Characterization of Poly(glycolide-co-D,L-lactide)/Poly(D,L-lactide) Microspheres for Controlled Release of GM-CSF

Dean K. Pettit,[1,6] J. Ronald Lawter,[2] W. James Huang,[2] Susan C. Pankey,[1,5] Nancy S. Nightlinger,[1] David H. Lynch,[3] Jo Ann C. L. Schuh,[4] Philip J. Morrissey,[4] and Wayne R. Gombotz[1]

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Purpose. This study describes the preparation and characterization of a controlled release formulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) encapsulated in poly(glycolide-co-D,L-lactide) (PLGA) and poly(D,L-lactide) (PLA) microspheres.

Methods. GM-CSF was encapsulated in PLGA/PLA microspheres by a novel silicone oil based phase separation process. Several different blends of PLGA and low molecular weight PLA were used to prepare the microspheres. The microspheres and the encapsulated GM-CSF were extensively characterized both *in vitro* and *in vivo*.

Results. Steady release of GM-CSF was achieved over a period of about one week without significant "burst" of protein from the microspheres. Analysis of microsphere degradation kinetics by gel permeation chromatography (GPC) indicated that low molecular weight PLA enhanced the degradation of the PLGA and thereby affected release kinetics. GM-CSF released from the microspheres was found to be biologically active and physically intact by bioassay and chromatographic analysis. Analysis of serum from mice receiving huGM-CSF indicated that the GM-CSF was biologically active and that a concentration of greater than 10 ng/mL was maintained for a period lasting at least nine days. MuGM-CSF was not detected following *in vivo* administration of muGM-CSF microspheres. The tissues of mice receiving muGM-CSF microspheres were characterized by infiltration of neutrophils, and macrophages which were in significant excess of those found in mice administered with placebo controls (i.e. microspheres without GM-CSF).

Conclusions. This study demonstrates the influence of formulation parameters on the encapsulation of GM-CSF in PLGA/PLA microspheres and its controlled release in biologically active form. The intense local tissue reaction in mice to muGM-CSF microspheres demonstrates the importance of the mode of delivery on the pharmacologic activity of GM-CSF.

[1] Department of Analytical Chemistry and Formulation, Immunex Corporation, Seattle, Washington 98101.

[2] Chemical and Pharmaceutical Development, Wyeth-Ayerst Research, Pearl River, New York 98195.

[3] Department of Immunobiology, Immunex Corporation, Seattle, Washington 98101.

[4] Department of Molecular Immunology, Immunex Corporation, Seattle, Washington 98101.

[5] Current address: Pharmaceutical Research Institute, Bristol-Meyers Squibb, Seattle, Washington 98101.

[6] To whom correspondence should be addressed. (e-mail: pettitd@immunex.com)

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INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine which exhibits plieotropic activity in the hematopoietic and immune systems. GM-CSF was originally cloned based on its ability to stimulate the proliferation and differentiation of hematopoetic precursors of the granulocyte/monocyte lineage (1,2). Subsequent studies with purified recombinant GM-CSF surprisingly revealed many additional effects on mature granulocytes and macrophages. For instance, GM-CSF can induce cytokine production and cytotoxic activity in these cells (3). In addition, GM-CSF enhances antigen processing and presentation by macrophages (4). GM-CSF is currently being used clinically to accelerate recovery of neutrophil levels following bone marrow transplantation (5), is in clinical trials in settings of infection in immunocompromised patients (6), and has been suggested as a vaccine adjuvant (7). The serum elimination half-life of human GM-CSF in humans is approximately one hour, and therefore daily injections are required to achieve therapeutic effects (8). The desire to reduce the frequency of GM-CSF administration has led to such formulation strategies as PEGylation (9), encapsulation in gelatin chondroitin sulfate microspheres (10), and liposomal carriers (11).

The controlled release of peptides and recombinant proteins with biodegradable microspheres prepared from poly(glycolide-co-D,L-lactide) (PLGA) and poly(D,L-lactide) (PLA) has been the subject of numerous investigations (12–14). However, although the delivery of peptides such as LHRH analogues from PLGA microspheres has been both technically and commercially successful (13), protein delivery with microspheres has been particularly challenging. Denaturation (e.g. aggregation, oxidation, deamidation, disulfide exchange, etc.) has lead to diminished biological activity and undesirable release kinetics of microencapsulated proteins. Denaturation following microsphere encapsulation has been associated with the physical/chemical attributes of the proteins (14–16), the conditions of the encapsulation process (17,18), or the microenvironment encountered during release of protein from the microspheres (15,19). Several specific strategies have been developed to help stabilize proteins through the process of encapsulation and *in vivo* release.

As with other implanted materials the administration of PLGA microspheres *in vivo* has been shown to elicit mild inflammation via the classic foreign body response (20). The intensity, duration, and ultimate resolution of the foreign body response may be affected by many factors including the size, shape, and chemical characteristics of the implanted microspheres (21). Also, the biological agent that is released from the microspheres may affect the local tissue response to the microspheres. For example, a PLGA encapsulated wound healing factor, bone morphogenic protein (BMP), has been shown to mediate the local inflammatory and wound healing response (22). Other agents that are to be released from microspheres, cytokines in particular, may be expected to significantly alter (enhance or exacerbate) the inflammatory and foreign body response when released from PLGA microspheres.

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In this study we have encapsulated both human (hu) and murine (mu) GM-CSF in PLGA/PLA microspheres. Our objective was to develop microspheres that could be administered via a single subcutaneous injection, and would release biologically active GM-CSF over a period of about one week.

