In Vivo Activity of Solid Phase Interleukin 2

Edward D. Crum and David R. Kaplan

ABSTRACT

Interleukin 2 (IL-2) coupled to polystyrene beads to form a solid phase of the cytokine was able to increase the cytotoxic activity of rat spleen cells in vitro. A single injection of the IL-2 coupled beads into the peritoneal cavities of normal rats also resulted in the in vivo activation of cytotoxicity of peritoneal exudate cells, whereas a single i.p. injection of the same amount of soluble IL-2 was not effective. When IL-2 coupled beads were mixed with methylcholanthrene-induced Mc7 or Mc107 sarcoma cells and injected into normal syngeneic Wistar rats, the growth of the tumor was suppressed. This effect was localized to the site of the injection. The in vivo activities were achieved with modest amounts of IL-2, less than has been previously reported to be well tolerated in rodents. IL-2 coupled to a solid matrix may be useful for delivering increased concentrations of the lymphokine to tumor dominant regions while maintaining low systemic levels and thereby increasing the therapeutic index.

INTRODUCTION

Interleukin 2 is a potent immunoregulatory lymphokine produced by T-lymphocytes (1). Among its major activities are enhancement of cytotoxicity of T-lymphocytes (1-3) and natural killer cells (1, 4-6) and induction of other potentially cytotoxic cytokines (1, 7, 8). Administration of IL-2 alone or in combination with adoptively transferred lymphoid cells has resulted in regression of established tumors in both animal models (9, 10) and human patients (11-14). However IL-2 has been associated with a variety of systemic and organ specific toxicities with a leaky capillary syndrome as an apparent common underlying mechanism (11-15). Although the pathophysiology of the capillary leakiness is not clear, induction of this state is dose and time dependent with IL-2 appearing to be a mediator rather than a direct toxin to capillary endothelium (16). High systemic levels of IL-2 may be necessary to achieve concentrations at the site of in vivo tumor growth that are adequate to induce local antitumor activity. A strategy of focusing the lymphokine at such a site without high systemic levels might enhance its therapeutic index.

Recently, it has been found that IL-2 can be coupled to solid matrices to form a solid phase of the lymphokine, and under prior in vitro conditions, IL-2 molecules are released from such preparations (4). Here, we report that IL-2 coupled to polystyrene beads has local biological activity in vivo. The immobilization of IL-2 on particulate carriers could be a useful approach for local or regional treatment of tumors while minimizing systemic toxicity.

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3 The abbreviations used are: IL-2, interleukin 2; HBSS, Hanks' balanced salt solution; PBS, fetal bovine serum; BSA, bovine serum albumin.


MATERIALS AND METHODS

Animals and Tumors. Wistar rats were purchased from the Trudeau Institute, Inc., Saranac Lake, NY. The syngeneic methylcholanthrene-induced sarcomas Mc7 (17) and Mc107 (18) were obtained from Professor R. W. Baldwin, University of Nottingham, England. Tumor tissue was stored at -80°C and passed in normal female rats by s.c. implantation. Suspensions of tumor cells for challenges were prepared by mincing and trypsinizing tumors removed after 10 to 18 days of in vivo growth (19).

Preparation of Solid Phase Interleukin 2. IL-2 was conjugated to polystyrene beads to form a solid phase of the lymphokine (2). Recombinant IL-2 (donated by Cetus, Emeryville, CA), with specific activity of 3 x 10^8 Cetus units or 18 x 10^6 IU/mg was used. Ten-μm polystyrene beads (Polysciences, Warrington, PA), were activated by treatment with carbodiimide following the manufacturer's recommendations. IL-2 (250 μg), or BSA, or 0.1 M ethanolamine in 0.1 M borate buffer, pH 8.5, were added to the activated beads and allowed to sit overnight at room temperature. The capacity of activated beads was in excess of the amount of protein present and therefore all IL-2 exposed to the beads was bound as detected by absence of IL-2 activity in the postcoupling supernatant by biological assay. After this conjugation step, the beads were blocked by exposure to 0.1 M ethanolamine for 30 min. Prior to use, beads were washed in 50-100 volumes of HBSS, and resuspended in fresh medium.

IL-2 Biological Assay. IL-2 was measured by a biological assay as previously described (20). The indicator cells were human T-cell clones 5 or 6 days after antigenic stimulation. Cyclosporin A (500 ng/ml) was included in the assay to prevent IL-2 secretion by the indicator cells. The sensitivity for the IL-2 bioassay was approximately 0.5 pm. This assay gave parallel straight lines in probit analysis for recombinant IL-2, and the results mirrored an enzyme linked immunosorbent assay for IL-2. The assay was calibrated with recombinant IL-2.

