Drug Development and Industrial Pharmacy, 24(9), 841–852 (1998)

RESEARCH PAPER

Preparation and Testing of Cyclosporine Microsphere and Solution Formulations in the Treatment of Polyarthritis in Rats

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ABSTRACT

We prepared a microencapsulated sustained-release formulation of cyclosporine A (CsA) and compared its efficacy to the solution formulation of cyclosporine A (Sandimmune, Sandoz) in an attempt to improve the treatment of rheumatoid arthritis. Microspheres containing cyclosporine were prepared with poly(lactic co-glycolic acid) (PLGA), a polymer in the submicron particle range of 0.22–0.8 μm. Studies were carried out to determine uptake rates and mechanisms of lymphocyte inhibition mediated by macrophages containing CsA microspheres in vitro. The results of these studies were used to establish whether lower doses of the microencapsulated cyclosporine could be used in in vivo studies in the polyarthritic rat model for rheumatoid arthritis. In vitro dissolution testing revealed that CsA was released extremely slowly from microspheres for up to 48 hr (0.002%). Radiolabeled ^{3}H CsA was incorporated into some PLGA microspheres or the microspheres were labeled using a ^{99m}Tc radioligand when needed, and radiolabeling efficiency was consistently above 50%. Uptake studies at various microsphere-to-macrophage ratios (1:1, 1:5, 1:10) were carried out using ^{99m}Tc radiolabeled microspheres and macrophages obtained from normal and polyarthritic rats. Normal macrophages behaved significantly differently from arthritic macrophages throughout the study. Arthritic macrophages cause increased amounts of CsA to be released (68% of the dose) into the culture medium past 24 hr compared to normal macrophages (48% of the dose). This factor may account for the significantly increased inhibition (68.2%) of mixed lymphocyte culture proliferation in the presence of arthritic macrophages containing CsA-loaded PLGA microspheres over normal macrophages (48.2%) that were pre-exposed to the same microsphere dose. The equivalent quantity of CsA as

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that contained in the microspheres when placed in solution or the same quantity of blank PLGA microspheres caused decreased levels of lymphocyte inhibition when compared to the effects of CsA microspheres in macrophages of normal cells, but significantly decreased levels of inhibition in arthritic cells. From the in vivo studies, it is evident that CsA microspheres, even at low dose levels, were highly effective in inhibiting polyarthritis in rats.

INTRODUCTION

Adjuvant-induced polyarthritis in rats is an animal model that has been extensively used to study the pathogenesis of rheumatoid arthritis (RA) and to identify drugs that have potential therapeutic efficacy (1). This model resembles the human disease both clinically and pathologically. The pathogenesis of RA is associated with a complex immuno-inflammatory response resulting in chronic proliferative synovitis of diarthrodial joints, although the etiology of this disease is unknown (2). Evidence supports the theory that both the cellular and humoral immune systems are involved in the pathogenesis of this disease. RA can be induced in rats by the injection of heat-killed *Mycobacterium butyricum* suspended in light mineral oil into the subplantar region of the hindpaw. At the site of injection, an acute inflammation appears within 24 hr and reaches its maximum intensity approximately 4 days post-adjuvant injection. During the next 6–7 days, the inflammation subsides, and the animals appear to be recovering. However, between days 10 and 12 post-adjuvant, a polyarthritis develops involving hindpaws, forepaws, the ears, and the tail. This animal model has also been shown to possess immunological abnormalities similar to those of the human disease (3) such as reduced response of spleen and peripheral blood lymphocytes to mitogen Con A and enhanced response to Con A from cells associated with sites of inflammation. Studies have also shown that cyclosporine A (CsA) can inhibit both adjuvant arthritis and collagen arthritis in rats when administered at the time of disease induction. The effects, however, on established arthritis in these animals appear to be different depending on the animal model. Matthews et al. (4) and Borel et al. (5) have shown that the administration of CsA to adjuvant arthritic rats starting on day 15 post-adjuvant injection decreases hindpaw swelling and increases body weight.

However, toxicity is a major concern associated with the use of CsA. Nephrotoxicity, hepatotoxicity, hirsutism, neurotoxicity, hypertension, and altered coagulability have all been reported with CsA (6). In RA patients treated with CsA, the most prevalent adverse effects include nephrotoxicity, hypertension, gastrointestinal intolerance, hypertrichosis, and tremors (7,8). These adverse effects may be due to high systemic levels of CsA. Recent reports by D'Souza et al. (9,10) indicate that the clearance of CsA is decreased in animal models of arthritis and diabetes. Therefore, when treating RA patients with CsA, the dose and blood levels of this drug should be carefully monitored because of drug toxicity, and because of the effect of the disease state on the disposition of the drug. To reduce systemic blood levels and associated toxicity, a sustained-release cyclosporine microsphere system employing subtherapeutic doses of CsA was evaluated.

One of the principal purposes of this study was to improve the treatment of rheumatoid arthritis by using CsA-containing microspheres. Because it is known that macrophages engulf foreign particles, it is possible that they may relocalize at points of chronic inflammation and allow the CsA released from the biodegrading microspheres to inhibit T lymphocyte function, thereby abolishing the inflammatory response in rheumatoid arthritis.

To study the effects of CsA-loaded microspheres, several types of microspheres were formulated and various in vitro and in vivo studies were carried out with the following objectives in mind:

1. To formulate, determine proper size, and evaluate dissolution characteristics of blank poly(lactic *co*-glycolic acid) (PLGA) microspheres and PLGA microspheres containing either cold CsA or ^{3}H CsA.
2. To radiolabel and evaluate the stability of uptake of PLGA microspheres containing amino polystyrene with ^{99m}Tc coupled radioligand into macrophages of normal and arthritic rats.
3. To characterize the in vitro release of CsA from macrophages (obtained from normal and arthritic rats) that have been presented with ^{3}H CsA microspheres, and to study the inhibitory effects of blank and CsA microspheres containing macrophages (normal and arthritic) or CsA in solution on mixed lymphocyte transformation in vitro.
4. To compare the effect of low dose (1 and 2.5 mg/kg) CsA microspheres and Sandimmune (Sandoz,

NJ) administered intraperitoneally (i.p.) on the inhibition of polyarthritis in rats, and to examine the effect of chronic i.p. administration of H CsA microspheres (1 mg/kg i.p.) on tissue accumulation in arthritic rats.

