Sustained Delivery of Interleukin-2 from a Poloxamer 407 Gel Matrix Following Intraperitoneal Injection in Mice

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Parenteral delivery of recombinant biologic response modifiers (BRMs) remains a challenge because of the brief intravascular half-life of most recombinant proteins and their associated rapid clearance from the circulation. Recombinant derived interleukin-2 (rIL-2) was formulated with Pluronic F-127, N.F. (poloxamer 407, N.F.) and the biological activity determined vs time at 4, 22, and 37°C. As assessed by rIL-2-induced peripheral blood lymphocyte (PBL) uptake of [^{3}H]thymidine, storage of rIL-2/poloxamer 407 (33% w/w) for 72 hr at 4 and 22°C did not result in an overall negative slope of the [^{3}H]thymidine vs time profiles. However, storage of an rIL-2/poloxamer formulation at 37°C for 72 hr resulted in an approximate 15% reduction in the biological activity as assessed by [^{3}H]thymidine incorporation. As assessed by bioassay ([^{3}H]thymidine uptake), the cumulative percentage rIL-2 released *in vitro* at 22°C after 8 hr from rIL-2/poloxamer 407 matrices containing either 30% (w/w) or 35% (w/w) poloxamer 407 was 81.8 ± 1.7 and 82.1 ± 4.7%, respectively. When ELISA was used to determine the amount of rIL-2 released vs time, the corresponding values for the cumulative percentage rIL-2 released were 82.6 ± 10.1 and 40.9 ± 8.8%. Cytotoxicity of rIL-2-stimulated PBLs cultured with poloxamer 407 (0.17%, w/w) toward malignant Daudi cells was significantly ($P < 0.05$) enhanced compared to controls. Finally, mice injected with the rIL-2/poloxamer 407 formulation (1×10^5 U/inj. q.d. × 3 days) demonstrated a bioequivalent effect of rIL-2-induced natural killer (NK) cell activity *in vitro* toward malignant murine YAC-1 cells at one-half the standard exogenously administered dose of rIL-2 known to generate enhanced NK lytic activity in mice (1×10^5 U/inj. b.i.d. × 3 days). No untoward systemic side effects were observed for mice injected i.p. with polymer vehicle alone (30%, w/w) (0.15 ml q.d. × 3 days), pH 7 phosphate-buffered saline (PBS) (0.15 ml q.d. × 3 days), rIL-2 formulated with poloxamer 407 (30%, w/w) (1×10^5 U/0.15 ml q.d. × 3 days and 0.5×10^5 U/0.15 ml q.d. × 3 days), or rIL-2 dissolved in PBS (1×10^5 U/0.15 ml b.i.d. × 3 days). Thus, poloxamer 407, N.F., did not denature rIL-2 when the latter was stabilized with human serum albumin (HSA), enhanced the *in vitro* lytic ability of human rIL-2-stimulated PBLs against malignant Daudi cells, and served as a sustained-release parenteral vehicle for rIL-2 when injected i.p. into mice. Thus, based on these preliminary findings, it appears that poloxamer 407, N.F., may potentially be useful for the formulation and sustained delivery of select protein pharmaceuticals following extravascular administration.

KEY WORDS: recombinant interleukin-2; Pluronic F-127; poloxamer 407; biological response modifiers; cancer immunotherapy.

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INTRODUCTION

Interleukin-2 (IL-2) is another protein that belongs to a class of potent immunoregulatory molecules that initiate the clonal expansion of sensitized or activated T cells (1). *In vivo* administration of exogenous IL-2 has been shown to activate natural killer (NK) cells (2) or lymphocyte-activated killer (LAK) cells (3), enhance alloantigen responsiveness (2), restore immune function in acquired immunodeficient states (4) or nude mice (5), and enhance antitumor effects, either alone (3) or with adoptively transferred effector cells such as long-term cultured T cells (6) or LAK cells (7). Treatments using IL-2 and killer cells, principally LAK cells, have met with encouraging results for select cancers in both experimental animal models and humans (8). The major consideration with the use of high-dose IL-2 therapy is the associated toxicity. Further, the *in vivo* use of IL-2 is also severely hindered because of IL-2's rapid clearance from the circulation, with detectable serum levels lasting only a few hours following parenteral administration of IL-2 to mice (9,10). It has been reported (9) that the magnitude of the *in vivo* effects of exogenously administered IL-2 is more positively correlated with the length of time IL-2 is detectable in the serum rather than the peak serum level achieved. In fact, prolonged, detectable serum IL-2 levels resulted in a greater antitumor effect than high serum levels of short duration, and prolonged elevations in serum IL-2 correlated with high levels of endogenous lymphocyte proliferation (9,11) and reduced number of metastases in multiple murine model systems (3,12). Hence, to circumvent the problem of systemic toxicity and increase the therapeutic efficacy of IL-2 by maintaining prolonged serum levels, we have evaluated a sustained-release, injectable formulation designed for extravascular administration of interleukin-2.

Recently it was reported that poloxamer 407 (Pluronic F-127) may be a suitable vehicle for inclusion into sustained-release, parenteral formulations designed to deliver proteins following extravascular administration (13). Poloxamer 407 belongs to a chemically similar family of ABA block copolymers. Since poloxamers are nonionic surfactants and possess excellent wetting, antifoaming, and solubilizing properties, many of these copolymers have been used in the food, cosmetic, agricultural, and pharmaceutical industry. In addition, certain members of the Pluronic family exhibit the property of reverse-thermal gelation, that is, the polymer exists as a mobile viscous liquid at reduced temperatures but forms a rigid semisolid gel network with an increase in temperature (14). The property of reverse-thermal gelation is probably best exhibited by poloxamer 407, which has been used to deliver macromolecular (nonpeptidic) drugs as well as a biologically active macromolecule (protein) to rats in a sustained fashion following intramuscular and intraperitoneal injection, respectively (13,15). Previously, it had been demonstrated *in vitro* that the loss in biological activity of a model protein based on activity determinations and viscometry was negligible when formulated with poloxamer 407 [14% (w/w)] (16). Poloxamer 407 was also shown to lack any inherent myotoxicity following either single or multiple intramuscular injections of an aqueous solution of the vehicle to rabbits (17). The lack of inherent musculoirritancy to-

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gether with the rapid sol-to-gel transition (18) make poloxamer 407 an ideal vehicle with which to sustain the delivery of interleukin-2 or other biological response modifiers following extravascular administration.

