL-2, the concentration of IL-2 increased to its maximum at 6h after injection, then began to decrease gradually. IL-2 was detected even on the third day after a single subcutaneous injection of one IL-2 mp. Augmentation of NK activity and generation of IL-2 activated killer cells were observed in the spleen from day 1–day 3 after a single subcutaneous injection of IL-2 mp into C57BL/6 mice. This activation was not observed following a single subcutaneous injection of the same amount of IL-2 in PBS. Adoptive immunotherapy by a single subcutaneous injection of IL-2 mp followed by intravenous injections of in vitro cultured IL-2 activated killer cells showed better results in decreasing the number of metastases of Lewis lung carcinoma in C57BL/6 mice than immunotherapy using IL-2 solution.(ABSTRACT TRUNCATED AT 250 WORDS)

KEYWORDS: IL-2, drug delivery system, immunotherapy, mouse

*PMID: 8506753 [PubMed - indexed for MEDLINE]
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Acta Med Okayama 47 (2) 79-84 (1993)

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A sustained release system for interleukin-2 (IL-2), and IL-2 mini-pellet (IL-2 mp), was developed by fusing IL-2 into a needle shaped collagen. Serum concentration of IL-2 after a single subcutaneous injection of the IL-2 mp into C57BL/6 mice remained elevated longer than after an injection of aqueous IL-2. IL-2 in the serum became undetectable by 6 h after a subcutaneous injection of 1×10^6 unit of IL-2 in phosphate-buffered saline (PBS). In contrast, after a single subcutaneous injection of IL-2 mp containing the same amount of IL-2, the concentration of IL-2 increased to its maximum at 6 h after injection, then began to decrease gradually. IL-2 was detected even on the third day after a single subcutaneous injection of one IL-2 mp. Augmentation of NK activity and generation of IL-2 activated killer cells were observed in the spleen from day 1 - day 3 after a single subcutaneous injection of IL-2 mp into C57BL/6 mice. This activation was not observed following a single subcutaneous injection of the same amount of IL-2 in PBS. Adoptive immunotherapy by a single subcutaneous injection of IL-2 mp followed by intravenous injections of *in vitro* cultured IL-2 activated killer cells showed better results in decreasing the number of metastases of Lewis lung carcinoma in C57BL/6 mice than immunotherapy using IL-2 solution. This slow delivery system of IL-2 appears promising in the application of adoptive immunotherapy now under investigation in human subjects because it augments biological effects by prolonging the duration of interaction of IL-2 and its receptive cells and decreases side effects by lowering maximal concentrations.

Key words : **IL-2, drug delivery system, immunotherapy, mouse**

Interleukin-2 (IL-2), originally described as a T cell growth factor, is a glycosylated lymphokine secreted from antigen or lectin stimulated T cells (1). IL-2 has therapeutic potential in treating cancers and infectious diseases because it promotes lymphocyte proliferation, and generates so-called lymphokine activated killer (LAK) cells *in vitro* and *in vivo* (2). Since the cloning of cDNA coding for IL-2, recombinant IL-2 from *Escherichia coli* has become available in large quantities and has been applied in clinical trials (3). LAK cells rapidly lose their viability in the absence of IL-2, and continuons presence of IL-2 is required for the maintenance of cytolytic activity and viability of LAK cells in adoptive immunotherapy (4). Unfortunately, IL-2 is rapidly cleared from the circulation, resulting in limitied bioavailability of IL-2 (5, 6). Moreover, serious side effects make it difficult to administer high doses of IL-2 to maintain sufficient IL-2 in the circulation (3). The development of methods, that would permit the maintenance of constant serum levels of IL-2, would provide an acceptable and presumably preferable alternative to conventional intravenous administration of high-dose IL-2.

Biological activity of IL-2 is limited by IL-2 receptor concentration, duration of exposure to IL-2, and IL-2 concentration (4). The slow release system of IL-2 serves to prolong the duration of exposure and is expected to

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Produced by The Berkeley Electronic Press, 1993

exhibit augmented biological effects. We have developed a formulation of IL-2, the IL-2 mini-pellet (IL-2 mp), to increase its serum half-life via a slow release system (7). This has been accomplished by mixing IL-2 with highly purified bovine collagen with little antigenicity and forming it into a hard needle-shaped pellet.

In this study, we examined the pharmacokinetics of IL-2 mp and compared its biological activity to aqueous IL-2 in mice. We further investigated the biological effect of IL-2 mp accompanied by adoptive transfer of LAK to determine if it effectively decreased lung metastases of Lewis lung carcinoma in C57BL/6 mice.