METHODS

In-Vitro Studies: Formulation of Cyclosporine-Containing Microspheres

Cold PLGA–CsA Microspheres

PLGA 50:50 and CsA were dissolved in methylene chloride. The solution was emulsified into distilled water containing polyvinyl alcohol (PVA) and Tween 80 using a biohomogenizer for 30 min. The contents were further emulsified using a Branson Sonifier (Fisher Scientific, Pittsburgh, PA) probe to achieve an average emulsion droplet size of less than 1 μm. This process was continuously monitored by sampling the emulsion every 5 min and observing the emulsion under a light microscope. The emulsion was allowed to gradually warm past this time by sonication at a power level of 40 for 30 min. This process was continuously monitored by sampling and observing the emulsion under a light microscope. Methylene chloride was removed under reduced pressure. The formed microspheres were recovered by centrifugation and were rinsed three times with fresh distilled water. PVA and Tween 80 were removed by dialyzing the microspheres in distilled water for 24 hr. Microspheres were placed in a desiccator and stored at −30°C till used.

PLGA–^{3}H CsA Microspheres

Formulation procedures were identical to the above procedure except that ^{3}H CsA (50 μl, 1 μCli/μl) was added to the cold CsA solution in methylene chloride.

Formulation of Non-Cyclosporine-Containing Microspheres

PLGA–Amino Polystyrene Microspheres

PLGA 50:50 (75 mg) and amino polystyrene (25 mg) were dissolved in 10 ml of 70:30 methylene chloride and 1-methyl 2-pyrrolidine. The solution was dispersed into 100 ml of distilled water containing Tween 80 and PVA using a propeller stirrer for 5 min at 800 rpm. The contents were further emulsified using a Branson Sonifier probe (at a power level of 60) for 15 min to achieve an average emulsion droplet size of less than 1 μm. This process was continuously monitored by sampling the emulsion every 5 min and observing the emulsion under a light microscope. The formed microcapsules were centrifuged out at 6000 rpm for 20 min, rinsed three times with 100-ml portions of fresh distilled water, placed in a desiccator, and stored at −30°C until used.

Formulation of Blank Microspheres

Microspheres of PLGA alone were prepared using the same procedure as described. Methylene chloride (10 ml) was used as the internal phase.

Sizing of Microspheres

Microspheres were sequentially filtered through 10, 2, 0.8, and 0.22 μm nylon HPLC filters to obtain a working size range for the in vitro and in vivo studies. Particles between 0.22 and 0.8 μm were used for all studies.

Radiolabeling of PLGA–Amino Polystyrene Microspheres

A bifunctional chelate 2, 3, 5, 6 tetra fluorophenol mercaptoacetyl diglycine-γ-butyrate (MAG_2-GABA-TFP, 0.3 mg; Neorx, Corp., Seattle, WA), was dissolved in 0.9 ml isopropyl alcohol. A 0.6-ml portion of this solution was acidified by addition of 0.16 ml of glacial acetic acid–0.2 N HCl (2:14). The acidified ligand (0.5 ml) was added to a tube containing premixed stannous gluconate complex (sodium gluconate 50 mg, stannous gluconate dihydrate, 1.2 mg/ml) and ^{99m}Tc (about 100 mCi/ml). The contents were incubated at 75°C for 15 min and then neutralized with 0.5 ml of bicarbonate buffer. A known amount of PLGA–amino polystyrene microspheres in saline suspension was added to the activated ligand and allowed to incubate for 30 min. The reaction contents were centrifuged and washed twice with pH 7 phosphate-buffered saline (PBS) to remove unreacted free ^{99m}Tc complex. A dose-calibrated aliquot of the microsphere suspension was used for various studies.

In Vitro Stability Studies on ^{99m}Tc-Radiolabeled PLGA–Amino Polystyrene Microspheres

Stability Studies in Culture Medium or Phosphate-Buffered Saline

A calibrated aliquot of radiolabeled microspheres was placed in triplicate sets of microcentrifuge tubes containing 1.5 ml of culture medium (RPMI 1640 [Gibco

Laboratories, MD] supplemented with 10% fetal calf serum [FCS, Gibco Laboratories]) or pH 7 PBS. At 4, 24, and 48 hr, the tubes were spun in an ultracentrifuge and the supernatant was replaced by fresh medium. The activity in the supernatant and the microspheres was measured by means of a gamma well counter at 48 hr. The activity associated with each fraction was expressed as a percentage of the total activity of all samples and the cumulative percent radiolabel released was calculated for each time point. A scatter plot of cumulative percent radiolabel released versus time was prepared and best fit equations were calculated. These curves were used to predict radiolabel release rates at other times.

Stability Studies in Rat Plasma

The above study was repeated using fresh rat plasma at each time point.

In Vitro Dissolution Kinetics of CsA From PLGA–CsA Microspheres

CsA (4.95 mg) containing PLGA microspheres of 0.22–0.8 μm diameter was placed in a dialysis bag (MW 14,000 cut off) before being incorporated within a USP basket assembly and stirred at 100 rpm in pH 7.3 phosphate buffer for 48 hr. Aliquots of the dissolution medium (2 ml) were sampled at 0.5, 1, 2, 4, 8, 24, and 48 hr. CsA concentrations were measured using a radioimmunoassay (RIA) kit (Incstar, Inc., Minneapolis, MN). A cumulative percent CsA release versus time plot was constructed.

Microsphere Uptake by Macrophages

Cell Isolation Procedures

Peritoneal exudate cells were isolated from unstimulated normal and arthritic rats. The cells were isolated using 50 ml of glucose nutritive medium (GKN) composed of Hanks' balanced salt solution (HBSS) supplemented with 0.2% glucose, centrifuged at 400 × *g*, resuspended briefly in 1 ml of distilled water in order to lyse red blood cells, and restored to isotonicity using 1 ml of 1.8% w/v NaCl. The cells were then plated in 100 ml culture flasks containing 25 ml of culture medium (RPMI 1640) supplemented with 10% FCS for 1.5 hr. Culture medium containing nonadherent cells was aspirated out, and the flask was rinsed twice with GKN. The adherent cells were gently scraped off the flask using a rubber policeman, counted in a hemocytometer, and tested for viability using trypan blue. The macrophages were then diluted in culture medium to obtain a final dilution of 0.5×10^7 cells in 2 ml. Two milliliters of cell suspension was plated in 25-ml culture flasks in a CO_2 incubator (37°C, 5% CO_2) for 1.5 hr before use.