The purpose of the present investigation was to characterize release of IL-2 *in vitro* from an IL-2/poloxamer 407 sustained-release formulation and to evaluate the effect of the poloxamer on IL-2 to induce the proliferation of peripheral blood lymphocytes (PBLs). In addition, the effect of poloxamer 407 on IL-2 to stimulate the generation of cytotoxic LAK cells *in vitro* was assessed using a human malignant cell line (Daudi). Finally, the sustained-release IL-2/poloxamer 407 formulation was tested to determine whether sustained release of the IL-2 from the polymer depot following intraperitoneal injection to mice would result in enhanced cytotoxicity of nonspecific NK cells obtained from freshly harvested splenocytes against a murine malignant cell line (YAC-1).

MATERIALS AND METHODS

Materials

Recombinant human interleukin-2 (rIL-2) was kindly provided by Hoffman-La Roche, Inc. (Nutley, NJ). Each vial contained 10^6 units of rIL-2, 25 mg of human serum albumin (HSA), and 5 mg of mannitol. Poloxamer 407, N.F., was a gift from BASF (Parsippany, NJ) and was used as received. Poloxamer solutions were made up in a pH 7.0 ± 0.01 phosphate buffer which consisted of 0.05 *M* potassium phosphate monobasic and 0.015 *M* sodium phosphate dibasic. Both buffer components were obtained from Fisher (Fair Lawn, NJ) and used as received.

Ficoll–Hypaque (Pharmacia) density-gradient separation was used to harvest human peripheral blood lymphocytes (PBLs) from whole blood. Growth medium employed in the bioassay of rIL-2 was RPMI-1640 (GIBCO Laboratories) and Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories). Cell density calculations were performed using a hemocytometer cell plate and Trypan blue (GIBCO Laboratories) to stain viable lymphocytes.

Bioassay of IL-2 was determined using flat-bottomed, 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA). Cells were pulsed with [^{3}H]thymidine (1 μCi of [^{3}H]thymidine; sp act, 1 μCi/umol; Amersham, Arlington Heights, IL). DNA fragments labeled with [^{3}H]thymidine residues were harvested on filter paper (Whittaker M.A. Bioproducts) using a Brandel cell harvestor (M-125, Gaithersburg, MD). Dried filter papers were combined with 3 ml of Aquasol scintillation fluid (Research Products, Inc., Rockford, IL) and counted in a liquid scintillation counter (Beckman Instruments Inc., Irvine, CA; Models LS 9000 and LS 8000) at 2 sigma error.

Malignant human and murine cell lines used in cytotoxicity assays were Daudi and YAC-1 (American Type Culture Collection, Rockville, MD), respectively. All Daudi and YAC-1 cells used in cytotoxicity assays were radiolabeled with ^{51}Cr purchased from Amersham (Arlington Heights, IL).

Mice (male, C57-Black, 7–8 weeks) for *in vivo* studies were obtained from Harlan, Inc. (Indianapolis, IN). Enzyme-linked immunosorbent assay (ELISA) kits for quantitative determination of rIL-2 were obtained from Assay Research, Inc. (College Park, MD).

Instruments

A Van Kel (Van Kel Industries, Model Vander Kamp 600, Edison, NJ), six-station, dissolution apparatus was used for providing constant hydrodynamic and temperature conditions during the *in vitro* release studies. An ultracentrifuge (International Equipment Company, Model Centra-7R) was used in harvesting PBLs. All absorbance values of individual wells contained in the 96-well ELISA plates were determined with an ELISA plate reader (Biotek, auto reader EL310).

Methods

Formulation of IL-2/Poloxamer 407 Matrices

Recombinant human interleukin-2 (1×10^6 U) was reconstituted under aseptic conditions with sterile pH = 7 phosphate buffer (1 ml) which had previously been autoclaved. Following reconstitution, 100 μl of the rIL-2 solution was added to each of six 10-ml glass vials containing approximately 0.4 g of sterile phosphate buffer. To three of the vials, 0.3 g of poloxamer 407 was added. The second set of vials (n = 3) each received 0.35 g of poloxamer 407. All of the vials were then brought to a final weight of 1.0 g using additional sterile phosphate buffer. All vials were subsequently placed on ice overnight to facilitate dissolution of the polymer by the "cold method" of incorporation (14). Thus, two sets of sustained-release rIL-2/poloxamer matrices were formulated for evaluation of IL-2 release at 22°C: a set (n = 3) containing 30% (w/w) poloxamer 407 and a second set (n = 3) containing 35% (w/w) poloxamer 407. An additional group (n = 2) of rIL-2/polymer matrices was formulated as described above for evaluation of rIL-2 release at 37°C.

In the second group of rIL-2/poloxamer matrices evaluated for IL-2 release at 37°C, the final weight of each of the matrices was 10 g. However, the concentrations of polymer used in each matrix comprising the second group were 30% (w/w) and 35% (w/w) as above. The second group of two rIL-2/poloxamer matrices contained 200,000 U of rIL-2 per matrix. The 10-fold increase in final matrix weight and 2-fold increase in the amount of rIL-2 incorporated into each matrix contained in the second group were selected to determine the effect of carrier protein (human serum albumin; HSA) on the rate of rIL-2 release from the polymer matrices *in vitro*. Therefore, the second group of rIL-2/poloxamer matrices represented formulations in which the weight percentage of HSA incorporated in the final formulation was fivefold less than the weight fraction of HSA included in the rIL-2/poloxamer matrices contained in the first group.

rIL-2 Bioactivity Determinations

Human peripheral blood lymphocytes were harvested using Ficoll–Hypaque density-gradient centrifugation according to standard techniques (19). Lymphocytes were then suspended in IMDM containing 10% fetal calf serum (FCS) for maintaining hematopoietic cells and counted using a he-

mocytometer to obtain an estimate of the lymphocyte density. Growth medium (IMDM) was then added to the PBL suspension to obtain a cell density of 1.5×10^6 cells/ml. PBLs were stimulated to proliferate by the addition of rIL-2 in either the presence or the absence of the polymer and were plated and placed in an incubator (37°C) having a 5% CO_2 atmosphere. Following 4 days of incubation, the plates were temporarily removed from the incubator and each well was pulsed with 10 μl of [^{3}H]thymidine solution. Plates were then returned to the incubator for 6–8 hr to allow proliferating PBLs to incorporate [^{3}H]thymidine. Following the 6- to 8-hr [^{3}H]thymidine uptake period, the contents of each well were passed through filter paper using the cell harvestor and [^{3}H]thymidine incorporation was determined by combining the dried filter paper with 3 ml of liquid scintillation cocktail and then counting the solution using a liquid scintillation counter.