Materials and Methods

Mice. Female C57BL/6 mice, aged 8–12 weeks, obtained from Shizuoka Agricultural Cooperative Farm for Experimental Animals, Hamamatsu, Japan, were used in the experiments. Caged in groups of 6 or less, the animals were kept in our experimental animal farm in compliance with animal care regulations.

Tumors. Lewis lung carcinoma cells (3LL) were maintained *in vivo* in syngeneic animals by serial intramuscular transplantation. Intramuscular tumors were excised and dissected free of necrotic tumor and connective tissue, minced, and subjected to treatment with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) at 37°C for 15 min. The dispersed tumor cells were washed twice with Hank's balanced salt solutions (HBSS) containing 10% fetal calf serum (FCS), filtered through a #150 mesh filter 3 times, and then adjusted to the desired cell concentration. JTC-11 cells, resistant to natural killer cells (NK), originated from murine Ehrlich's tumor (8) and have been maintained in our laboratory in complete medium (CM) of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 5 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO), 10 mg/ml of gentamicin and 5% FCS. NK-sensitive YAC-1 lymphoma cells were maintained in tissue culture in the same medium (CM) as described above.

IL-2 and its slow release formulation (IL-2 mp). The human recombinant IL-2 used for the present experiment was supplied by Ajinomoto Company, Ltd, (Tokyo, Japan). The biological activity of IL-2 is expressed in units, as defined previously (9). Sustained release formulation of IL-2 (IL-2 mp) was produced by Sumitomo Pharmaceutical Company, Ltd., (Osaka, Japan), in the manner previously reported (7). Briefly, 2×10^8 units (U) of IL-2 was homogenously mixed with 25 g of 2% water solution of highly purified bovine dermal collagen (Koken Company, Ltd., Tokyo, Japan); the mixture was dried , minced, molded to cylindrical shape, and then cut into mini-pellets as reported previously (10). Each mini-pellet was made to contain 1×10^6 U of IL-2.

Pharmacokinetics of IL-2 mp and aqueous IL-2 in mice. In pharmacokinetic studies, one group of the C57BL/6 mice was injected subcutaneously in the left side of the back, with one IL-2 mp via a 16-G elastic needle with a plunger. The same amount of IL-2 dissolved in phoshate-buffered saline (PBS) was also administered intravenously or subcutaneously to another group. The blood of 3 to 5 mice, anesthetized with ether, was collected in series by cardiac puncture. After clot formation, the samples were centrifuged at 3,000 rpm for 5 min, and the sera were harvested and stored at −20°C until IL-2 assay. IL-2 activity was determined by using an IL-2 dependent cell line, CTLL-2, as described above. The results were expressed as the mean concentration of 3–5 mice.

Spleen cell suspension. Spleens, which were removed from mice given different forms of IL-2, were dissociated mechanically by using the blunt end of a plastic syringe plunger in HBSS. The dissociated cells were filtered twice through a #150 mesh. Erythrocytes were lysed with hypotonic buffer. The cells were washed and resuspended in CM at a desired concentration.

For the preparation of LAK cells, 1×10^6 spleen cells/ml were incubated for 3 days in CM supplemented with 100 U/ml of IL-2 in T75 flasks (Falcon, Cockeysville, MD). The harvested LAK cells were washed in HBSS with 5% FCS and suspended in PBS. The viable cells were counted by the trypan blue dye exclusion method and adjusted to a desired concentration.

Cytotoxicity assays. A 4 h chromium release assay was performed to determine *in vitro* cytotoxicity. Target cells were labeled in 1.0 ml of CM containing 500 μCi of $Na^{51}CrO_4$ for one hour at 37°C. The assays were performed in triplicates of 96 wells (Falcon, Cockeysville, MD). Each well contained 1×10^4 labeled target cells and various numbers of effector cells in a volume of 0.2 ml of CM. After 4 h of incubation at 37°C in 5% CO_2, supernatants were harvested. The killing percentage was calculated as follows: (experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm) × 100.

In vivo induction of cytotoxic cells in spleens from mice treated with IL-2 mp. Mice were subcutaneously injected once with either one IL-2 mp or its equivalent amount of IL-2 (1×10^6 U) in PBS. Spleen cells were prepared as described above, 0 to 7 days after treatment. Both NK activity and LAK activity were evaluated simultaneously using YAC-1 and NK resistant JTC-11 cells as targets, respectively. The results were expressed as means of triplicate results.