Study Protocol

Microsphere uptake studies were carried out over 1 hr. Six flasks were used for each time point. A stock suspension of ^{99m}Tc-radiolabeled microspheres in RPMI 1640 supplemented with 10% FCS was prepared so that 1 ml of stock suspension contained 5×10^7 microspheres. Serial dilutions of the stock solution were carried out to obtain microsphere suspensions of 2.5×10^7 and 0.5×10^7 microspheres/ml. Two milliliters of each microsphere suspension was placed in sets of 24 flasks each, containing plated macrophages (1×10^7 cells). At 15, 30, 45 min, and 1 hr culture medium containing nonphagocytosed microspheres was withdrawn from six flasks, the flasks were rinsed twice with 1-ml portions of fresh culture medium, and the culture medium and the rinses were placed in a tube. Adhered cells were dissolved using 2 ml of 2% w/v sodium dodecyl sulfate (SDS). The flasks were rinsed twice with 1-ml portions of SDS and the dissolved cells along with the washes were measured for ^{99m}Tc radiolabel activity using a gamma counter (Tracor Analytic, Pharmacia LKB Nuclear, MD).

Percent uptake (percent activity of the cell fraction) was expressed as a percent of the total activity in each flask according to the formula:

$$\% \text{ Uptake} = \frac{\text{activity of cell fraction}}{\text{activity of cell fraction} + \text{activity of culture medium}} \times 100$$

Release Profiles from Macrophages Containing CsA Microspheres In Vitro

Peritoneal exudate cells were obtained from normal and arthritic rats after i.p. injection of 45 ml chilled HBSS. Contaminating red blood cells were lysed by pulsing with 1 ml of distilled water followed by 1 ml of 1.8% w/v NaCl solution. The cells were plated in culture flasks containing RPMI 1640 supplemented with 10% FCS for 2 hr before removal of nonadherent cells. After three rinses, adherent cells were scraped off the flasks, counted, and tested for viability using trypan blue. One-milliliter aliquots of 5×10^5 macrophages in culture medium (RPMI 1640 supplemented with 10% FCS) were placed

in 1.1-ml culture tubes (Skatron, Inc., Norway). Aliquots (0.1 ml) containing 5×10^5 ^{3}H CsA microspheres (0.22–0.8 μm diameter) were added to each tube. The tubes were lightly capped and placed in a CO_2 incubator (37°C, 95% humidity) for 1 hr to allow the macrophages to engulf the microspheres. CsA release studies were then carried out. At 0.5, 1, 2, 4, 8, 20, 24, 48, 72, and 96 hr, groups of six tubes were harvested using a cell harvester (Skatron Inc.) equipped with 9-mm glass fiber filtermats that retain particles over 1 μm in size. The filter disks were transferred to scintillation tubes and allowed to dry overnight. Acetone (1 ml) was added to each tube to dissolve CsA and microspheres engulfed within cells. SDS (200 μl) followed by 10 ml of scintillation cocktail (Beckman, CA) was added to each tube. Counts (cpm) were obtained using a liquid scintillation counter (Beckman LS 2000) and interpreted as percent of the control (lymphocytes exposed to thymidine only) counts.

In Vitro Inhibition of Mixed Lymphocyte Culture Proliferation by Macrophages Containing Microspheres

Macrophages were isolated from normal and arthritic male Sprague Dawley rats as previously described. Aliquots of 1×10^5 macrophages in 0.4 ml of culture medium were used in all experiments. Mixed lymphocyte bands from diluted peripheral blood (1:2 in isotonic saline) from separate normal and arthritic rats were obtained using a density gradient procedure (ficoll 24 parts, 9% w/v and hypaque, 10 parts, 40% w/v). Contaminant red blood cells were lysed by pulsing with hypotonic saline and the lymphocytes were counted and tested for viability using trypan blue. Aliquots of 1×10^3 lymphocytes in 0.4 ml of culture medium were used for all experiments.

Blank and CsA-containing microspheres (1×10^5/0.1 ml) and CsA solution (1.1387 μg/0.1 ml) were preincubated with macrophages for 1 hr in a CO_2 incubator prior to use. Lymphocyte suspensions (0.4 ml) were added to each group of six tubes. ^{3}H thymidine (0.1 ml, 20 μCi/ml) was added after 24 hr to all tubes. Thymidine incorporation into transformed lymphocytes was measured at 48 hr after addition to the tubes. Details of the protocol are listed in Table 1. Cells were recovered using a cell harvester and counts per minute of ^{3}H thymidine were obtained using a liquid scintillation counter. Percent inhibition of ^{3}H thymidine uptake was interpreted as the in vitro percent inhibition of lymphocyte activity by blank microspheres, CsA in solution, or CsA within microspheres.

Table 1

Protocol Details

Groups	Study Description
1	Lymphocytes
2	CsA solution + lymphocytes
3	Blank microspheres in macrophages + lymphocytes
4	CsA microspheres in macrophages + lymphocytes

In Vivo Studies

Animals

Male Sprague Dawley rats (200–225 g, 7 weeks old) were used in all experiments. They were housed in individual cages and allowed free access to food and water.

Induction of Arthritis

Rats were injected into the subplantar region of the right hindpaw with 4 mg *Mycobacterium butyricum* (Difco Laboratories, PA) suspended in 0.1 ml of light mineral oil. The development of arthritis in both hindpaws was followed plethysmographically by mercury displacement on days 21, 28, 35, and 42 (weeks 0, 1, 2, and 3) post-adjuvant injection. Blood samples were drawn on the same days to measure whole blood CsA trough levels and to obtain serum alkaline phosphatase and blood urea nitrogen levels.

Dosing Protocol

To determine the ability of CsA (Sandimmune) and CsA microspheres to inhibit the established disease, CsA microspheres (1 and 2.5 mg/kg) and CsA in oil (Sandimmune, and 2.5 mg/kg, 6–10 rats) were administered i.p. every 2 days to separate groups of rats commencing day 21 post-adjuvant injection. A single CsA microsphere dose of 1 mg/kg i.p. was administered to a group of six rats. Microspheres were suspended in isotonic saline and Sandimmune (100 mg/ml) was diluted with olive oil. Doses were reconstituted to half the desired strength per milliliters. Twice the body weight in microliters was injected into rats.