Effect of Poloxamer 407 on rIL-2 Activity

To determine the effect of poloxamer 407 on rIL-2-induced proliferation of PBLs, 700-μl samples of the PBL suspension (above) were placed into 48 6-ml sterile polypropylene tubes. The tubes, containing 700 μl of the PBL suspension, were then split into three groups. To each tube in groups 1, 2, and 3 was added 100 μl of reconstituted rIL-2 at a concentration of either 5, 50, or 500 U/ml, respectively. To one tube in each group of 16 tubes was then added 100 μl of polymer solution [0, 0.0056, 0.028, 0.056, 0.083, 0.11, 0.17, 0.22, 0.28, 0.33, 0.39, 0.44, 0.50, 0.56, 0.61, and 0.67% (w/w)], to bring the total volume of an individual tube to 900 μl.

The contents of each tube from each group were then plated in triplicate on a 96-well costar plate and assayed 4 days later for lymphocyte proliferative response as described above. Since preliminary pilot studies had suggested potential diminution in rIL-2-induced proliferation of PBLs with increasing concentrations of poloxamer 407, the following concentrations of poloxamer 407 were also evaluated: 1.0, 1.5, 2.0, 2.5, and 3.0% (w/w).

To assess whether the HSA (protein stabilizer) included in the lyophilized formulation of rIL-2 afforded protection against potential denaturation by poloxamer 407, a study was conducted in which the IL-2's ability to stimulate proliferation of PBLs was determined following various incubation times in poloxamer 407 (33%, w/w). These studies were performed by incorporating rIL-2 (1.6×10^5 U) in 3 g of either poloxamer 407 (33%, w/w) or sterile PBS and storing the rIL-2 formulations at 4, 22, and 37°C. In addition, solutions of PBS and poloxamer 407 (33%, w/w) were also stored at the above temperatures to serve as controls (no rIL-2). At 0, 24, 48, and 72 hr, the rIL-2/PBS, rIL-2/poloxamer 407, PBS, and poloxamer 407 formulations stored at 22 and 37°C were placed on ice for 15 min and an aliquot (100 μl) was obtained. The 15-min incubation period on ice was necessary for the rIL-2/poloxamer and poloxamer 407 formulations, since at 22 and 37°C, the formulations existed as a semisolid. Similarily, samples at the above time points were obtained for rIL-2 formulations and control solutions stored at 4°C. All collected samples were then stored at −70°C until the completion of the 72-hr sampling period.

Following the 72-hr incubation study, the samples from each formulation were thawed and individually diluted with sterile growth medium such that the concentrations of poloxamer 407 and rIL-2 were 0.23% (w/w) and 384 U/ml, respectively. Control solutions were treated identically to test formulations with regard to dilution. Aliquots (200 μl) of the diluted rIL-2/PBS, rIL-2/poloxamer 407, PBS, and poloxamer 407 formulations were then plated in triplicate with a suspension of freshly harvested human PBLs (1.5×10^6 cells/ml) and the biological activity of rIL-2 was assayed as described above. Results were then plotted individually for each group as the [^{3}H]thymidine incorporated vs formulation type at each storage temperature.

Finally, a study was conducted to assess the stability of the rIL-2 in the presence of PBLs incubated in a solution of poloxamer 407 (0.33%, w/w) over 96 hr. This study was different from the 72-hr stability study at 4, 22, and 37°C described above because the rIL-2-stimulated lymphocytes (500 U/ml) were in contact with the polymer for 96 hr. Four groups were evaluated for effect of poloxamer 407 on proliferating PBLs. Two groups served as controls, namely, growth medium and a 0.33% (w/w) solution of poloxamer 407. The remaining two groups were PBLs stimulated to proliferate with either rIL-2/PBS (500 U/ml) or rIL-2/poloxamer 407 (0.33%, w/w, poloxamer 407; 500 U rIL-2/ml). Twenty wells of a 24-well costar plate contained the four groups of PBLs. At 0, 24, 48, 72, and 96 hr, one well from each of the four groups (4 wells/time point) was analyzed for rIL-2 activity using bioassay.

Release of rIL-2 in Vitro

Prior to the *in vitro* release studies conducted at 37°C and room temperature (22°C), the IL-2/polymer solutions were gently swirled (75 rpm) on an orbit shaker for 1 hr at 4°C to assure a homogeneous dispersion of the rIL-2 in the polymer vehicle. The glass vessels containing the rIL-2/polymer formulations were subsequently placed in the dissolution tester in approximately 80 ml of distilled water thermostated at either 37 or 22°C. Following formation of the rIL-2/polymer gel matrices, 1.0 ml of pH 7 phosphate buffer maintained at 22°C was then placed directly above and in contact with the gelled rIL-2/polymer formulations to form an upper aqueous receptor phase. Thus, the release of rIL-2 from the rIL-2/poloxamer matrices was evaluated using a membraneless diffusion system in which the receptor phase was in direct contact with the gelled IL-2/poloxamer formulation. The upper receptor phase was continuously slowly stirred (15 rpm) during each 1-hr release period of the *in vitro* release studies to avoid any shear-related denaturation of the rIL-2 released from the semisolid polymer matrix. To maintain sink conditions, the receptor phase directly above and in contact with each matrix was completely removed hourly and replaced with fresh buffer. For rIL-2/poloxamer matrices evaluated for release of rIL-2 at 37°C, 10 ml of the receptor phase was used. Receptor phase samples collected during the release studies conducted at 22 and 37°C were divided into two equal-volume fractions and were immediately frozen at −70°C for subsequent bio- and immunoassay of rIL-2. For receptor phase samples analyzed for rIL-2 using bioassay, the extent of [^{3}H]thymidine uptake observed

when PBLs were stimulated to proliferate with individual receptor phase samples (200 μl) was compared to [^{3}H]thymidine incorporation by PBLs stimulated to proliferate with known concentrations of freshly reconstituted rIL-2. The percentage of IL-2 released into each volume of receptor phase for each 1-hr collection interval was then calculated and the results are expressed as the cumulative percentage of rIL-2 released vs time.

Quantitation of rIL-2 Release by Immunoassay

To determine the amount of rIL-2 released from the sustained-release rIL-2/polymer matrices during the 8-hr release study, an immunoassay kit was also used. The ELISA assay kit used for determination of rIL-2 in receptor phase fractions employed rIL-2 which had been purified by Hoffmann-La Roche, Inc., as the standard. The individual receptor phase aliquots from each 1-hr release interval for each formulation tested at 22°C were appropriately diluted and then assayed according to the manufacturer's instructions. The assay kit had a calibration curve that extended from 98 pg/ml to 100 ng/ml. Eighteen hours after addition of the alkaline phosphatase substrate solution provided with the kit, the absorbance of the colored solution in each well of the ELISA plate was read at 405 nm using an ELISA reader (Biotek, auto reader EL 310). The absorbance values from the calibration standards were then processed using a computer software program (Biotek, Apple Computer Software) that generated a log-logit fit of the absorbance data. Each receptor phase was tested in triplicate from each collection time period from three rIL-2/polymer formulations to assess overall variance in the assay system and release rate between individual rIL-2/polymer sustained-release matrices. Results of these studies were then correlated with predictions of the percentage of IL-2 released from the rIL-2/polymer matrices based on bioassay.