Adoptive immunotherapy method. Female C57BL/6 mice were injected with 1×10^6 3LL cells in the left hind food pad. The primary tumor was surgically resected 10 days after the inoculation. Animals were devided into 5 experimental groups according to the treatment they received; a) no treatment, b) 1×10^8 of LAK, c) IL-2 mp, d) 1×10^6 U of aqueous IL-2 and 1×10^8 of LAK, e) IL-2 mp and 1×10^8 of LAK. LAK cells were administered via the tail vein on the 11th and 14th day after tumor inoculation. An IL-2 mp or 1×10^6 U of IL-2 in PBS was injected subcutaneously once on day 11. After the animals were killed on the 21st day, lungs were insufflated with Indian ink, removed, and bleached. The number of metastases was computed as the number of white nodules counted. Each group consisted of 6 mice.

Statistical analysis. The significance of the differences was assesssed with Student's *t* test with Welch approximation.

Results

Pharmacokinetics of IL-2 mp in mice. IL-2 mp released biological active IL-2 into serum with a longer half-life time (T 1/2) and a lower maximal serum concentration than the same amount of aqueous IL-2 (Fig. 1). Serial evaluation of serum concentration of IL-2 revealed that IL-2 concentration increased at a constant rate until 6h after injection, when the serum level was about 1×10^4 U/ml. The IL-2 level then decreased gradually until the third day. On the third day after single subcutaneous injection of IL-2 mp, 7.4 ± 4.2 U/ml of IL-2 was detected in the serum. Five days after injection, IL-2 was no longer detectable in the serum. T 1/2 was 6h for IL-2 mp. The IL-2 levels after subcutaneous injection of 1×10^6 U of IL-2 in PBS were also evaluated. The concentration peaked immediately after injection and rapidly decreased; serum IL-2 could not be detected after 3h. For aqueous IL-2 given subcutaneously, T 1/2 was 15 min. The serum half life of IL-2 from IL-2 mp was prolonged by 24 fold. When the same amount of IL-2 in PBS was administered intravenously, IL-2 in the serum disappeared more rapidly, and T 1/2 was 8min (data not shown).

NK activity and IL-2-activated killer cell activity in the spleen after injection of IL-2 mp. NK activity in the spleen of IL-2 mp treated animals showed an increase 24h after injection, and this NK activity continued to increase gradually until the third day after treatmeant (Table 1). NK activity on the day 3 was as high as 38.9 %. NK activity in the spleens of mice treated with soluble IL-2 showed a slight increase at 24h post-injection, but remained at a low level during the observed period. IL-2-activated killer cell activity was induced by 24h, increased on the day 2 and remained on a plateau until the day 3 after a single IL-2 mp treatment (Table 2). Spleen cells from mice treated with aqueous IL-2 showed no IL-2 activated killer cell activity. This augmentation of NK activity and IL-2-activated killer cell activity was not observed on day 5 or 7 after IL-2 mp treatment.

Adoptive immunotherapy by combination of IL-2 mp and IL-2-activated killer cells. To confirm the beneficial effects of sustained release of IL-2 by the mp, adoptive immunotherapy using injections of IL-2 mp or IL-2 in PBS followed by intravenous injection of *in vitro* generated IL-2-activated killer cells was performed (Fig. 2). The number of 3LL metastatic foci in the lungs of C57BL/6 mice was effectively reduced by treatment with a combination of IL-2 mp and IL-2-activated killer cells. Treatment of lung metastases by either LAK or IL-2 mp