In Vivo Activation of Spleen Cells by IL-2 Coupled Beads. Spleens removed aseptically from normal rats were minced and passed through fine mesh and gauze. The erythrocytes were osmotically lysed with Tris buffered ammonium chloride. The lymphoid cells were washed with HBSS with 1% FBS, resuspended at 2 x 10^6/ml in RPMI 1640 medium, and placed in culture at 37°C in a humidified 5% CO2 atmosphere for 5 days with IL-2 coupled beads, control beads, or soluble IL-2. The cultured cells were harvested by scraping and washing the wells, washed with HBSS/1% FBS and resuspended in complete culture medium for cytotoxicity assay.

Collection of Peritoneal Cells. The peritoneal cavities of rats were lavaged with 15-20 ml ice-cold HBSS containing 1 unit heparin/ml. Recovered cells were washed twice with HBSS/1% FBS and then resuspended in complete culture medium at appropriate concentrations for cytotoxicity assay.

Measurement of in Vivo Tumor Growth. Single cell suspensions of tumor at concentrations of 3 x 10^6 to 5 x 10^6 were mixed with equal volumes of suspensions of IL-2 coupled beads, control beads, soluble IL-2, or medium immediately prior to injection. One-tenth ml of the mixtures was injected into the calf muscle of the hind legs of normal rats. Serial caliper measurements were made of the anterior-posterior and medial-lateral diameters of the legs at the injection sites. The increase in the average leg diameter over base line values provided an objective measurement of tumor growth in each rat (19).
Cytotoxicity Assay. YAC-1 mouse lymphoma cells were labeled with Cr by incubating 3 x 10^6 cells suspended in 1 ml complete culture medium containing 100 µCi Na^24CrO_4 (200–900 Ci/g; ICN Radiochemicals, Irvine, CA) at 37°C for 90–120 min. The cells were washed 3 times and diluted with complete culture medium to a concentration of 5 x 10^5/ml. One hundred µl of lymphoid cells at appropriate concentrations in complete culture medium to give effector:target ratios of 100:1 to 12.5:1, or medium only to determine spontaneous Cr release, or 1% sodium dodecyl sulfate to determine total Cr release, were dispensed into the appropriate wells of 96 round-bottomed well tissue culture plates (Corning, Corning, NY). One hundred µl containing 5 x 10^5 labeled YAC-1 target cells were then added to all wells. Plates were incubated for 4 h at 37°C in humidified 5% CO_2 atmosphere. Supernatants were harvested from each well at the end of incubation by using a Skatron supernatant harvesting system (Skatron, Inc., Sterling, VA) and counted in a gamma counter. All lymphoid cells were tested in triplicate. Spontaneous and total releases were each obtained by using the average values from 12 replicate wells. Cytotoxic activity was quantitated as follows:

\[
\text{% of specific release of } Cr = \frac{\text{Experimental release cpm} - \text{spontaneous release cpm}}{\text{Total release cpm} - \text{spontaneous release cpm}} \times 100
\]

The percentage of specific release for each effector population was converted to lytic units, defined as the number of effectors per 10^6 total cells that cause 20% lysis of 5 x 10^5 targets.

Statistical Analysis. Tumor sizes in different groups were compared by the nonparametric sum of ranks test. Values of cytotoxicity of test and control effector cells were compared by unpaired t tests.

RESULTS

In Vitro Activation of Rat Spleen Cells. Recombinant human IL-2 coupled to Sepharose or polystyrene beads to form a solid phase releases soluble, biologically active IL-2 under proper conditions in vitro. Prior to in vivo testing of IL-2 coupled to polystyrene beads in rats, it was necessary to determine whether this preparation was able to activate rat lymphoid cells in vitro. Spleen cells from normal rats were cultured with varying concentrations of IL-2 coupled beads, control beads coupled with BSA or ethanolamine, or soluble IL-2. The cultured spleen cells were then assayed for cytotoxicity against YAC-1 cell targets by chromium release. Table 1 shows a representative experiment in which spleen cells cultured for 5 days with IL-2 coupled to polystyrene beads demonstrated an increase in cytotoxic activity that was equal to that induced with soluble IL-2. Control beads coupled with BSA as shown in Table 1 or with ethanolamine (data not shown) did not induce increased cytotoxicity. The coculture of IL-2 coupled beads and spleen cells did not result in the presence of soluble cytotoxic products in the culture medium as assessed by cytotoxicity against YAC-1 cells (data not shown).