MATERIALS AND METHODS

Materials

Four PLGA polymers with monomer ratios of approximately 50/50, and one PLA polymer were used in this study for the preparation of microspheres (Table I). PLGA copolymers were produced by ring-opening polymerization with dodecanol as the initiator; therefore, the carboxyl end-groups are blocked. PLA was synthesized by polycondensation; consequently the carboxyl groups are not blocked. Polydimethylsiloxane (Dow Corning 360 fluid, 350 centistokes) and octamethylcyclotetrasiloxane (Dow Corning 244 fluid) were obtained from Dow Corning (Midland, MI). Reagent grade methylene chloride was obtained from J.T. Baker. Recombinant huGM-CSF and muGM-CSF were expressed in yeast and purified at Immunex as described (23,24).

Microsphere Preparation

Microspheres were prepared according to the procedures previously outlined (25). Briefly, solutions of GM-CSF (hu or mu in 100 mM tris, pH 7.4) were concentrated to approximately 85 mg/mL using Amicon Centriprep™ concentrators. Mixtures of PLGA and/or PLA totaling 0.4 grams were dissolved in 3.6 mL of methylene chloride. The GM-CSF solution (250 μl) was then added to the polymer solution and the mixture was homogenized. Five milliliters of polydimethylsiloxane were added with further stirring. The microspheres thus formed were hardened by pouring the mixture into 2 liters of octamethylcyclotetrasiloxane which was stirred for an additional hour. The microspheres were collected by filtration, sieved to eliminate large particles, and then dried under a vacuum at 37°C for 5 days.

Table I. Poly(glycolide-co-D,L-lactide) (PLGA) and Poly(D,L-lactide) (PLA) Polymers

Polymer designation	Inherent viscosity	M_w[c]	Source
PLGA1	0.71[a]	40,400	Cytec Industries Co.
PLGA2	0.40[b]	22,300	Boehringer Ingleheim (RG503)
PLGA3	0.38[a]	21,700	Cytec Industries Co.
PLGA4	0.30[b]	13,700	Boehringer Ingleheim (RG502)
PLA	0.19[b]	6,100	Boehringer Ingleheim (R104)

Note: All PLGA were 50/50 lactide/glycolide.

[a] Inherent viscosity measured at 30°C in hexafluoroisopropanol at a concentration of 0.5 g/dL.

[b] Inherent viscosity measured at 25°C in chloroform at a concentration of 0.1 g/dL. Inherent viscosity of 0.19, 0.30, and 0.40 determined in chloroform at 25°C at a concentration of 0.1 g/dL correspond to about 0.26, 0.44, and 0.58 in hexafluoroisopropanol at 30°C at a concentration of 0.5 g/dL.

[c] Weight average molecular weight was determined by gel permeation chromatography in tetrahydrofuran versus polystyrene standards.

Microsphere Characterization

Each batch of microspheres was characterized for particle size distribution with a Malvern Particle Size Analyzer (Malvern Instrument Co., Model 2600C). Approximately 10 mg of microspheres were suspended in 0.5 mL of PBS and shaken vigorously in a 1 mL cuvette prior to size distribution analysis. The protein loading efficiency and total protein loading of GM-CSF into the microspheres was determined by amino acid analysis (Beckman Instruments, 6300 Amino Acid Analyzer). In this technique approximately 10 mg of microspheres were hydrolyzed in 1 mL of 6 M HCl in glass tubes which were flame sealed and heated at 110°C for 22–24 hours. The tubes were opened, the acid was boiled off, and 100 mM citrate buffer (pH 2.0) was added prior to analysis. Protein concentrations determined by this method were confirmed by solvent extraction of microspheres in methylene chloride and quantitation at 280 nm.

The release of GM-CSF was measured *in vitro* in specially designed cells (26). Microspheres were weighed into the cells which were then placed in culture tubes containing phosphate buffered saline (PBS) (50–75 mg of microspheres per 6 mL of PBS). A vacuum was drawn on the culture tubes in order to remove any air trapped in the cells and to assure complete wetting of the microspheres. The culture tubes were agitated on a shaker table at 37°C. At time intervals ranging from 2 hr through 10 days, the release medium was retrieved and replaced with fresh PBS. The release media samples were assayed by the BioRad total protein assay (BioRad, Hercules, CA) and the cumulative release data were calculated.

Placebo Microsphere Degradation Studies

Placebo microspheres were prepared with tris buffer not containing GM-CSF and were from 100% PLGA1, 100% PLA, or a blend of 80% PLGA1 and 20% PLA. Each of these batches of microspheres were incubated in PBS, pH 7.4 at 37°C for periods of 1, 2, 4, 6, 8, 10, and 14 days. Following incubation, the microspheres were dried by speed vac and dissolved in tetrahydrofuran (THF). Polymer molecular weight distributions were measured by gel permeation chromatography (GPC) using a Waters HPLC system equipped with a styragel HR4E column (Waters, Milford, MA). Polystyrene standards (Polysciences, Warrington, PA) were dissolved in THF and used to calibrate the system (1 mL/min flow rate; retention times varied from 5 to 10 minutes for polystyrene standards weighing 90,000 to 500 Da respectively).

Protein Characterization

HuGM-CSF released from microspheres *in vitro* was characterized by size exclusion (SE) and reverse phase (RP) chromatography. All chromatography was carried out on a Waters HPLC system (Millipore Corp. Milford, MA). The conditions for SE chromatography utilized a Biosil SEC 125 column (BioRad, Richmond, CA) with PBS pumped at 1 mL/min. RP chromatography was carried out using a 1%/min gradient of acetonitrile/trifluoroacetic anhydride (TFA) (0.1%) and saline (1 M NaCl)/TFA(0.1%) pumped at 1 mL/min on a C18 column (10 μm particle size, 300 Å pore size, Vydac, Hesperia, CA). Bioactivity of protein in the release media was measured by

^{3}H-thymidine uptake in TF-1 cells (for huGM-CSF) or FDCP2.1D (for muGM-CSF) as described (27,28).