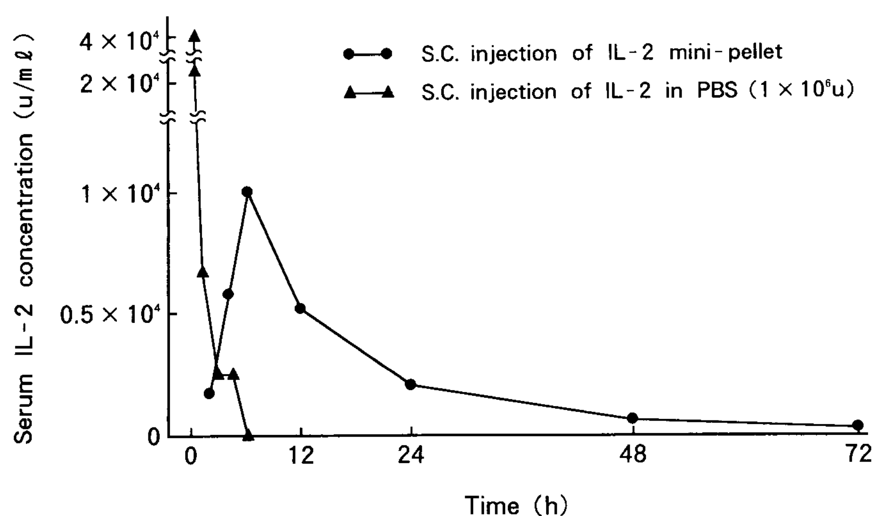

Fig. 1 Pharmacokinetics of interleukin-2 (IL-2) after single subcutaneous injection of IL-2 mini-pellet (IL-2 mp) or IL-2 in PBS. IL-2 mp containing 1×10^6 u of IL-2 or the same amout of IL-2 dissolved in PBS was subcutaneously injected into C57BL/6 mice. Serum samples from three to five mice at each time point were analyzed for IL-2 concentration using CTLL-2 cells. Results were presented by means of each sample and S.D. was less than 20 % of value.

Table 1 Natural Killer (NK) activity of splenocytes treated with interleukin-2 mini-pellet (IL-2 mp) or IL-2 in PBS

Effector splenocytes	Ratio Effector/Target cells	Percent cytotoxicity					
		No injection	Days after injection				
			1	2	3	5	7
IL-2 mini-pellet	50		23.1	38.5	38.9	10.1	9.7
	25		19.8	33.7	32.7	6.7	5.6
IL-2 in PBS	50		12.5	11.8	8.0	9.0	7.2
	25		10.6	8.6	7.3	4.6	5.7
No treatment	50	8.3					
	25	6.7					

Table 2 Lymphokine activated killer (LAK) activity of splenocytes treated with interleukin-2 mini-pellet (IL-2 mp) or IL-2 in PBS

Effector splenocytes	Ratio Effector/Target cells	Percent cytotoxicity					
		No injection	Days after injection				
			1	2	3	5	7
IL-2 mini-pellet	50		16.0	29.0	28.0	8.2	5.2
	25		10.5	25.5	24.3	7.3	4.8
IL-2 in PBS	50		4.5	3.5	3.4	1.3	2.3
	25		3.8	2.7	3.3	1.2	2.2
No treatment	50	3.9					
	25	2.7					

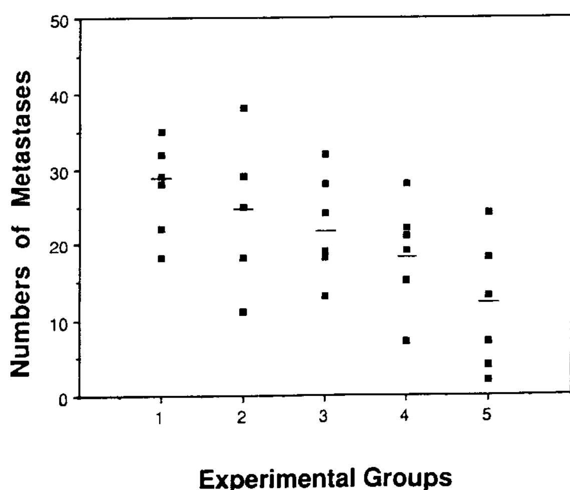

Fig. 2 Immunotherapy of lung metastases using interleukin-2 (IL-2) and lymphocyte activated killer (LAK). Lewis lung carcinoma in C57BL/6 mice was treated with a) no treatment, b) 1×10^8 of LAK, c) IL-2 mp, d) 1×10^6 U of aqueous IL-2 and 1×10^8 of LAK, or e) IL-2 mp and 1×10^8 of LAK, under the experimental protocol described in Materials and Methods section.

alone showed no significant effect on the inhibition of the number of metastatic foci in this model. When LAK cells were injected, accompanied with a single injection of either aqueous IL-2 or IL-2 mp, the number of the metastatic foci were significantly reduced ($p < 0.05$ and $p < 0.01$, respectively). The combination of LAK and IL-2 mp was superior to that of LAK and aqueous IL-2 ($p < 0.05$). None of the animals treated with IL-2 mp showed any apparent side effects, such as weight loss or gain.

Discussion

The newly developed slow delivery system of IL-2 (IL-2 mp) was able to sustain a serum concentration of IL-2 in mice. Single injection of IL-2 mp induced activated killer cells effectively *in vivo*. Immunotherapy employing IL-2 mp in combination with LAK cells showed a better result than therapy using the same amount of aqueous IL-2 and LAK cells.