In Vivo Induction of Cytotoxic Activity within Peritoneal Cavity. To determine if IL-2 coupled to polystyrene beads could activate cytotoxic cells in vivo, we injected bead preparations or soluble IL-2 into the peritoneal cavities of normal rats. Four days later the peritoneal cavities were washed out and the recovered cells were assayed for cytotoxicity against YAC-1 targets. Table 2 shows the results of a representative experiment. Only cells obtained from rats receiving 150,000 IU IL-2 coupled to polystyrene beads had a level of cytotoxic activity greater than rats given injections of medium only. Rats receiving a single i.p. injection of 150,000 IU soluble IL-2 or polystyrene beads coupled with ethanolamine had no increase in the level of cytotoxicity of peritoneal cells above that demonstrated in medium injected into control rats.

Suppression of Growth of Tumor Cells Mixed with IL-2 Coupled Beads. To determine if IL-2 coupled beads could activate effector mechanisms able to suppress in vivo tumor growth, we mixed bead preparations or soluble IL-2 with single cell suspensions of Mc7 or Mc107 tumor immediately prior to injection into the hind legs of normal rats and measured tumor growth. Experiments shown in Fig. 1 demonstrate that the growth of tumor cells mixed with 42,000 IU IL-2 coupled to polystyrene beads was suppressed. The same number of control beads coupled with BSA or ethanolamine, soluble IL-2, or a combination of control beads and soluble IL-2 had no influence on the growth of admixed tumor cells.

Mc7 tumor cell suspensions alone or mixed with IL-2 coupled beads were examined for exclusion of trypan blue dye immediately after mixing, at 1 h after mixing, and after overnight incubation at 37°C. There were no differences in tumor cell viabilities between suspensions containing or not containing IL-2 beads (data not shown), indicating that the beads lacked immediate direct cytotoxic effects on the tumor cells.

Fig. 2 demonstrates the growth of Mc7 cells mixed with 10-fold dilutions of IL-2 coupled polystyrene beads. Tumor growth from mixtures containing 150,000 IU of IL-2 coupled beads was completely suppressed. Growth of mixtures containing 15,000 IU IL-2 coupled beads was partially suppressed and no suppression was present in mixtures containing 1,500 IU IL-2 coupled beads. These results indicate that the tumor growth suppression by IL-2 coupled beads was dose dependent.

Limitation of Tumor Suppressive Activity of IL-2 Coupled Beads to Local Injection Site. The possibility was examined that injection of IL-2 coupled beads mixed with tumor cells activated systemic cytotoxic mechanisms. In the experiment shown in Fig. 3, rats were given injections of Mc7 cells mixed with IL-2.

<table>
<thead>
<tr>
<th>Table 1 In vitro activation of cytotoxicity with IL-2 coupled beads</th>
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<tr>
<td><strong>Spleen cells cultured with</strong></td>
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<tr>
<td>12,000 IU/ml IL-2 beads</td>
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<tr>
<td>6,000 IU/ml IL-2 beads</td>
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<tr>
<td>3,000 IU/ml IL-2 beads</td>
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<tr>
<td>BSA beads</td>
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<tr>
<td>9,000 IU/ml soluble IL-2</td>
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<td>No IL-2</td>
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* Normal rat spleen cells at 2 x 10^6/ml in complete medium cultured for 5 days.
* Assayed against Cr labeled YAC-1 cells: 5 x 10^5 targets/well at effector:target cell ratio of 100:1, 50:1, 25:1, 12.5:1, and expressed in lytic units. Mean ± SEM of 4 separate cultures; each culture assayed in triplicate at each effector:target cell ratio.
* Concentration of IL-2 coupled to polystyrene beads (0.037 IU of IL-2/bead) in culture.
* % P < 0.025 compared to lytic activity of spleen cells cultured without IL-2.
* BSA coupled to polystyrene beads added to culture at a concentration of 1.62 x 10^5 beads/ml (same concentration of beads as in 6,000 IU IL-2 coupled bead culture).