Cytotoxicity Studies

Cytotoxicity toward human malignant Daudi cells of rIL-2-induced lymphocytes which had previously been incubated in the presence or absence of polymer were assessed *in vitro*. Two 24-well, flat-bottomed Costar plates were prepared as described below. To each 2-ml well of the 24-well Costar plate was added 1.56 ml of a PBL suspension harvested above (1.5×10^6 lymphocytes/ml) and 0.22 ml of a freshly reconstituted solution of rIL-2 (50 U/ml). So that the volume of solution in each well would be approximately 2 ml, 0.22 ml of either a sterile solution of PBS (12 wells), a sterile solution of 1.5% (w/w) poloxamer 407 (6 wells), or a sterile solution of 3.0% (w/w) poloxamer 407 (6 wells) was added.

The plates were then placed in an incubator (5% CO_2, 37°C). After 4 days, the plates were removed from the incubator and the lymphocytes from each of the three groups for an individual plate were pooled and resuspended to provide a cell density of 1.0×10^6 cells/ml. The rIL-2-induced killer cells (E = effectors) from each group were then combined with previously radiolabeled Daudi cells (T = targets) at E:T ratios of 50:1, 25:1, 12:1, and 6:1 and plated on 96-well, round-bottomed Costar plates. The Daudi cells were radiolabeled with sodium chromate (^{51}Cr; 100 μCi/1.5×10^6 cells). Following radiolabeling, the Daudi cells were washed three times with 5 ml of IMDM, the radioactive supernatants discarded, and the ^{51}Cr-containing Daudi cells resuspended in growth medium to obtain a cell density of 15,000 Daudi cells/100 μl. To determine the extent of spontaneous and maximum cell lysis, two additional sets of six wells each on a separate Costar plate contained target cells combined with either growth medium (PSG-IMDM) or a 3.0% solution of Triton X-100. The plates were then returned to the incubator. Following a 4-hr incubation period, the 96-well plates were spun (1500 rpm) for 5 min, 100 μl of the supernatant was removed and placed in glass scintillation vials, and the vials were counted in a gamma counter. Results are expressed as the percentage specific lysis vs E:T ratio.

In Vivo *Studies*

A set of *in vivo* experiments was conducted in which the novel rIL-2/polymer formulation was injected i.p. into four groups of mice to assess the cytotoxicity of rIL-2-induced nonspecific killer cells toward a murine malignant cell line (YAC-1). Mice (male, black, C57, 7–8 weeks) were injected i.p. with 0.15 ml of either sterile PBS (n = 3 mice), 35% (w/w) poloxamer 407 in sterile PBS (n = 3 mice), rIL-2 reconstituted with sterile PBS [1×10^5 U/0.15 ml (n = 3 mice)], or rIL-2 formulated in 35% (w/w) poloxamer 407 [0.5×10^5 U/0.15 ml (n = 3 mice) and 1×10^5 U/0.15 ml (n = 3 mice)]. All mice in each group were injected once a day with the above solutions except the group of mice that received i.p. injections of rIL-2/PBS. The latter group of mice received an injection of rIL-2 in PBS two times a day. All injections were continued for 3 days. Following 3 days of administration, the mice were sacrificed and the spleens excised from each animal. Harvested splenocytes collected from each of the mice in each group were then pooled and lymphocytes isolated by standard techniques employing centrifugation of splenocyte suspensions on a 1.08 *M* Ficoll–Hypaque density gradient. Following centrifugation, the cytotoxicity of the isolated lymphocytes toward a murine malignant cell line (YAC-1) was assessed for each group of mice at E:T ratios of 50:1, 25:1, 12:1, and 6:1. Results are expressed as percentage specific lysis vs E:T ratio.

Data Analysis

The mean counts per minute (cpm) for each of the three determinations of [^{3}H]thymidine uptake were averaged. To determine the net mean value of [^{3}H]thymidine taken up by the PBLs, the mean values for the [^{3}H]thymidine taken up by PBLs that were incubated with receptor phase containing only poloxamer 407 [approximately 2.0% (w/w)] were subtracted from the mean values of [^{3}H]thymidine incorporated by PBLs incubated with receptor phase aliquots containing rIL-2 and poloxamer 407 from the *in vitro* release study. The mean values of the net [^{3}H]thymidine taken up by PBLs stimulated to proliferate with receptor phase fractions collected from the 30% (w/w) and 35% (w/w) rIL-2/poloxamer 407 formulations evaluated *in vitro* were then converted to units of rIL-2 activity via a calibration curve employing freshly reconstituted rIL-2. The cumulative percentage rIL-2 released versus time was determined by dividing the mean activity value (units) by the total number of units of rIL-2

initially incorporated into the rIL-2/poloxamer 407 matrices and multiplying the resultant quotient by 100.

The mean value of the percentage specific lysis of rIL-2-induced lymphocytes toward Daudi cells at different E:T ratios was analyzed by one-way analysis of variance (ANOVA) to determine statistical significance of poloxamer groups compared to controls. Multiple comparisons of means testing was performed using the method of Scheffé.

RESULTS

IL-2 Bioactivity Following Incubation in Poloxamer 407

Figure 1 is a representative plot of [^{3}H]thymidine incorporated vs sampling time for rIL-2 formulations stored at 4°C. Similar trends were observed at 22 and 37°C. As can be seen in Fig. 1, test formulations which contained poloxamer 407 and were stored at 4°C for 72 hr did not result in an overall negative slope of the [^{3}H]thymidine uptake vs time profiles. However, as reflected by the [^{3}H]thymidine vs time profile for the rIL-2/poloxamer formulation stored at 4°C, the extent of [^{3}H]thymidine incorporation by proliferating PBLs was less than that observed with the corresponding rIL-2/PBS formulation (Fig. 1).