In Vivo Administration

Eight-week-old female mice (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen free environment.

HuGM-CSF Microspheres

Microspheres containing 0.91% huGM-CSF (wt/wt) were prepared with 100% PLGA3 (endotoxin levels of water from rinsed microspheres were lower than detection limits by our assays). These microspheres were suspended in a solution containing 3% methyl cellulose, 4% mannitol, and 0.1% polysorbate 80. Three mice per group (one group was used for each time point) were injected subcutaneously with either huGM-CSF microspheres (55 mg of microspheres containing 500 μg of huGM-CSF), placebo microspheres (55 mg of microspheres per mouse), or soluble huGM-CSF (50 or 500 μg per mouse). All injection volumes were 500 μl. Mice were sacrificed at 1, 2, 6 hr, 1, 3, 5, 7, and 9 days post injection and serum samples were isolated by centrifugation and frozen. Concentrations of huGM-CSF were determined by ELISA using monoclonal capture antibodies (M8, Immunex designation), and polyclonal detection antibodies (P5, Immunex designation). Serum samples were also analyzed for huGM-CSF bioactivity by TF-1 bioassay. The specific activity of serum huGM-CSF was determined by calculating the percentage of residual activity of the serum samples relative to a non-encapsulated huGM-CSF control.

MuGM-CSF Microspheres

Microspheres containing 0.69% muGM-CSF (wt/wt) were prepared from a blend of 80% PLGA1 and 20% PLA (endotoxin levels of water from rinsed microspheres were lower than detection limits by our assays). Three mice per group (one group was used for each time point) were injected with muGM-CSF microspheres (25 mg of microspheres containing 175 μg of muGM-CSF), or placebo microspheres (25 mg of microspheres per mouse). All injection volumes were 500 μl in suspension media containing 3% methyl cellulose, 4% mannitol, and 0.1% polysorbate 80. Mice were sacrificed at 2, 4, 7, 9, 11, 14, 28, 44, and 95 days after administration of the microspheres. Tissues at the injection sites were harvested, embedded in paraffin, thin sectioned, and stained with hematoxylin and eosin. Serum samples were also collected and analyzed for muGM-CSF concentration by ELISA using monoclonal capture antibodies (M1, Immunex designation), and polyclonal detection antibodies (P4, Immunex designation).

In separate studies, muGM-CSF microspheres were administered to mice through subcutaneous injection at doses of 1 μg, 0.3 μg, and 0.1 μg (muGM-CSF weight). Placebo microspheres were also administered in order to measure the local tissue response to microspheres alone. The kinetics of local tissue swelling were measured by applying calipers to the tissue at the injection site and measuring the "diameter" or thickness of the swollen tissue. Tissue surface area swelling in mm^2 due to administration of the microspheres was calculated from the caliper measurement. Measurements were taken at 7, 14, 21, and 28 days post administration.

RESULTS

Microsphere Characterization

The median size of the microspheres typically measured 20 to 80 μm, with 90% of the microsphere preparation or more measuring less than 100 μm in diameter (data not shown). The microsphere diameter could be controlled by the concentration of polymer in the emulsion and the speed of stirring during the addition of polydimethylsiloxane. Incorporation efficiencies for both hu and muGM-CSF were typically >95%.

The *in vitro* release kinetics for huGM-CSF microspheres prepared from PLGA1, PLA or a blend of 80% PLGA1 and 20% PLA are shown in Figure 1. HuGM-CSF was released rapidly from PLA microspheres, in a burst fashion, but very slowly from those prepared from PLGA1. When the two polymers were blended together, an intermediate release profile was achieved. Based on these results additional blends of polymers were used to prepare microspheres (PLGA2, PLGA4, and PLA). The *in vitro* release kinetics for this group of microspheres is shown in Figure 2. A clear trend in the early rate of release of huGM-CSF was achieved for the microspheres prepared from polymer blends. As the proportion of high molecular weight PLGA1 was exchanged for PLA, the early rate of release of huGM-CSF was enhanced. This effect was shown to be incrementally controlled by the proportion of each of the polymer components. The release of GM-CSF was found to be incomplete (less than 100% after 14 days) for microspheres containing lower proportions of PLA.

Determination of residual GM-CSF contained within the microspheres following *in vitro* release experiments were carried out on microsphere formulations which did not release 100% of their GM-CSF. These mass loss experiments confirmed the accuracy of the *in vitro* release measurements to within ±5%, (data not shown).

Placebo Microsphere Degradation

Weight average molecular weights of polymer from placebo microspheres produced from 100% PLA, an 80%/20%

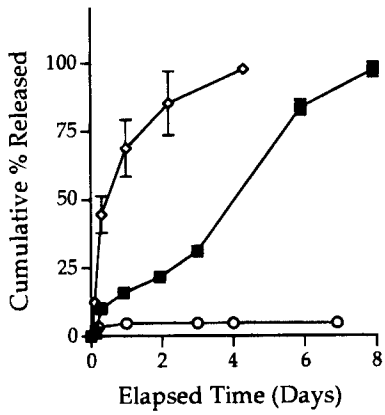

Fig. 1 Release of huGM-CSF from microspheres prepared from 100% PLA (◇), an 80%/20% mixture of PLGA1 and PLA (■), or 100% PLGA1 (○). Each of the microspheres was loaded with approximately 0.5% GM-CSF by wt.