<table>
<thead>
<tr>
<th>Table 2 In vivo activation of cytotoxicity with IL-2 coupled beads</th>
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<tr>
<td><strong>Injections (i.p.) into cell donors</strong></td>
</tr>
<tr>
<td>150,000 IU IL-2 beads</td>
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<tr>
<td>150,000 IU soluble IL-2</td>
</tr>
<tr>
<td>Ethanolamine beads</td>
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<td>Medium</td>
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* All i.p. injections in 3 ml of HBSS.
* Peritoneal cavities lavaged 4 days after i.p. injections. Cells assayed against YAC-1 targets. Cytotoxicity expressed in lytic units; mean ± SEM of 4 rats/group.
* IL-2 coupled to 4 x 10^6 polystyrene beads injected into each rat.
* IL-2 coupled to 4 x 10^6 polystyrene beads injected into each rat.
Fig. 1. Suppression of growth of tumor cells mixed with IL-2 coupled to polystyrene beads. Normal rats given injections i.m. of mixtures containing $2 \times 10^7$ tumor cells. A, Mc7 cells plus 42,000 IU IL-2 coupled polystyrene beads (Δ); Mc7 cells plus BSA coupled beads (○); Mc7 cells only (●). B, Mc107 cells plus 42,000 IU IL-2 coupled polystyrene beads (Δ); Mc107 cells plus ethanolamine coupled beads (○); Mc107 cells only (●). C, Mc7 cells plus 42,000 IU IL-2 coupled polystyrene beads (Δ); Mc7 cells plus 42,000 IU soluble IL-2 (○); Mc7 cells only (●). D, Mc7 cells plus 42,000 IU IL-2 coupled polystyrene beads (Δ); Mc7 cells plus BSA coupled beads plus 42,000 IU soluble IL-2 (○); Mc7 cells only (●). Five rats/group; points, median tumor diameters; bars, tumor diameter ranges in mm in each group. In all 4 experiments shown, sizes of tumors from mixtures containing IL-2 coupled beads were smaller than other 2 groups ($P < 0.01$) at 2, 3, and 4 weeks after injection.

Fig. 3, rats were given injections of Mc7 cells mixed with IL-2 coupled beads in one leg and the identical number of Mc7 cells only into the opposite leg. A control group of rats was given injections of the same number of Mc7 cells only and tumor growth was measured and compared. Only tumor cells mixed directly with IL-2 coupled beads were suppressed in the rats given injections of that preparation. Tumor growth in the opposite leg of these animals and in the control group was the same. Since growth of the tumor inoculum not including beads would have been retarded if injection of IL-2 coupled beads had systemically activated antitumor mechanisms, the measurable tumor suppressive effect was limited to the site of injection.

DISCUSSION

In this study, IL-2 coupled to polystyrene beads to form solid phase lymphokine, demonstrated both in vitro and in vivo biological activity in rats. Previous studies of IL-2 coupled to Sepharose or polystyrene beads have shown that in the presence of a variety of proteins, up to 80% of the bound IL-2 is released.
ACTIVITY OF IL-2 COUPLED BEADS

Fig. 2. Dose dependency of tumor suppression by IL-2 coupled to polystyrene beads. Normal rats given i.m. of 2 x 10^7 MC7 cells mixed with polystyrene beads coupled to 150,000 IU IL-2 (A); 15,000 IU IL-2 (O); 1,500 IU IL-2 (D); or 2 x 10^7 MC7 cells only (B). Five rats/group; points, median tumor diameters; bars, tumor diameter ranges in mm in each group. Sizes of tumors from mixtures containing 150,000 IU IL-2 coupled beads were smaller than MC7 only (P < 0.01) at 2, 3, and 4 weeks after injection. Sizes of tumors from mixtures containing IL-2 coupled to polystyrene beads (A). The same number of MC7 cells only injected i.m. into the opposite leg of the same rats (C) (group 1) and i.m. into 5 different rats (A) (group 2). Points, median tumor diameters; bars, tumor diameter ranges in mm. Growth of tumor in the leg opposite from that given injections of IL-2 coupled beads plus MC7 cells (group 1 rats) was the same as that of growth in control, group 2 rats. Sizes of tumors from mixtures containing IL-2 coupled beads were smaller than the MC7 only groups (P < 0.01) at 2, 3, and 4 weeks after injection.

Fig. 3. Local tumor suppressive activity of IL-2 coupled to polystyrene beads. Five normal rats given injections i.m. of 2 x 10^7 MC7 cells mixed with 27,000 IU IL-2 coupled to polystyrene beads (A). The same number of MC7 cells only injected i.m. into the opposite leg of the same rats (C) (group 1) and i.m. into 5 different rats (A) (group 2). Points, median tumor diameters; bars, tumor diameter ranges in mm. Growth of tumor in the leg opposite from that given injections of IL-2 coupled beads plus MC7 cells (group 1 rats) was the same as that of growth in control, group 2 rats. Sizes of tumors from mixtures containing IL-2 coupled beads were smaller than the MC7 only groups (P < 0.01) at 2, 3, and 4 weeks after injection.

over a period of days. This phenomenon has been interpreted to result from a gradual dissolution of aggregated IL-2 bound to the bead matrix. Thus, it seems likely that the activation of rat spleen cells cultured with IL-2 coupled beads was mediated by the measured release of soluble IL-2 from the solid phase matrix. Control beads did not increase cytotoxicity, indicating that the bead matrix itself lacked activating potential.