Positive controls (rats with adjuvant arthritis) were administered blank PLGA microspheres (1 and 2.5 mg/kg) suspended in saline. Negative controls (normal rats) also received blank PLGA microspheres (1 and 2.5 mg/kg) suspended in saline or olive oil.

Assay Procedures

Blood samples (1 ml) were obtained each week by tail snips in ethylenediaminetetra-acetate- (EDTA) coated tubes. The samples were centrifuged at room temperature and 25 µl of the plasma was assayed for CsA content using an RIA kit (Cyclo-trac, Incstar). Serum alkaline phosphatase and serum blood urea nitrogen (BUN) levels were measured using colorimetric kits (Sigma Chemical Co., St. Louis, MO).

Calculations

Percent inhibition in arthritis was calculated using the formula below:

$$\% \text{ Inhibition} = \left(1 - \frac{V - V_{Neg}}{V_{Pos} - V_{Neg}}\right) \cdot 100 - \text{average percent inhibition at week 0}$$

where V = paw volume of test rat; V_{Neg} = average paw volume of negative controls on same day, and V_{Pos} = average paw volume of positive controls on same day.

Average percent inhibition of arthritis and standard error were calculated for hindpaws of animals in each group. Graphs of the percent inhibition versus time (weeks) were constructed for the five dosing regimens (microspheres: 1 and 2.5 mg/kg/2 days, 1 mg/kg once, and Sandimmune 1 and 2.5 mg/kg/2 days) for each hindpaw using the above formula.

Dose Accumulation Study

Rats were dosed with ^{3}H CsA microspheres (0.22–0.8 µm) 1 mg/kg/2 days for 3 weeks. At the end of 3 weeks, the rats were sacrificed and various tissues (brain, thymus, lungs, heart, kidneys, liver, spleen, rear paws, muscle, bone, mesenteric lymph nodes, stomach, small intestine, appendix, and large intestine) were removed. Tared quantities of tissue (approximately 0.5 g) were oxidized in an automated tissue oxidizer (Tricarb, Hewlett Packard, Avondale, PA) and counted using a liquid scintillation counter (LS 2000, Beckman). All values were extrapolated if required to total tissue and represented as a percent of the total recovered activity.

Statistics

A 2-factor repeated measures ANOVA was used to calculate levels of significance (p values) for within-subject and between-subject comparisons. Comparisons between serum levels were made using regular ANOVA. A $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Formulation of PLGA Microspheres

Spherical CsA-and amino polystyrene-containing microspheres were successfully formulated in the 0.22–0.8 µm range.

Drug Content

Microspheres were found to contain 9.38% w/w of cold CsA. The target encapsulation efficiency was 10% w/w. This corresponds to an overall encapsulation efficiency of over 99%. The lipophilic nature of CsA would help account for high entrapment efficiency.

In Vitro Dissolution Kinetics of CsA from PLGA–CsA Microspheres

Dissolution rates of CsA from the microspheres were extremely slow in the 48-hr study (Table 2). Only 0.002% of the available CsA in the microspheres leached out into the dissolution medium at 48 hr. A small burst effect is observed up to 8 hr. This proved that CsA was effectively entrapped within the PLGA matrix.

Radiolabeling of PLGA–Amino Polystyrene Microspheres and Radiolabel Stability

At least 50% radiolabeling efficiency was achieved using the MAG_2–GABA–TFP radiolabel technique in all studies. The amino polystyrene was incorporated into the microsphere in order to provide an amine (NH_2) func-

Table 2

In Vitro CsA Dissolution Study

Time (hr)	Cumulative Percent CsA Released
0.5	0.00041
1	0.00029
2	0.00029
4	0.00039
8	0.00037
24	0.00123
48	0.00216

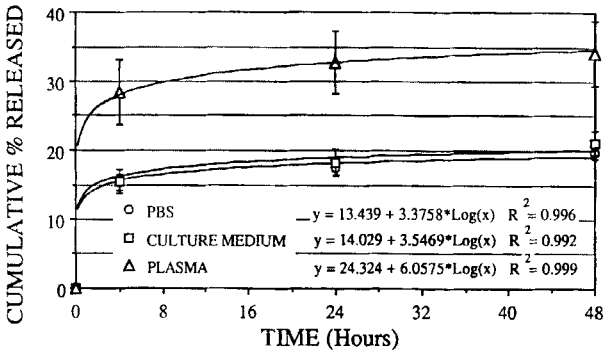

Figure 1. Radiolabel stability studies in PBS, HBSS, and rat plasma.

tional group for radioligand attachment. An amide linkage was formed between this group and the radioligand. A graph of cumulative percent radiolabel released versus time for ^{99m}Tc-radiolabeled microspheres in PBS, culture medium, and rat plasma is shown in Fig. 1. The curve-fitted equations predict that more than 90% of extractable radiolabel is released into the supernate within 1 hr. From the above data it was decided to allow all ^{99m}Tc-radiolabeled microspheres to incubate in PBS for 1 hr in order to leach out unbound radiolabel before use in all further studies requiring ^{99m}Tc radiolabel.