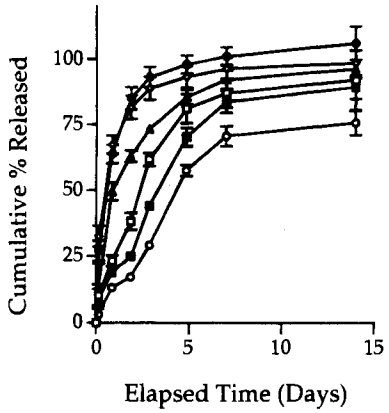

Fig. 2. Release of huGM-CSF from microspheres prepared from blends of PLGA2, PLGA4, and PLA. The composition of PLGA2/PLGA4/PLA used to prepare the microspheres were: 25/15/60 (●); 25/20/55 (◇); 20/30/50 (▲); 30/20/50 (□); 25/30/45 (■); and 25/35/40 (○).

mixture of PLGA1 and PLA, and 100% PLGA1 are shown as a function of incubation time (Fig. 3). These data clearly show that the blending of PLA and PLGA 1 significantly enhances the rate of polymer degradation relative to microspheres prepared from PLGA alone.

Protein Characterization

The characteristics of huGM-CSF released from microspheres prepared from 80% PLGA1 and 20% PLA are shown in Figure 4. The release profile for this formulation is shown in Figure 4A. The RP and SE chromatograms are shown in Figures 4B and 4C, respectively. The three distinct peaks shown in the RP chromatograms are characteristic of GM-CSF produced in yeast (the earliest two peaks represent protein that contains N- or a combination of N- and O-linked glycosylation, respectively, and the latest retention peak is due to unglycosylated GM-CSF, unpublished). No appreciable changes in the RP or SE chromatograms were evident over the one week *in vitro* release period. The chromatograms at day two suggest some degradation of unglycosylated GM-CSF. The bioassay data shown in Figure 4D support that the biological activity of GM-CSF is essentially unchanged over the seven day release period.

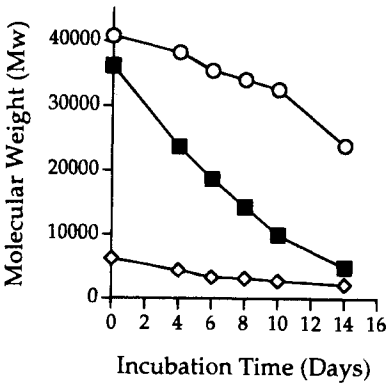

Fig. 3. Changes in the weight averaged molecular weights (M_w) of placebo microspheres incubated in PBS given as a function of incubation time. The microspheres were prepared from 100% PLA (◇), an 80%/20% mixture of PLGA1 and PLA (■), or 100% PLGA1 (○).

No measurable changes in the pH of the release medium were noted at any of the time points (data not shown).

Pharmacokinetics of hu and muGM-CSF

The serum levels of huGM-CSF in mice following subcutaneous administration of huGM-CSF in solution or in microspheres are shown in Figure 5. Following injection of huGM-CSF solutions serum levels were detectable for several hours. The serum elimination half-life was estimated to be approximately 1.5 hr in this model. Following injection of huGM-CSF microspheres an initial spike of huGM-CSF was observed which had a duration of less than four hours which was followed by a relatively constant level of approximately 10–100 ng/mL over the remaining nine-day period of investigation. It should be noted that despite 54% sequence homology between hu and muGM-CSF, huGM-CSF does not bind to muGM-CSF receptor and has no measurable biological activity on murine cell lines (29).

MuGM-CSF was not detected in mouse serum following subcutaneous administration of muGM-CSF microspheres at any of the time points (two to nine days post administration). The limit of detection for this ELISA assay is 1 ng/mL.

Local Tissue Characterization

Histopathology

Tissue sections observed at the injection site consisted of clusters of microspheres separated by a border of infiltrating and reactive cells. For mice administered with placebo microspheres the cellular infiltrate consisted of macrophages, and infrequently polymorphonucleocytes (PMNs) and lymphocytes (see Figures 6B and 6D). Conversely, the response to microsphere encapsulated muGM-CSF was characterized by a more extensive cellular infiltrate of PMNs and macrophages, and more granulation tissue and fibrosis (see Figures 6C and 6E). The intense local cellular response to muGM-CSF microspheres was observed throughout the 95 day period of histological analysis. As a result of this enhanced reactivity, the size of the injection site mass was consistently larger in the presence of muGM-CSF microspheres, compared to similar time-points for placebo microspheres.

Aside from the differences in the intensity of reaction and the specific cell types present, both placebo microspheres and microspheres encapsulated with muGM-CSF followed a similar course progressing through: i) cellular infiltration between and around microspheres, associated with edema (days 2 and 4); ii) resolution of the edema, formation of granulation tissue and early degradation of the microspheres (day 7); iii) granuloma formation characterized by a central core of degenerated microspheres and PMNs, surrounded by a layer of multi-nucleated giants cells and PMNs, and then a fibrous capsule (days 9, 11 and 14); and iv) infrequent or complete absence of identifiable microspheres, maturation and resolution of the granuloma (days 28, 44, and 95). Although intact PLGA microspheres were difficult to identify in tissue sections, the inflammatory reaction around the microspheres delineated clusters of discrete, round spaces approximating the size of the microspheres, with a translucent material lining the rim of the spaces. As the reaction progressed, these spaces became less discrete, translucent mate-