The i.p. injection of IL-2 coupled beads resulted in the in vivo activation of nonspecific cytotoxic cells in that compartment. A single injection of the same amount of soluble IL-2 lacked this activity. Presumably the soluble IL-2 dissipated systemically, leaving an insufficient concentration for an adequate duration to have a biological effect at this site. Ethanolamine coupled control beads also lacked the capacity to induce increased levels of cytotoxicity, indicating that the activity of the IL-2 coupled to polystyrene beads was not a result of a nonspecific inflammatory response to the bead matrix.

IL-2 coupled beads were also able to suppress the growth of admixed tumor cells. The result could not be explained by an immediate direct lethal effect of the beads upon the tumor cells. Rather, it is most likely that the IL-2 coupled beads activated cytotoxic cells at the injection site. Whether these were nonspecific cytotoxic cells recruited from the host or present within the freshly trypsinized tumor preparation, specifically sensitized T-lymphocytes within the inoculum, or local effector cells stimulated to produce cytotoxic cytokines has not yet been investigated. Control beads did not alter tumor growth, indicating that bead matrix was not responsible for the antitumor effects.

Just as a single injection of soluble IL-2 into the peritoneal cavity failed to induce increased cytotoxicity at that site, the same amount of soluble IL-2 alone or mixed with control beads failed to suppress the growth of admixed tumor cells. Since IL-2 coupled to polystyrene beads was more biologically active in vivo than the same amount of soluble IL-2, it is likely that the gradual release of IL-2 from the solid phase matrix served to focus the lymphokine at the site of injection and maximize the time of exposure for the relevant effector cells. The unimpaired growth of tumor distant from the site of IL-2 coupled beads admixed with tumor (Fig. 3) further supports the idea that injected bead activity was predominantly local rather than systemic.

With the development of techniques to produce recombinant IL-2, relatively large quantities of this lymphokine have become available for clinical testing. Administration of IL-2 with or without adoptively transferred lymphoid cells has resulted in regression of tumors in animal models (9, 10) and human patients (11-14). In preclinical models, a direct IL-2 dose-tumor response relationship has been demonstrated (9), influencing the design of clinical trials to use high doses of IL-2. Unfortunately, toxicity is also dose related, often limiting the amount of IL-2 that patients can tolerate, perhaps to less than optimal therapeutic quantities (11-15). Effector mechanisms responsible for IL-2 induced tumor regression are likely multiple, and consequently the dominant mechanisms are unclear. Diffuse activation of cytotoxic effector cells and cytokine production (22) driven by high systemic concentrations of IL-2 may be necessary to achieve antitumor activity. On the other hand, the necessity for high systemic levels may be related to achieving adequate concentrations of IL-2 in the region of a tumor. In support of the latter possibility is the demonstration of tumor regression with repeated local injection of IL-2 into regions of tumor growth (23, 24) and the observed antitumor
activity after infusion of IL-2 into the peritoneal (25) or pleural spaces (26) of patients.

The in vivo activity demonstrated by IL-2 coupled beads in this study was achieved with modest amounts of the lymphokine. No obvious toxic effects were noted in rats given injections of bead preparations. In vivo activation of cells in the peritoneal cavity of 200-g rats was achieved by a single injection of 25,000 Cetus units of IL-2 coupled to polystyrene beads. It is likely that this dose would not be toxic since it is considerably less than the well tolerated dose of 48,000 Cetus units/200 g infused into rats i.v. over 24 h (27).

Therefore these studies indicate that IL-2 coupled to polystyrene beads to form a solid phase of the lymphokine has both in vitro and in vivo biological activity. In vivo, the preparation appears to locally focus the actions of IL-2. The activity is induced with amounts of IL-2 less than those previously shown to be well tolerated in rodents. Regional perfusion of IL-2 coupled to an appropriate solid matrix might be useful for treatment of dominant areas of tumor growth, for example in lung, liver, or peritoneal cavity. IL-2 coupled to a matrix designed to be retained at such sites would deliver increased concentrations of the lymphokine to the region while maintaining low systemic levels, thereby increasing the therapeutic index.

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REFERENCES


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