Effect of Poloxamer 407 on rIL-2-Induced Lymphocyte Proliferation

The effect of poloxamer 407 on rIL-2-induced lymphocyte proliferation is shown in Fig. 2. The [^{3}H]thymidine uptake vs poloxamer 407 concentration profile over the poloxamer 407 concentration range of 0.028% (w/w) to 0.67% (w/w) for PBLs stimulated to proliferate with rIL-2 (50 U/ml) appeared to decline compared to the control [0.0% (w/w) poloxamer 407; i.e., PBS] value of [^{3}H]thymidine incorporation, as did the profile for PBLs stimulated to proliferate with 500 U/ml of rIL-2 [0.083% (w/w) to 0.67% (w/w)]. This apparent trend of decreased [^{3}H]thymidine uptake by rIL-2-stimulated lymphocytes with increasing concentrations of poloxamer 407 in the cell culture is shown more dramatically as the polymer concentration exceeds 0.67% (w/w) (Fig. 3). It can be seen in Fig. 3 that at approximately 1.5% (w/w) poloxamer 407 contained in the cell culture, PBLs stimulated to proliferate with rIL-2 attained a proliferative response (as reflected by [^{3}H]thymidine uptake) that was equivalent to suboptimal rIL-2 stimulation of PBLs. This experiment was conducted once to confirm the trend observed at lower concentrations of poloxamer 407 [i.e., <0.67% (w/w)]. Enhanced proliferation of rIL-2-stimulated PBLs (500 U rIL-2/mL) was observed in the present study when lymphocytes were cultured in a medium containing 0.33% (w/w) poloxamer 407 compared to lymphocytes grown in medium containing only rIL-2 [500 U/ml] (Fig. 4).

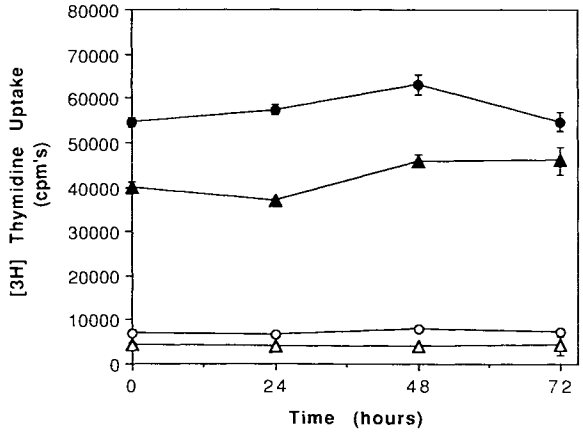

Fig. 1. Biological activity of rIL-2 when rIL-2 was incubated in 33% (w/w) poloxamer 407 *in vitro* at 4°C. Bioassay using rIL-2-stimulated PBLs was performed subsequent to collection of all samples from each time point. (▲) rIL-2/poloxamer; (●) rIL-2/PBS; (△) poloxamer 407; (○) PBS.

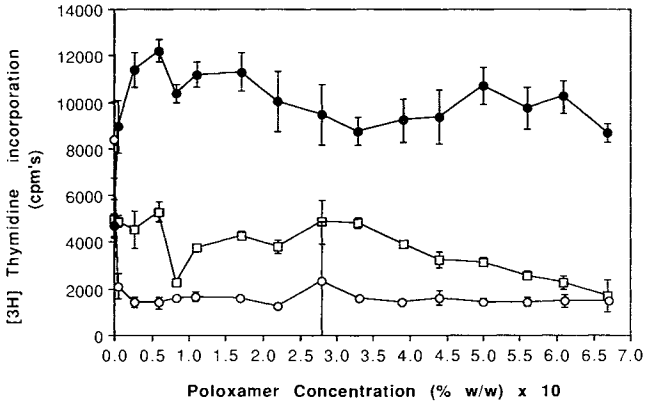

Fig. 2. Effect of poloxamer 407 on the proliferation of rIL-2-stimulated human peripheral blood lymphocytes (PBLs) *in vitro:* (○) 5 U rIL-2/ml; (□) 50 U rIL-2/ml; (●) 500 U rIL-2/ml.

IL-2 Release *in Vitro*

As shown in Figs. 5 and 6, release of IL-2 from IL-2/poloxamer 407 gel matrices *in vitro* at 22°C followed zero-order release kinetics. A constant rate of release was also observed for rIL-2/poloxamer 407 matrices evaluated at 37°C. Based on bioassay (Fig. 5), the cumulative percentages of rIL-2 released from the rIL-2/polymer matrices maintained at room temperature were 81.8 ± 1.7 and 82.1 ± 4.7% for the 30% (w/w) and 35% (w/w) rIL-2/poloxamer formula-

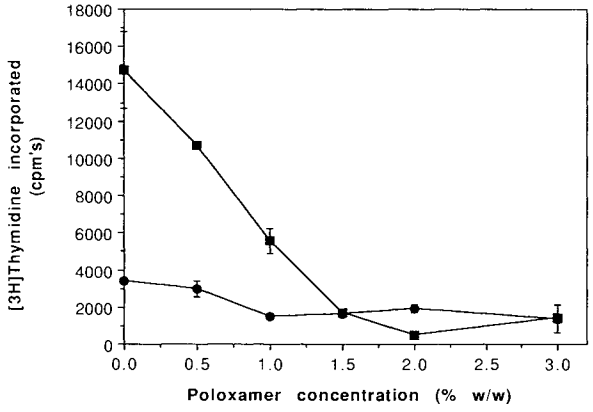

Fig. 3. The effect of increased concentrations of poloxamer 407 (cf. Fig. 2) on the proliferation of rIL-2 stimulated human PBLs *in vitro:* (●) 25 U rIL-2/ml; (■) 250 U rIL-2/ml.

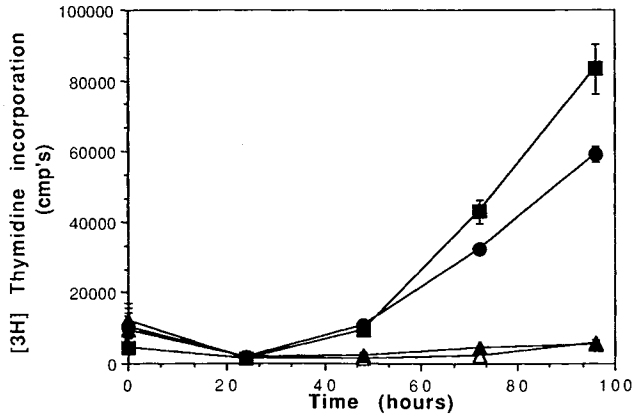

Fig. 4. The effect of poloxamer 407 (0.33%, w/w) on rIL-2-induced (500 U/ml) proliferation of human PBLs *in vitro*. Poloxamer 407 was present in the PBL culture medium for 4 days. Bioassay using rIL-2-stimulated PBLs was performed on samples at each time point. (■) rIL-2/poloxamer 407; (●) rIL-2/PBS; (▲) poloxamer 407 alone; (△) PBS.