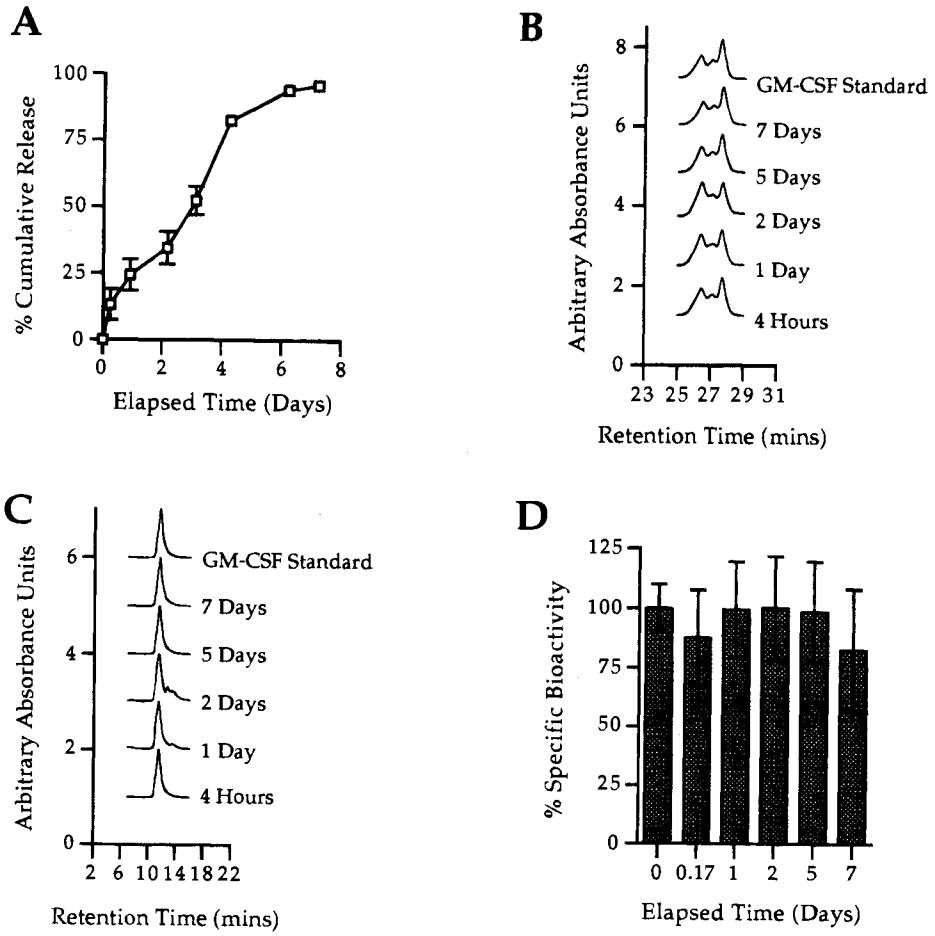

Fig. 4. Characterization of huGM-CSF released *in vitro* from microspheres prepared from a blend of 60% PLGA1, 20% PLGA2, and 20% PLA included: (A) *in vitro* release kinetics; (B) SE-HPLC, A220 chromatogram following injection of 200 μl of release media and flow through column at 1 mL/min with PBS; (C) RP-HPLC, A220 chromatogram following injection of 200 μl of release media and flow through column at 1 mL/min; and (D) specific activity of released material by TF-1 bioassay (data shown as average ± standard error of the mean, n = 5 − 6).

rial was lost, PMN and neutrophils and granular material filled the spaces, and finally the spaces were replaced by inflammation and granulation tissue.

Local Tissue Swelling

Following administration of three different doses of muGM-CSF microspheres local tissue swelling was found to be dose dependent (Figure 7). The peak in swelling areas occurred 14 days following administration of the microspheres, and at each dose the local tissue swelling was significantly reduced or immeasurable by day 28.

DISCUSSION

During the development and characterization of GM-CSF microspheres numerous formulations were prepared and tested with the objective of producing microspheres that could deliver biologically active GM-CSF over a period of one week. Several strategies were employed to control the release kinetics of GM-CSF from the microspheres including: utilization of degradable polymers of various molecular weights, blending of polymers of various molecular weights and types, the addition of excipients or other release rate modifiers, and variations in the encapsulation process (e.g. changing the GM-CSF loading percentage or microsphere size). Of these, polymer blending provided the simplest and most reproducible method for incrementally controlling the release kinetics of GM-CSF. The results of polymer blending on huGM-CSF release kinetics (Figs. 1 and 2) are consistent with the observations published by others (30–32). Specifically, the addition of a low molecular weight PLA to higher molecular weight PLGA served to enhance the initial *in vitro* release rates. The shapes of the release kinetic curves were further modified by the addition of other intermediate molecular weight PLGAs (compare Figs. 1 and 2).