Microsphere Uptake by Macrophages

Figure 2 describes ^{99m}Tc–PLGA microsphere uptake profiles of normal and arthritic macrophages in vitro. Maximal uptake at the end of 1 hr was exhibited by normal macrophages. Normal macrophages exhibited greater than 90% viability at all dose levels at the end of 1 hr. No significant dose-related differences were observed in the case of normal macrophages (ANOVA, repeated measures, $p < 0.05$), but the highest dose ratio (10:1) in the case of the arthritics exhibited significant differences with time when compared to the other doses (ANOVA, repeated measures, $p < 0.05$). Uptake profiles were found to be significantly different for normal and arthritic macrophages at each dose ratio (ANOVA, repeated measures, $p < 0.05$). For the arthritic macrophages, the highest uptake was observed at 15 min (27% dose, 10:1 microsphere-to-macrophage ratio) and was found to be significantly different from the other dose ratios at that time and also through the rest of the study. In the context of macrophage phagocytic ability in the presence of microspheres, it may be possible to draw

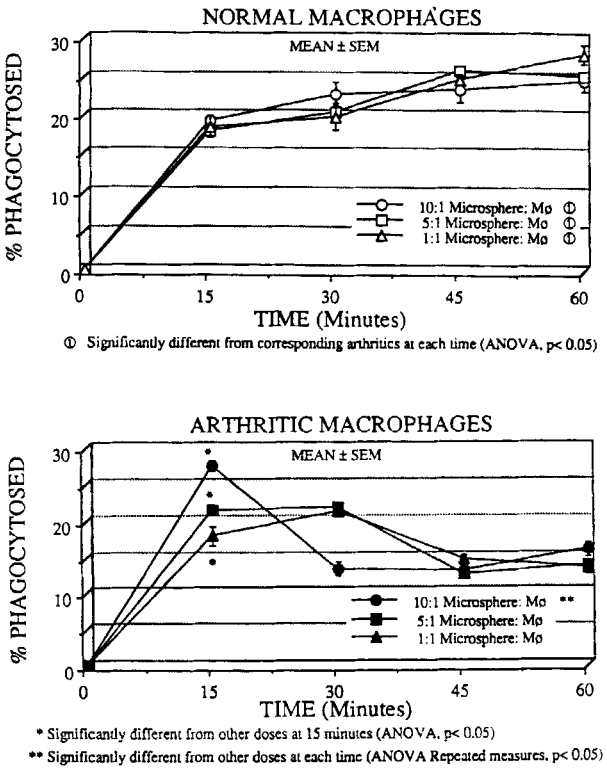

Figure 2. Microsphere uptake study by normal and arthritic macrophages.

some conclusions as to the decreased phagocytic uptake at 1 hr of macrophages derived from arthritic rats in comparison to normal rats. It may be that the high microsphere loading of arthritic macrophages, due to their increased state of activation, contributed to cell cytoxicity. In fact, an increased cytotoxicity associated with the 10:1 microsphere-to-macrophage ratio (69% arthritic cells viable at the end of 1 hr compared to more than 90% viable at the start) supports this theory. Because CsA-containing microspheres were not employed in this study, no suppression of macrophage activation or release of toxic metabolites was achieved. From the previous paragraphs it may be concluded that in the microsphere uptake studies: (*a*) Macrophages from normal cells were not in an activated state and therefore did not engulf microspheres rapidly. (*b*) Macrophages from arthritic rats seemed to be in a higher state of activation and therefore ingested increased numbers of microspheres initially. Subsequently, the macrophages either died or lost their ability to adhere to the culture flask and were discarded in the supernatant along with microspheres that were not

yet ingested. This is in agreement Tabata et al. (11,12), who observed that after ingesting more than 25 μg of polymer, macrophages were incapable of adhering to culture plates.

In Vitro Release of CsA from Microspheres within Macrophages

CsA levels within macrophages (normal and arthritic) decreased exponentially in the first 24 hr but were almost constant thereafter up to the end of the study period (96 hr). Normal macrophages retained a higher percentage of CsA either entrapped within microspheres or released within the cytoplasm of the macrophage in this time period compared to arthritic macrophages (Fig. 3). Higher levels (52% CsA dose) in normal macrophages compared to arthritic macrophages (32% CsA dose) may be explained on the basis of macrophage activation. Activated macrophages possess a more acidic environment which could lead to increased catalytic degradation of PLGA. Because activated macrophages are also more phagocytic, an increased amount of CsA released into the cell cytoplasm from the engulfed microspheres may be excreted extracellularly during the process of phagocytosis. The initial exponential decline is characteristic of drug diffusion (Fig. 3), however, the drug levels do not fit the classical Higuchi plot equations for drug diffusion from an insoluble matrix, nor do they fit a first-order exponential equation. Therefore, it must be assumed that some other process or combination of processes is in operation. A burst effect may be in operation in the first 24 hr. Note that the drug content of CsA microspheres used in the present studies was relatively high (More than 9% w/w) compared to microspheres prepared by other researchers (maximum 7%). At this level of drug loading, a substantial burst effect occurs and the normal biphasic drug release pattern from the PLGA microsphere is disrupted, leading to a merging of the release profiles contributed by diffusion and subsequent erosion. This study proves that a substantial amount of CsA is available for action within 24 hr, especially in the case of arthritic macrophages (60% dose).

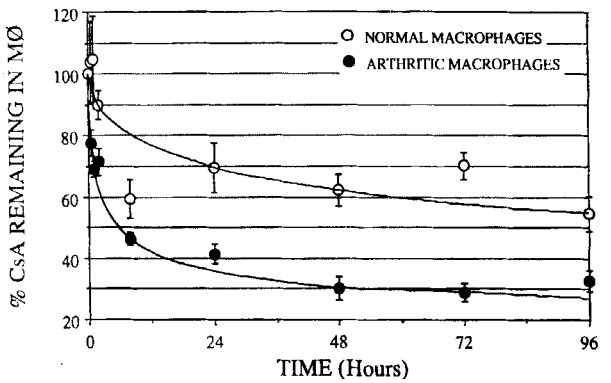

Figure 3. In vitro release study of CsA from microspheres within macrophages.

In Vitro Inhibition of Mixed Lymphocyte Activity by Macrophages Containing Microspheres

Figure 4 shows the effect of various pretreatments on transformation of normal and arthritic lymphocytes after addition of thymidine at 24 hr. In studies involving normal cells, no significant differences in lymphocyte transformation were observed between the effect of CsA in solution or that of macrophages previously presented with CsA microspheres. However CsA microspheres within arthritic macrophages produced significantly increased inhibition ($p < 0.05$) of lymphocyte transformation (68.17 ± 11.2%) compared to the effect of CsA in