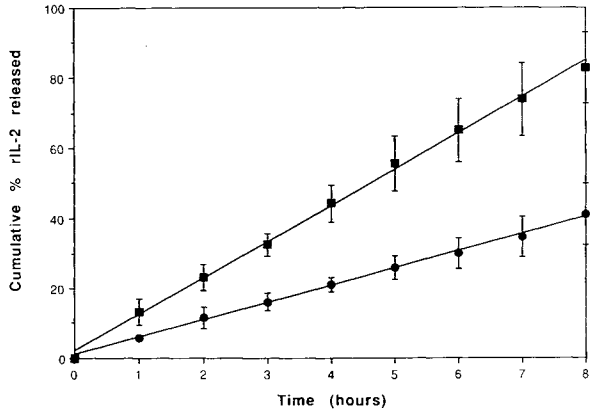

Fig. 6. Cumulative percentage rIL-2 released from rIL-2/poloxamer 407 matrices *in vitro* at 22°C. IL-2 released *in vitro* was determined by ELISA. (■) rIL-2/poloxamer 407 matrices containing 30% (w/w) poloxamer 407; (●) rIL-2/poloxamer 407 matrices containing 35% (w/w) poloxamer 407.

tions, respectively, and were not significantly different ($P > 0.05$). When receptor phase aliquots collected from *in vitro* release studies using rIL-2/poloxamer 407 matrices maintained at 22°C were analyzed for rIL-2 using ELISA (Fig. 6), the cumulative percentage rIL-2 released at 8 hr was 82.6 ± 10.1 and 40.9 ± 8.8% for the 30% (w/w) and 35% (w/w) rIL-2/poloxamer formulations, respectively.

In Vitro Cytotoxicity Studies

A representative percentage specific malignant cell lysis vs E:T ratio profile demonstrating the influence of poloxamer 407 incorporated in the incubation medium (PBL suspension) on the ability of lymphocytes activated with rIL-2 (50 U/ml) to lyse a malignant cell line *in vitro* is shown in Fig. 7. It can be seen in Fig. 7 that the percentage specific lysis of malignant Daudi cells by rIL-2-induced nonspecific killer cells was enhanced when PBLs were cultured in the presence of either 0.17 or 0.33% (w/w) poloxamer 407 compared to the control PBLs. The enhanced cytotoxicity was most apparent at E:T ratios of 12:1, 25:1, and 50:1.

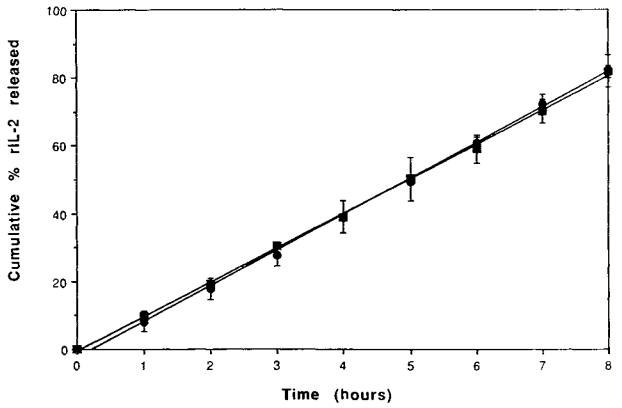

Fig. 5. Cumulative percentage rIL-2 released from rIL-2/poloxamer 407 matrices *in vitro* at 22°C. IL-2 released *in vitro* was determined by bioassay. (■) rIL-2/poloxamer 407 matrices containing 30% (w/w) poloxamer 407; (●) rIL-2/poloxamer 407 matrices containing 35% (w/w) poloxamer 407.

In Vivo Studies

The effect of sustained-release rIL-2 on splenic natural killer cytotoxicity toward YAC-1 cells is shown in Fig. 8. The percentage specific lysis at E:T ratios of 6:1, 12:1, 25:1, and 50:1 was approximately equivalent for mice treated with rIL-2/poloxamer 407 (0.5×10^5 U/0.15 ml q.d. × 3 days) and mice treated with pH 7 PBS (0.15 ml q.d. × 3 days). In contrast, mice treated with poloxamer 407 vehicle alone (0.15 ml q.d. × 3 days) had values of the percentage specific lysis that were less than corresponding values for mice injected with sterile PBS at E:T ratios of 6:1, 12:1, and 25:1. However, mice treated with the sustained-release rIL-2/poloxamer 407 formulation (1.0×10^5 U/0.15 ml q.d. × 3 days) had values of the percentage specific lysis in close

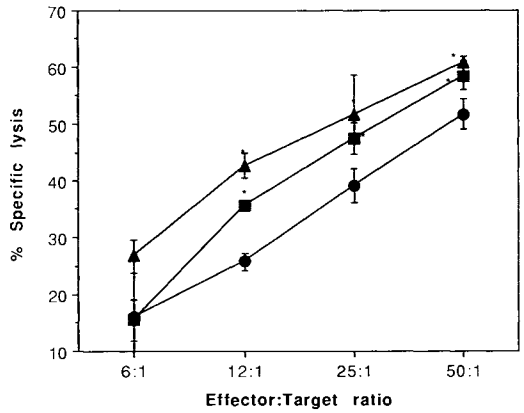

Fig. 7. The effect of poloxamer 407 on the generation of rIL-2-induced (50 U rIL-2/ml) nonspecific killer cells against malignant Daudi cells *in vitro*. (●) Human PBLs incubated in sterile PBS; (▲) human PBL's incubated with 0.17% (w/w) poloxamer 407; (■) human PBLs incubated with 0.33% (w/w) poloxamer 407. (*) Mean value significantly different ($P < 0.05$) than the mean value for PBS control.

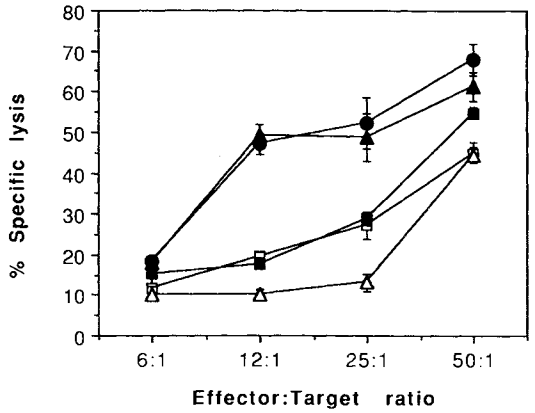

Fig. 8. The effect of poloxamer 407-formulated rIL-2 on splenic NK activity of C57 B/6 mice *in vitro*. (▲) Mice injected i.p. with rIL-2/PBS (1×10^5 U/0.15 ml) b.i.d. × 3 days; (△) mice injected with poloxamer 407 vehicle (0.15 ml) q.d. × 3 days; (□) mice injected i.p. with PBS (0.15 ml) q.d. × 3 days; (■) mice injected i.p. with rIL-2/poloxamer 407 (0.5×10^5 U/0.15 ml) q.d. × 3 days; (●) mice injected i.p. with rIL-2/poloxamer 407 (1.0×10^5 U/0.15 ml) q.d. × 3 days. On day 4, NK cytotoxicity of freshly isolated splenocytes was tested against malignant murine YAC-1 cells.

agreement to mice treated with rIL-2/PBS (1.0×10^5 U/0.15 ml b.i.d. × 3 days).