Studies carried out with placebo microspheres further support the role of low molecular weight PLA on the dissolution of PLGA1/PLA blended microspheres. Placebo microspheres prepared from PLGA1 alone degraded in PBS slowly in comparison with microspheres prepared from a blend of 80% PLGA1 with only 20% PLA (Fig. 3). Two mechanisms for the enhanced rate of degradation for the blended polymer microspheres are plausible. First, the presence of the PLA with its hydrophilic

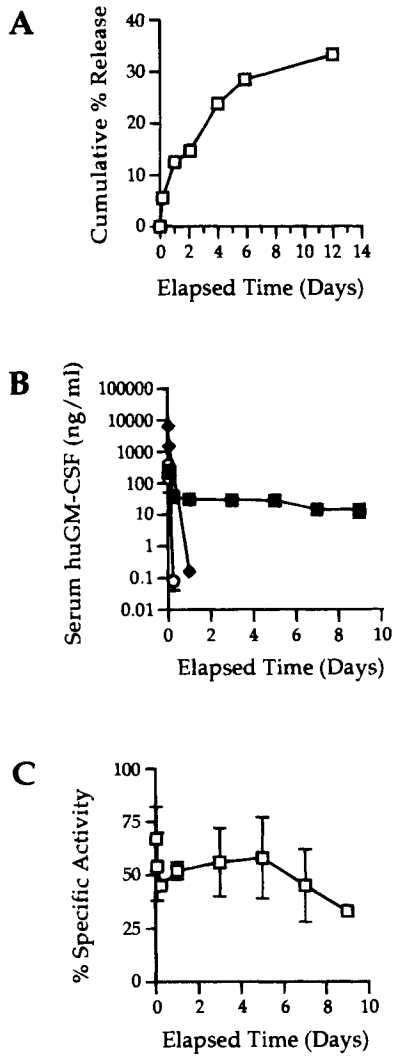

Fig. 5. Characterization of the *in vitro* release kinetics of huGM-CSF used in the mouse pharmacokinetics study (A). The microspheres used in this study were prepared from 100% PLGA3. Serum huGM-CSF levels were determined at periods ranging from 1 hr to 9 days following subcutaneous injection of huGM-CSF microspheres at 500 μg total dose (■), or subcutaneous injection of soluble huGM-CSF at 50 g (○) or 500 μg (♦) (B). The specific activity of the huGM-CSF in serum (activity in serum/activity of standard) was also determined at each time point (C).

carboxyl end group may increase the rate and quantity of water taken up by the polymer blend relative to pure end-blocked PLGA. Since water is a reactant, the degradation rate of the polymer matrix should increase. Secondly the carboxyl end groups of PLA may catalyze the degradation reaction. A combination of both mechanisms may also be possible.

The integrity and biological activity of huGM-CSF released *in vitro* were assessed by RP and SE chromatography, and TF-1 bioassay, respectively. Only minor changes were observed in the RP and SE chromatograms of huGM-CSF released from microspheres *in vitro* (Figs. 4B and 4C). These chromatograms suggest possible degradation of unglycosylated huGM-CSF from the day 2 sample; however, the low concentration of huGM-CSF released at this time point necessitated a concentration step prior to RP and SE analysis which may have contributed to their slightly altered chromatograms. Only a slight reduction in the overall specific bioactivity was measured for released huGM-CSF over the 7-day incubation period. Microsphere encapsulated GM-CSF was also directly analyzed following extraction with methylene chloride (data not shown). In these experiments SDS-PAGE and bioassay indicated no deterioration of the protein, suggesting that the subtle changes observed in the RP and SE chromatograms following the *in vitro* release studies may be related to the acidic micro-environment generated during degradation of the microspheres. While additional studies are warranted to investigate the physical condition of huGM-CSF released from microspheres in finer detail (e.g. peptide mapping to investigate subtle chemical alterations) the results obtained suggest that neither the encapsulation process nor the incubation in an aqueous environment as huGM-CSF is being released from the microspheres has serious adverse effect on the integrity or activity of the molecule.

The fact that huGM-CSF is released *in vitro* in relatively intact and biologically active form is surprising given the literature concerning the problems encountered with denaturation of PLGA encapsulated proteins (14–19). Our experience suggests that huGM-CSF may be particularly resistant to denaturation processes encountered during microsphere encapsulation and *in vitro* release studies. Encapsulation of other proteins in our laboratory by the phase separation process (data not shown) have not in general prevented protein denaturation or aggregation suggesting that the physico-chemical properties of huGM-CSF itself, rather than the encapsulation process, may account for the results shown here.

Large doses of huGM-CSF microspheres were administered to mice in order to determine whether serum levels of huGM-CSF could be maintained by the slow release of huGM-CSF from the microspheres. In this experiment, injection of soluble huGM-CSF was rapidly cleared from mice with serum elimination half-lives of approximately 1.5 hr (Fig. 5). Mice injected with huGM-CSF microspheres were found to have serum levels of huGM-CSF which were maintained between 10 and 100 ng/mL for at least nine days following administration. Furthermore, when the serum samples were tested using a TF-1 bioassay it was found that the specific biological activity of circulating huGM-CSF was maintained at approximately 50% of the non-encapsulated huGM-CSF control. The low specific activity of the material released *in vivo* may have been due to degradation of the huGM-CSF in some manner which was not modeled by the *in vitro* release studies (e.g. local degradation at the site of injection, circulating proteases, or neutralizing antibodies).

In contrast to the pharmacokinetics results obtained for huGM-CSF microspheres, systemic levels of muGM-CSF could not be measured following subcutaneous injection of muGM-CSF microspheres. The inability to measure circulating muGM-CSF may have been due in part to the relative insensitivity of the ELISA assay (the muGM-CSF assay is 1000 fold less sensitive than the huGM-CSF assay), however, even given the differences in assay sensitivity it is apparent that the systemic availability of muGM-CSF released from muGM-CSF microspheres is significantly lower than that of huGM-CSF released from huGM-CSF microspheres in the mouse. A previous investigation with radiolabeled muGM-CSF encapsulated and released from gelatin-chondroitin sulfate microspheres indi-