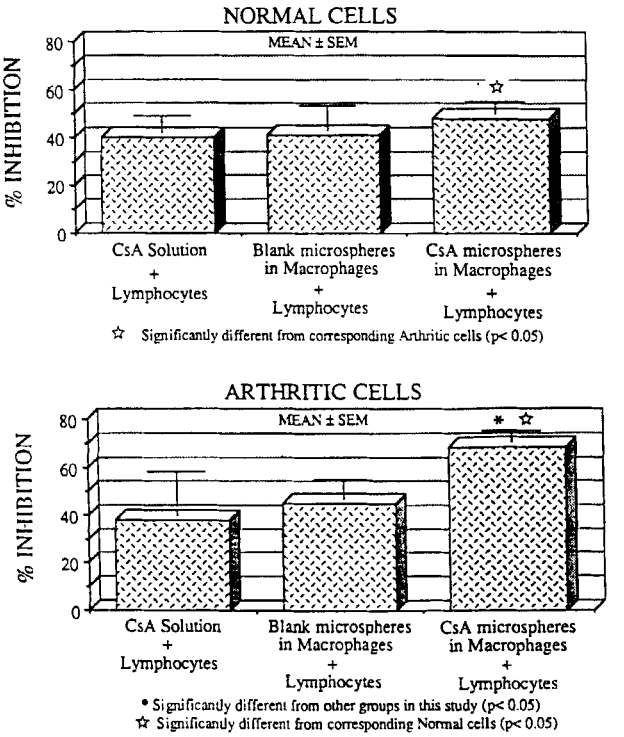

Figure 4. Lymphocyte transformation studies using normal and arthritic cells.

solution (38 ± 32.07%) or blank microspheres within macrophages (44.7 ± 20.51%). In addition, this group was found to be significantly different from the corresponding group of normal cells (CsA microspheres in normal macrophages + normal lymphocytes, 48.2 ± 11.3%). The increased inhibition may stem in part from higher CsA levels associated with arthritic macrophages (see Fig. 3), which may in turn be due to the higher activation levels of these macrophages. Note that blank microspheres within macrophages produced about the same levels of inhibition as those of CsA solution in both normal and arthritic macrophages.

The following conclusions may be drawn from the in vitro study. Microspheres of PLGA and PLGA containing CsA or amino polystyrene were formulated and used to study in vitro phagocytosis, CsA release, and effect on lymphocyte transformation. Radiolabeled microspheres were taken up differently by normal and arthritic macrophages, possibly because of the differences in activation. Release of ^{3}H CsA from macrophages engulfing PLGA microspheres was different past 24 hr in arthritic and normal macrophages. Higher levels of CsA released by arthritic macrophages compared to normal macrophages may account for the significant increase in inhibition of lymphocyte proliferation in the presence of arthritic macrophages compared to normal macrophages. These studies confirm that submicron-size CsA-loaded microspheres are taken up in sufficient quantities into arthritic macrophages, and that CsA is released in sufficient amounts in a 96-hr period to cause in vitro inhibition of lymphocyte proliferation.

In Vivo Studies

Treatment of Polyarthritis

The results for inhibition in arthritis after CsA microsphere and Sandimmune treatment are shown in Fig. 5. Percentage inhibition of arthritis in the left and right hindpaw was maximum at week 3 for the 2.5 mg/kg/2 days multiple dosing regimen (198.67% left paw, 78.52% right paw) (28,29). At week 3, inhibition of arthritis with the 1 mg/kg/2 days multiple-dosing regimen was lower in both paws (118.46% left, 74.32% right) and for the 1 mg/kg dose given once (23.92% left, 34.56% right). In the latter cases, peak inhibition also occurred at week 3 for both hindpaws. During the study period (3 weeks) the groups that were administered Sandimmune 1 mg/kg/2 days and 2.5 mg/kg/2 days exhibited maximum inhibition at week 2 (55.77% left, 7.64% right and 145.95% left, 10.08% right, respectively). For both of these doses, a drastic reduction in effectiveness (negative inhibition)

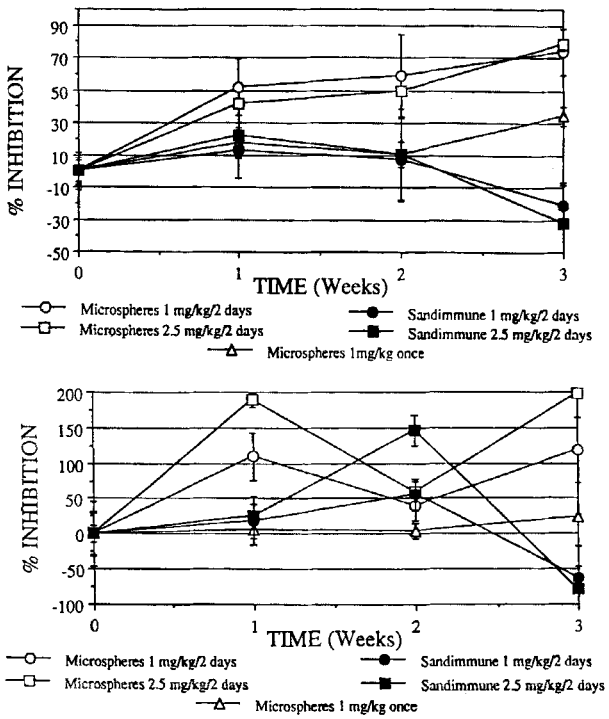

Figure 5. Inhibition of arthritis in the right (top) and left (bottom) paw after treatment with different cyclosporine formulations.

was observed at week 3. Paw data (injected and noninjected, i.e., right and left paw) for the 1 mg/kg/2 days and 2.5 mg/kg/2 days microsphere dosing regimen were significantly different from the corresponding Sandimmune-treated regimens (ANOVA, repeated measures, $p < 0.05$) with the exception of the noninjected (left) paw data for 1 mg/kg/2 days microsphere dosing regimen, which was not found to be significantly different from Sandimmune 1 mg/kg/2 days. The 1 mg/kg one dose CsA microsphere treatment was found to be significantly different from the 1 mg/kg/2 days CsA microsphere dose for both paws. The above data reflect greatly improved inhibition of polyarthritis when compared to the conventional oral dosing regimens utilizing higher doses (6.25–75 mg/kg) of CsA (13).