Visual observations of the mice treated with the doses mentioned above revealed that mice treated with rIL-2/PBS (1.0×10^5 U/0.15 ml b.i.d. × 3 days) appeared to have no respiratory distress by the third day compared to the mice treated with the sustained-release rIL-2/poloxamer 407 formulation (1.0×10^5 U/0.15 ml q.d. × 3 days). All mice from the other groups appeared normal as assessed by visual observation. Of particular interest is that a preliminary study (data not shown) involving mice that were injected with a rIL-2/poloxamer 407 formulation (1.0×10^5 U/0.15 ml b.i.d. × 3 days) resulted in a uniformly lethal dose after three injections compared to mice treated with either PBS or poloxamer 407 vehicle only.

DISCUSSION

Biologic response modifiers such as interleukin-2 are demonstrating therapeutic potential for use in human malignancies, especially when combined with various cellular therapies. However, rIL-2 and other recombinant-derived proteins continue to be administered primarily by the intravenous route of administration. Since it is well documented that the length of time that therapeutic levels of rIL-2 are maintained in the blood are more positively correlated with increased antitumor efficacy than transient high blood levels of rIL-2 (9), a sustained-release formulation of rIL-2 for parenteral administration would be beneficial. In addition, further prolongation of therapeutic blood levels of rIL-2 is possible by extravascular dosing. We have evaluated a controlled-release formulation of rIL-2/poloxamer 407 *in vitro* and *in vivo* and have demonstrated augmentation in the cytotoxic response of freshly isolated splenic NK cells toward YAC-1 cells when IL-2 is administered as a sustained-release formulation to mice.

Several attempts have been made to sustain the blood levels of IL-2 to increase therapeutic efficacy. As an example, Donohue and Rosenberg (10) used a natural polymer (gelatin) to attempt to prolong the serum levels of IL-2 following subcutaneous (s.c.) and intraperitoneal (i.p.) injection of an IL-2/gelatin formulation to mice. More recently, Dunn *et al.* (20) reported the slow release of IL-2 from an ethylene–vinyl–acetate (EVA) copolymer. Similarily, slow release of rIL-2 from an ALZET miniosmotic pump was more effective in augmenting the therapeutic efficacy of immune spleen cells in adoptive chemoimmunotherapy (ACIT) than a single injection of the same total dose of rIL-2 (21). A more practical approach for the sustained administration of rIL-2 involved implantation locally into the tumor site of a biodegradable IL-2 minipellet fabricated with atelocollagen derived from natural bovine skin (22). Others have employed Pluronic F-127 as a slow-release system for rIL-2 because of the ease of administration of a rIL-2/poloxamer formulation into any site of the body by use of a syringe (23) and the fact that the polymer depot need not be removed from the body at the completion of therapy. It was concluded that rIL-2/Pluronic F-127 sustained-release formulations may potentially produce an antitumor effect by activation or expansion of immune T cells (23) and that there exists the possibility that Pluronic F127-induced monocytes following parenteral injection may cooperate with rIL-2 in inhibiting tumor growth (23).

rIL-2 Bioactivity Following Incubation with Poloxamer 407

The findings in the present investigation using rIL-2/poloxamer 407 sustained-release formulations would suggest that poloxamer 407 may be a useful sustained-release vehicle for the extravascular administration of various biological response modifiers such as rIL-2. Since the rIL-2/poloxamer 407 sustained-release formulation is prepared without the use of toxic organic solvents or heat, rIL-2 was not inactivated during the formulation process. While the present study demonstrated (Fig. 3) that inclusion of poloxamer 407 at concentrations exceeding 1.5% (w/w) resulted in decreased [^{3}H]thymidine uptake by rIL-2-stimulated PBLs, this finding would be anticipated using any natural or synthetic polymer substance in a cell culture. However, the present study demonstrated that the inclusion of poloxamer 407 (33%, w/w) in growth medium containing rIL-2 does not cause denaturation and subsequent loss in biological activity of rIL-2 when stored at 4 and 22°C for 72 hr since the rIL-2 was not incubated with lymphocytes until day 4. Thus, if denaturation of all or a fraction of rIL-2 had occurred while rIL-2 was incubated for 72 hr with poloxamer 407 vehicle only, then the [^{3}H]thymidine uptake vs incubation time profiles in Fig. 1 should have resulted in a distinctly negative slope. This conclusion would support the findings of others. For example, it is well-known that nonionic detergents, in contrast to ionic detergents, are relatively inactive with regard to protein denaturation (24). Moreover, an interesting aspect of detergents is that at very low concentrations (<1.0%), they protect proteins from heat coagulation and denaturation by urea and guanidinium chloride (24). This protection factor has been exploited with regard to formulation strategies for newer protein pharmaceuticals. Ap-

proaches that have been reported to enhance the physical stability of recombinant proteins include the use of low-ionic strength buffers, avoidance of transition metals, the use of carbohydrates, poly alcohols, and certain amino acids, and the incorporation of surfactants (detergents) (25). However, the latter approach is generally reserved for nonionic surfactants.

Effect of Poloxamer 407 on rIL-2 Induced Lymphocyte Proliferation

The present study would suggest that increasing concentrations of poloxamer 407 in rIL-2-stimulated PBL cultures causes a reduction in the ability of proliferating lymphocytes to incorporate [^{3}H]thymidine (Fig. 3). The trend of reduced [^{3}H]thymidine uptake by rIL-2-stimulated lymphocytes with increasing concentrations of poloxamer 407 in the cell culture medium might potentially result from several mechanisms. First, cell-to-cell contact is typically a prerequisite for cell proliferation in an *in vitro* cell culture. A surface active agent added to the cell culture may reduce the surface tension of the growth media and disrupt the cell-to-cell adhesiveness. However, the apparent increase in [^{3}H]thymidine uptake by rIL-2-stimulated (500 U/mL) PBLs at lower concentrations of poloxamer 407 (Figs. 2 and 4) may potentially result from either an increase in lymphocyte membrane permeability to [^{3}H]thymidine by poloxamer 407 or an increase in the total number of lymphocytes able to incorporate [^{3}H]thymidine. The former hypothesis appears unreasonable since altered membrane permeability to creatine phosphokinase (CPK) was not observed by Johnston and Miller (17) when rabbits were injected intramuscularly with poloxamer 407. In addition, Hunter *et al.* (26) did not observe changes in the permeability of erythrocyte membranes to small cations when erythrocytes were exposed to hydrophilic members of a structurally similar group of polymers.