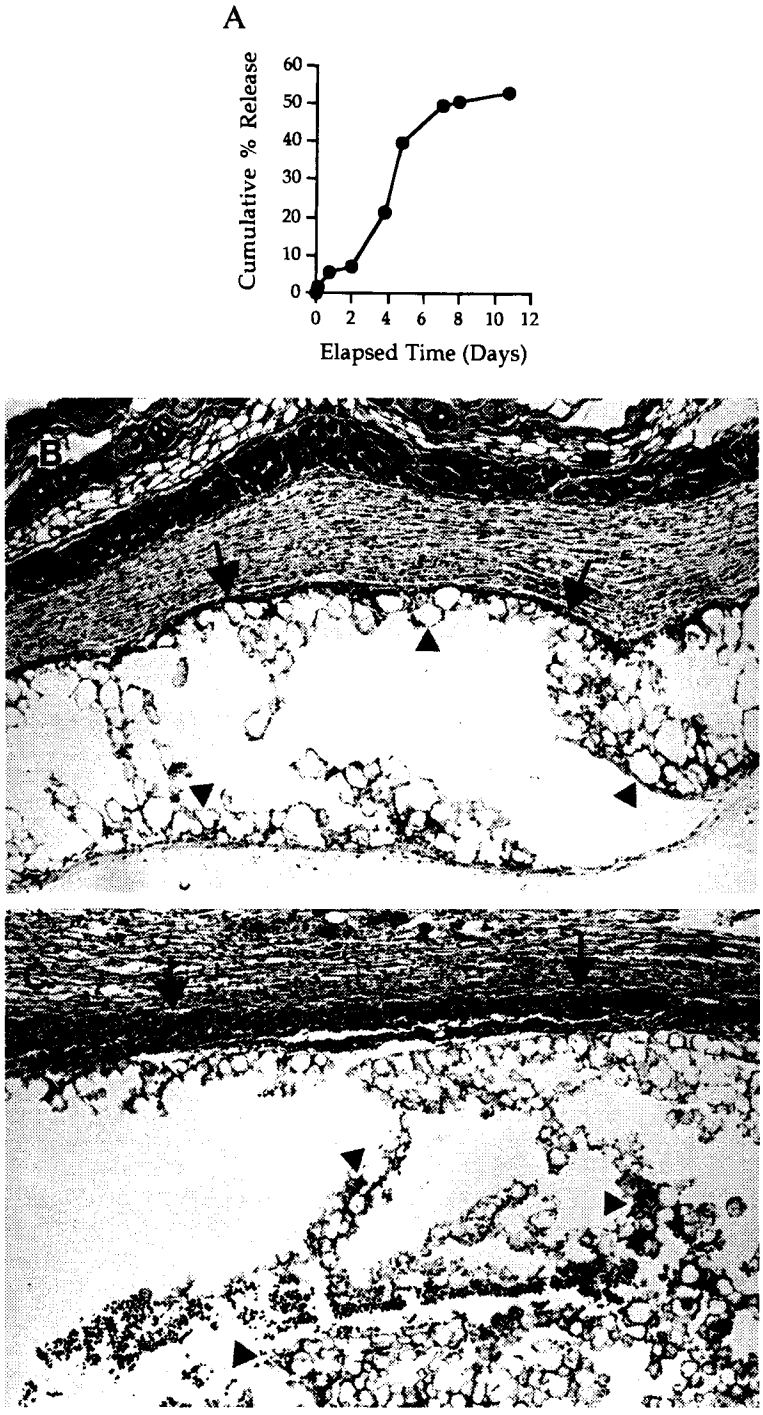

Fig. 6. Local tissue reaction in mouse to muGM-CSF microspheres prepared from a blend of 60% PLGA1, 20% PLGA2, and 20% PLA. The *in vitro* release kinetics of muGM-CSF from the microspheres is shown (A). The local tissue response to placebo (B) and muGM-CSF (C) microspheres are compared at day 2 (magnification = 25x). Compared to placebo, the muGM-CSF microspheres induced a larger mass at the subcutaneous implantation site, and a greater cellular infiltration within (arrowheads) and at the edge of the mass induced by the microspheres (arrows). The cellular infiltrate consisted predominately of macrophages in varying states of activation in response to placebo microspheres (D); whereas muGM-CSF microspheres (E) induced an intense infiltrate of neutrophils with fewer activated macrophages (day 7, magnification = 150x).