Serum CsA, BUN, and Alkaline Phosphatase Levels

Serum CsA levels are represented in Fig. 6. Note that at lower doses (1 mg/kg/2 days and 1 mg/kg one dose), serum levels were consistently below 63 ng/ml throughout the study, whereas the 2.5 mg/kg doses for the mi-

crosphere- and Sandimmune-treated doses were highest at week 2 (103.23 and 200.66 ng/ml, respectively). Borel (5) suggested a 50% inhibitory dose of 50 ng/ml for inhibition of mixed lymphocyte culture reactivity in vitro. The levels observed in this study are much lower than those observed by other researchers using CsA. Bioavailability of CsA was calculated to be 57.6% at 24 hr after a single i.p. dose in rats. Nooter et al. (14) also observed rapid blood uptake and tissue distribution of CsA following i.p. injection of CsA in rats. However, following i.p. injection of Sandimmune (20 mg/kg) in rabbits, D'Souza et al. (15) observed lower CsA blood levels of 986 ng/ml and a decreased bioavailability of 22.5%.

Serum BUN levels for all dose regimens are shown in Fig. 6. A significant elevation of BUN levels was observed at week 1 for the microsphere-treated groups, and an increased BUN level was observed for the 2.5 mg/kg/2 days treated group at week 3 when compared to baseline week 0 levels of untreated arthritic controls. Sandimmune (1 mg/kg/2 days) produced elevated BUN levels at weeks 2 and 3, whereas BUN levels of the Sandimmune 2.5 mg/kg/2 days treated group were normal throughout the study.

Serum alkaline phosphatase (ALKP) levels (Fig. 6) were elevated in all treated groups at week 1 except for the 1 mg/kg one dose microsphere treatment regimen. It is possible that CsA did not augment the disease-induced rise in inflammatory mediators associated with polyarthritis. In fact, low-dose treatment of CsA actually helped normalize abnormal ALKP levels in all treated groups.

Dose Accumulation Study

Figure 7 shows the tissue accumulation of ^{3}H CsA microspheres in arthritic rats chronically dosed with 1 mg/kg microspheres i.p. for 3 weeks. Data are represented as the percentage of total recovered activity. Maximum accumulation of CsA on a per-organ basis occurred in the liver followed by the spleen, lymph nodes, lungs, thymus, and kidneys. On a per-gram basis, maximum accumulation was apparent in the lymph nodes, thymus, and spleen. The chronic study revealed that microspheres were preferentially sequestered into the lymphatic tissue during transfer into the systemic circulation and the reticuloendothelial system thereafter. Kidney accumulation was low (1.22% dose/g). Therefore nephrotoxicity (increased BUN levels) because of chronic accumulation of CsA was not observed.

From the in vivo studies, it is evident that CsA micospheres, even at low dose levels, were highly effective

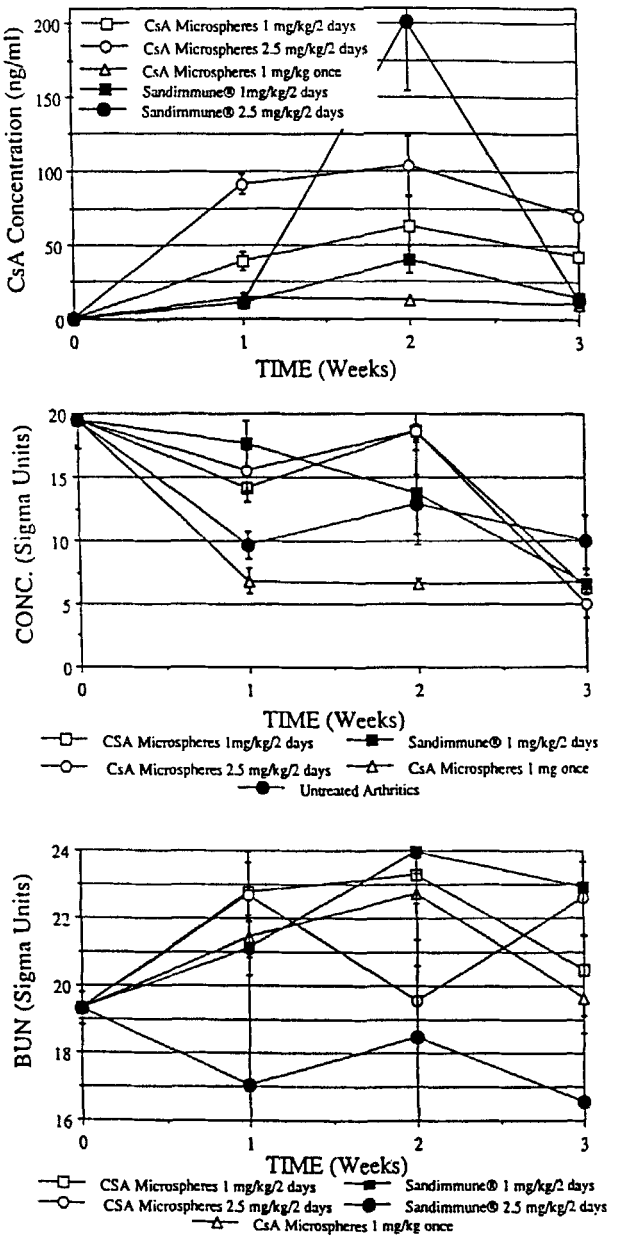

Figure 6. Serum CsA levels (top), ALKP levels (middle), and serum BUN levels (bottom) as a function of time after treatment with different CsA formulations in the rat.

in inhibiting polyarthritis in rats. Also, serum CsA levels were consistently below 104 ng/ml for the microsphere-treated groups. The Sandimmune 2.5 mg/kg/2 days treated group exhibited a higher serum level of CsA. At week 3 BUN levels were in most cases not significantly different from those of baseline arthritics at week 0, indicating that kidney toxicity occurred throughout the study