Since the report of successful treatment of malignancies in mice (2, 6), immunotherapy by IL-2 alone or IL-2 in concert with LAK cells has been extensively studied (3). IL-2 appears to mediate its antitumor effect through the activation of endogenous and/or exogenous LAK cells *in vivo* because prior irradiation of the host abrogates the antitumor effect of IL-2 (11). This observation indicates that one key to successful cancer immunotherapy using IL-2 is dependent upon the effective induction and expansion of effector cells *in vivo*.

The activation of lymphocytes is limited by the number of IL-2 receptors, the concentration of available IL-2, and the duration of IL-2 and IL-2 receptor interaction (4). IL-2, when administered *in vivo*, is rapidly cleared from the systemic circulation (5); both the duration and amount of available IL-2 are very limited due to this reason. High doses and frequent administrations of IL-2 are necessary to compensate which can result in serious side effects (3).

It has been shown that a low but sustained concentration of IL-2 by frequent intraperitoneal injections was superior to a high brief peak level of IL-2 in the expansion of endogenous lymphoid and transferred LAK cells (12, 13). These proliferative responses directly paralleled the therapeutic effect of LAK cells and IL-2.

Instead of frequent intraperitoneal or intravenous administration of IL-2 to maintain a sustained level, we injected IL-2 mp which was designed to release IL-2 in a slow delivery fashion. Single IL-2 mp injection released biological active IL-2 in a graduated fashion for as long as 3 days in the systemic circulation. Previous studies show that at least several units of IL-2 are required to exhibit its biological effects (12). At the end of day 3 after a single IL-2 mp injection, this level was well maintained. The highest concentration of IL-2 was much lower in the case of IL-2 mp than in that of the same amount of aqueous IL-2. This graduated kinetics of IL-2 may be preferable in addressing the dilemma of high dosage side effects.

As was expected from the kinetic study (Fig. 1), *in vivo* induction of LAK cells and activation of NK cells were observed in the spleen from the mice treated with a single injection of IL-2 mp. This was not observed in the animals treated with the same amount of aqueous IL-2. It was reported that intraperitoneal administration of high dose IL-2 every 8 h maintained the IL-2 concentration and induced LAK cells in the peripheral blood mononuclear cell (12).

Five days after the administration of a single IL-2 mp, the concentration of IL-2 sufficient to maintain LAK cells was no loger detected. This means that either the IL-2 mp should be injected every 3rd day in this animal model, or the content of IL-2 in a mini-pellet should be increased, which is not difficult to achieve. The data obtained here indicates that IL-2 mp is a more favorable alternative to induce *in vivo* induction of LAK cells than the conventional administration of IL-2.

Several attemps have been made to increase bioavailability of IL-2 in serum. Gelatin and IL-2 mixtures prolong half-life, but with significant side effects (5). PEG covalently bound to IL-2 showed significant prolongation of clearance time and favorable results in immunotherapy (14). Both of these experiments were performed by intravenous administration of IL-2. In contrast, IL-2 mp was given by subcutaneous injection, and the maintenance of serum level of IL-2 was achieved by gradual release of IL-2 from mp rather than by prevention of rapid secretion by the kidneys. This approach to the administration of IL-2 mp should be suitable for intratumor injection or embolization of feeding arteries, and other alternatives, such as PEG, may be useful in the treatment of hematological malignancies or in LAK therapy.

The effective access of adopted LAK cells is also important factor for a successful cancer treatment. Current adoptive immunotherapy requires the maintenance of specified serum levels of IL-2 to enable LAK cells to survive long enough to reach the tumors. Site directed administration of IL-2 has an advantage in LAK therapy because it can maintain enough IL-2 for a longer period in the circulation, and at the same time it can also maintain the local level of IL-2 after LAK cells reach the tumor site. Site directed maintenance of IL-2 also enables LAK cells to exhibit their lytic activity for a longer time. We also observed that site directed administration of IL-2 mp can activate lymphocytes adjacent to IL-2 mp and that perforin-positive lymphocytes were induced by day 7 after subcutaneous injection of IL-2 mp (15). This suggests that not only exogenously generated LAK cells, but also endogenously generated LAK cells or tumor infiltrating lymphocytes can participate in the eradication of tumor cells.

IL-2 mp, based on its performance in this mouse model, may have significant potential in cancer.

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Received September 2, 1992; accepted November 11. 1992.