The possibility of increased numbers of lymphocytes, and hence increased [^{3}H]thymidine uptake, when PBLs were stimulated with rIL-2 in the presence of poloxamer 407 appears more tenable. This may potentially result from increased stability afforded to rIL-2 by poloxamer 407 and, thus, increase the effectiveness of the cytokine to stimulate the proliferation of PBLs. For example, many of the recombinant-derived interferons are stabilized with various surfactants. In fact, some of the more common surfactants that have been employed as stabilizers in commercially available parenteral formulations are polysorbate 80, poloxamer, Brij, and various other nontoxic, nonionic surfactants (25). Because proteins often denature at the air/liquid or liquid/liquid interface, surfactants may help to reduce interfacial tension, thus reducing the propensity for protein unfolding. In addition, the incorporation of a surfactant may aid in solubilization of the protein to reduce the amount of protein at the interface. Nonionic surfactants, such as poloxamer 407, tend to be less structure perturbing to proteins than ionic surfactants since the critical micelle concentration is generally higher for the latter group of surfactants (25). In addition, nonionic surfactants cause fewer directional interactions because electrostatic binding is absent (25). Thus, since nonionic surfactants and, in particular, polyols tend to promote stabilization of recombinant proteins (25), the increased [^{3}H]thymidine uptake observed with rIL-2-stimulated PBLs at lower concentrations of poloxamer 407 may have potentially resulted from increased stability of rIL-2 and enhanced effectiveness of the cytokine to direct cell proliferation.

rIL-2 Release *in Vitro* from rIL-2/Poloxamer 407 Matrices

Release of rIL-2 *in vitro* from sustained-release rIL-2/poloxamer 407 formulations in the present study proceeded at a constant (zero-order) rate. The cumulative percentages of rIL-2 released *in vitro* from rIL-2/poloxamer 407 (30%, w/w) matrices evaluated at 22°C, as assessed by both bio- and immunoassay, were in close agreement (Figs. 5 and 6). The reason for the discrepancy between the cumulative percentages of rIL-2 released from rIL-2/poloxamer 407 (35%, w/w) matrices as determined by bio- and immunoassay (Figs. 5 and 6) is unknown, however, previous preliminary studies using the ELISA assay (data not shown) to quantitate various known concentrations of freshly reconstituted rIL-2 in the presence of poloxamer 407 [0.0, 1.0, 2.0, and 3.0% (w/w)] demonstrated no interference by the polymer in the quantitation of rIL-2 standards. Since we have demonstrated in the present studies that the presence of poloxamer 407 in a culture of rIL-2-induced PBLs may influence (increase or decrease) the extent of [^{3}H]thymidine incorporated, the amount of rIL-2 released from rIL-2/poloxamer 407 (35%, w/w) matrices *in vitro* at 22°C was probably more accurately predicted by immunochemical detection (ELISA).

In Vitro Cytotoxicity Studies

One of the most significant findings in the present study was the enhanced cytotoxicity of human PBLs toward Daudi cells following incubation in a poloxamer solution when compared to PBLs cultured in sterile PBS. Similar to enhanced rIL-2-induced proliferation of PBLs observed when dilute concentrations of poloxamer were included in the cell cultures, enhanced cytotoxicity appears to be a concentration-dependent phenomenon. Enhanced cytotoxicity observed in the present study was inversely related to poloxamer concentration in the cell culture (Fig. 7). Enhanced cytotoxicity with a lower concentration of poloxamer in the cell culture probably does not result from the poloxamer altering permeability of the Daudi cell to ^{51}Cr but, rather, may potentially result from the increased ability of rIL-2 to induce the proliferation of PBLs. In addition, target cells were also plated with a solution of the poloxamer during cytotoxicity studies to determine if poloxamer alone could induce Daudi cell lysis. The poloxamer, in contrast to a 3% (v/v) solution of Triton X-100 used to determine maximal cell lysis, did not cause Daudi cells to lyse to an extent that was significantly different than target cells incubated with growth media. This would support the premise that the poloxamer was not altering Daudi cell membrane permeability to ^{51}Cr. In contrast to enhanced proliferation of PBLs when cultured with poloxamer 407 and stimulated with rIL-2 (as reflected by increased amounts of [^{3}H]thymidine uptake), enhanced cytotoxicity of PBLs toward Daudi cells was probably not caused by increased numbers of lymphocytes which had

been incubated with poloxamer 407. Rather, enhanced cytotoxicity potentially resulted from more effective lytic activity of the lymphocytes, since all lymphocytes incubated in poloxamer solutions were first centrifuged to remove the polymer and the exact number of lymphocytes combined with the targets was known at each E:T ratio.

In Vivo Efficacy

Increased therapeutic efficacy of rIL-2 is postulated to be directly correlated with the length of time that a therapeutic plasma level of rIL-2 is maintained rather than the peak serum level obtained (9). If increased therapeutic efficacy of rIL-2, especially when combined with cellular therapies, is the goal with cancer immunotherapy, then the present investigation has successfully addressed the first requirement. Although exact pharmacokinetic parameters for rIL-2 administered extravascularly as an injectable rIL-2/poloxamer formulation will be the focus of future research in this area, our data directly and indirectly (mortality/visual observations) suggest that sustained levels of rIL-2 were achieved. We observed a bioequivalent effect of rIL-2-induced NK-cell activity at one-half the total dose with no untoward systemic side effects from the rIL-2 or polymer vehicle when administered to mice by intraperitoneal injection. Thus, poloxamers may be used to create injectable rIL-2 formulations that provide sustained delivery of rIL-2 without systemic side effects.

Implications for Protein Pharmaceuticals

The therapeutic implications of the sustained-release formulation we have evaluated are numerous. Specifically, other BRMs could be formulated with the polymer and injected extravascularly. The sustained-release poloxamer depot is easily administered without the need for surgical implantation, contains no toxic additives, and potentially protects the formulated protein from significant denaturation by proteases contained in interstitial fluids. The latter advantage results from the protein being dispersed in a polymer matrix that allows the protein to come into contact with interstitial fluids only after its diffusion through the polymer network. Thus, the sustained-release poloxamer system should increase the therapeutic efficacy of a number of diseases presently being treated with protein pharmaceuticals.

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