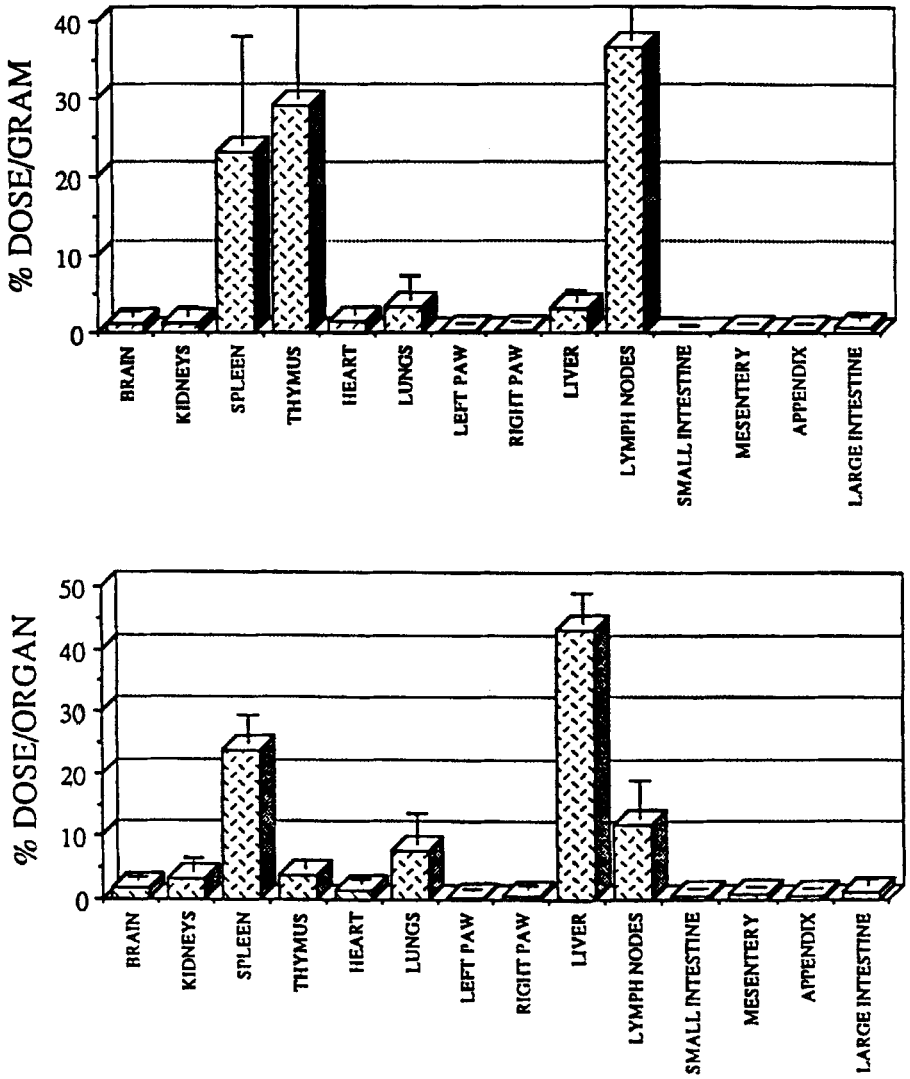

Figure 7. Biodistribution of CsA in the rat after administration of tritiated CsA contained in microspheres for 3 weeks.

in all microsphere groups. ALKP levels were higher at week 1 compared to other weeks but were normal thereafter, indicating that hepatotoxicity was reduced during treatment. Finally, chronic dosing with ^{3}H CsA microspheres revealed that lymph nodes accumulated large quantities of CsA microspheres.

The results of this study prove that i.p. injection's of low-dose CsA microspheres were extremely effective in the treatment of polyarthritis in rats. The mechanism of action is related to macrophage-mediated lymph node accumulation of CsA microspheres, followed by systemic inhibition of circulating T lymphocytes.

REFERENCES

1. R. T. Owen, Adjuvant induced polyarthritis—an overview, Methods Find. Exp. Clin. Pharmacol., 2, 199 (1980).
2. S. H. Pollock, H. W. Matthews, and W. H. Spruell, Medical aspects of rheumatoid arthritis, U.S. Pharm., 4, 33 (1979).
3. S. C. Gilman, Immunological abnormalities in rats with adjuvant-induced arthritis—II. Effect of antiarthritic therapy on immune function in relation to disease development, Int. J. Immunopharm., 9, 9 (1987).
4. H. W. Matthews, S. H. Pollock, and P. F. Han, Effect

of arthritis in rats and rheumatoid arthritis on mitogenic responsiveness and the cyclic nucleotide system of lymphocytes isolated from various tissues, Fed. Proc. Amer. Soc. Biol., 46, 122 (1987).
5. J. F. Borel, Comparative study of in vitro and in vivo drug effects on cell-mediated cytotoxicity, Immunology, 31, 631 (1976).
6. S. C. Gilman, Lymphoid abnormalities in rats with adjuvant-induced arthritis. I. Mitogen responsiveness and lymphokine synthesis, Ann. Rheum. Dis., 43, 847 (1984).
7. M. Dougados and B. Amor, Cyclosporin A in rheumatoid arthritis: Preliminary clinical results of an open trial, Arth. Rheum, 30, 83 (1987).
8. M. E. Weinblatt, J. S. Coblyn, and P. A. Fraser, Cyclosporin: a treatment of refractory rheumatoid arthritis, Arth. Rheum., 30, 11 (1987).
9. M. J. D'Souza, H. W. Matthews, and S. H. Pollock, Cyclosporine disposition in adjuvant-induced arthritis, FASEB, 2, 7328 (1988).
10. M. D'Souza, L. Fowler, H. Solomon, and S. H. Pollock, Pharmacokinetics of cyclosporine in streptozotocin-induced diabetic rats, Drug Metab. Dispos. 16, 778 (1989).
11. Y. Tabata and Y. Ikada, Synthesis of gelatin microspheres containing interferon, Pharm. Res., 6, 296 (1989).
12. Y. Tabata and Y. Ikada, Protein precoating of polylactide microspheres containing a lipophilic immunopotentiator for enhancement of macrophage phagocytosis and activation, Pharm. Res. 6, 422 (1989).
13. S. H. Pollock, H. Matthews, B. Collier, P. J. DeSouza, J. Lhose, and M. J. D'Souza, Comparison of the anti-arthritic effect of cyclosporine A and cyclosporine G in rats, Pharm. Res., 7, S-162 (1990).
14. K, Nooter, F. Schultz, and P. Sonneveld, Evidence for a possible dose dependent pharmacokinetics of cyclosporine A in the rat, Res. Commun. Chem. Pathol. Pharmacol., 43, 407–415 (1984).
15. M. J. D'Souza, R. Venkataramanan, J. Augustine, M. Zemaitis, R. Ptachcinski, and G. Burckart, Combined effect of cyclosporine and prednisolone on oxidative drug metabolism, Pharm. Res., 4, 114S (1987).