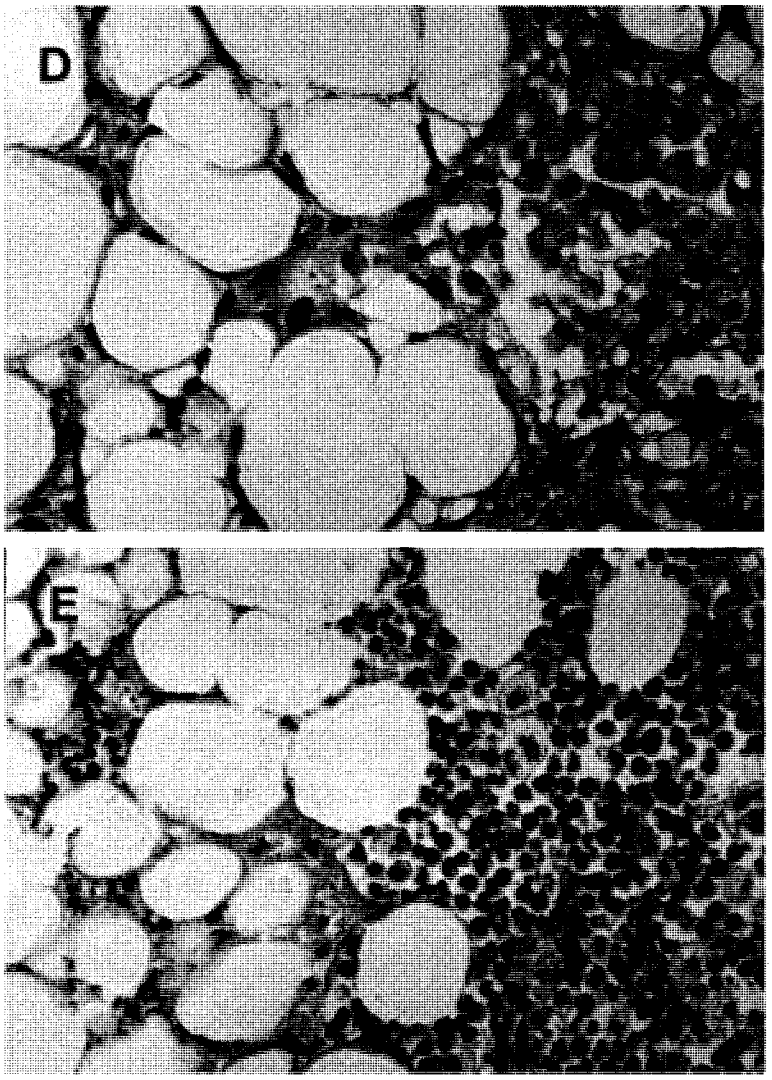

Fig. 6. Continued.

cates that muGM-CSF can be found in the circulation following smaller doses of muGM-CSF, however, no comparisons with huGM-CSF were reported in this study (10). The difference in the quantity of circulating mu vs. huGM-CSF most likely relates to the fact that huGM-CSF does not bind to murine cell receptors and is therefore more systemically available (29).

The response of mice to subcutaneous administration of placebo microspheres resulted in the formation and resolution of a granuloma typical of a response to a foreign body (nonphagocytosable material). The findings in this study were similar to those previously described for PLGA microspheres administered to rats, with the exception that in this study microspheres degraded earlier (day 28 in mice versus day 120 in rats), and in mice the observation of subcuticular fat, and regions of fat necrosis during the resolution phase of the foreign body response was more common than in rats (20). Differences in formulation and administration of more microspheres in the rats may account for the apparent enhanced rate of degradation of microspheres and resolution of the granuloma with fatty tissue changes in the mice in the current study. For the microspheres containing muGM-CSF, the intense influx of neutrophils and macrophages resulted in a subcutaneous mass that was larger, responded with a broader band of granulation tissue and fibrosis, and a slight lag in resolution of the granuloma when compared to placebo microspheres.

The kinetics of tissue swelling in response to muGM-CSF microspheres indicated a peak in swelling by day 14 (Figure 7). This correlates with the histopathological appearance of the tissue with loss of edema, no additional influx of cells, increasing encapsulation of the mass and degradation of many microspheres by day 14, followed by maturation and resolution of the granuloma by day 28 and beyond.

GM-CSF is known to be a powerful immunological modulator and is capable of chemotactic attraction of neutrophils, macrophages, and monocytes (33). The intense muGM-CSF specific cellular reaction induced at the site of administration may have also limited release of muGM-CSF to the systemic circulation, accounting for our inability to detect systemic muGM-CSF, and the prolonged foreign body response. Similar *in vivo* results have been observed by one of the authors in our laboratories (PJM, unpublished) with muGM-CSF encapsulated in ethylene vinyl acetate implants, suggesting that the intense

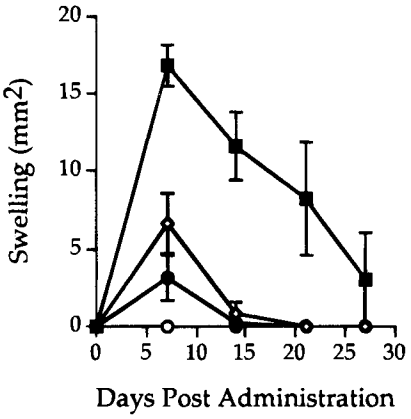

Fig. 7. Local tissue swelling kinetics following administration of muGM-CSF microspheres containing (■) 1 μg, (◇) 0.3 μg, (●) 0.1 μg, or (○) 0 μg (i.e. placebo control) muGM-CSF.

reaction to encapsulated muGM-CSF is related to the depot release action from the muGM-CSF microspheres rather than the type of polymeric vehicle in which the muGM-CSF is encapsulated.

The intense local tissue response to GM-CSF administered in combination with a foreign body implant (microspheres) may preclude their use as a single injection, controlled release therapy in indications that require maintaining systemic levels of GM-CSF. However, the local tissue response generated by GM-CSF microspheres over a short period of time may be useful in the application of vaccine adjuvancy where the recruitment and local control of antigen presenting cells is desired. Unlike traditional adjuvants such as alum which mediate a humoral immune response, GM-CSF is known to also stimulate the cell mediated immune response. Golumbek and coworkers have taken advantage of this difference and reported the use of muGM-CSF microspheres prepared from collagen and chondroitin sulfate as a vaccine adjuvant in a murine melanoma model (10). In their studies macrophages and dendritic cells (antigen presenting cells which mediate the cellular immune response) were observed in the vicinity of muGM-CSF microspheres, presumably attracted to the site by the action of slow release GM-CSF.

In this report we have demonstrated the encapsulation of GM-CSF into PLGA/PLA microspheres and have characterized these microspheres both *in vitro* and *in vivo*. These studies have shown the influence of formulation parameters on release kinetics and have demonstrated the local tissue response when GM-CSF is released in depot form. The results of these studies support further investigation of GM-CSF microspheres in controlled release indications.

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