Prolonged Anti-Inflammatory Action of DL-Lactide/Glycolide Copolymer Nanospheres Containing Betamethasone Sodium Phosphate for an Intra-Articular Delivery System in Antigen-Induced Arthritic Rabbit

Eijiro Horisawa,[1,3] Tsuyoshi Hirota,[1] Satoko Kawazoe,[1] Jun Yamada,[1] Hiromitsu Yamamoto,[2] Hirofumi Takeuchi,[2] and Yoshiaki Kawashima[2]

Received November 6, 2001; accepted January 4, 2002

Purpose. The objective of the present study was to develop prolonged anti-inflammatory action of DL-lactide/glycolide copolymer (PLGA) nanosphere incorporating a water-soluble corticosteroid (betamethasone sodium phosphate; BSP). Another aim was to demonstrate the biocompatibility and biologic efficacy of these BSP-loaded nanospheres directly administered into ovalbumin-induced chronic synovitis in the rabbit.

Methods. BSP-loaded nanospheres were prepared by an emulsion solvent diffusion method in oil (caprylate and caprate triglyceride). The drug releasing properties of the nanospheres were measured *in vitro* in phosphate buffer saline (PBS: pH7.4), and *in vivo* in rat air-pouch (pseudo synovial fluid). The BSP-loaded nanosphere suspensions were administered into the joint cavity in a model of antigen-induced arthritic rabbit and evaluated by measuring the joint swelling, and the biocompatibility was appraised by histologic microscopy.

Results. The BSP-nanospheres were a unimodally-dispersed particulate system with a mean diameter ranging from 300 to 490 nm, and BSP was efficiently entrapped in the lipophilic copolymer (PLGA), although its hydrophilic properties. The drug release-rate from the nanospheres in PBS was controlled by the molecular weight and the lactic/glycolic acid (LA/GA) ratio of the polymers. The *in vitro* releasing study demonstrated that sustained drug release occurred for over three weeks. In the antigen-induced arthritic rabbit, the joint swelling decreased significantly by administering BSP-loaded nanospheres during a 21-day period after intra-articular challenge. With regards to the prolonged anti-inflammatory efficacy, serum antibody to ovalbumin showed a sustained reduction during the period, and the steroidal effect appeared by the degradation of the polymer in the synovium. The BSP-nanosphere administered was phagocytosed by the synovial activated-cells and the cartilage degradation was almost prevented.

Conclusions. Direct intra-articular injection of a PLGA nanosphere system with a water-soluble steroid provided a prolonged pharmacological efficacy in the joints of arthritic rabbits. The local anesthetic in the knee-joints was evaluated to be safe and without biologic damage.

KEY WORDS: PLGA nanosphere; emulsion solvent diffusion method; intra-articular delivery system; antigen-induced arthritis; prolonged anti-inflammatory action.

[1] Maruho Co., Ltd., Pharmaceutical Research Laboratories, 2763, Takamiya-cho, Hikone, Shiga 522-0201, Japan.

[2] Gifu Pharmaceutical University, Department of Pharmaceutical Engineering, 5-6-1, Mitahora-higashi, Gifu 502-0003, Japan.

[3] To whom correspondence should be addressed. (e-mail: horisawa_ahl@mii.maruho.co.jp)

INTRODUCTION

Conventionally, as a therapy for the arthritis (osteogon-arthritis: OA, rheumatoid arthritis: RA), it is well known that local therapy by injecting anti-inflammatory agents (1–5) is effective. The direct injection of colloidal steroid-crystals into the joints is effective for the RA disease (4). However, it was reported that the drug rapidly disappeared from the articular cavity (5). From the view point of clinical therapy for QOL, the development of a prolonged-release system injected into the articular joints is desired. Therefore, to achieve a sustained anti-inflammatory effect, the administration of drug-encapsulated liposomes (6–10), microspheres prepared with serum albumin (11–13), gelatin/chondroitin 6-sulfate (14) and polylactic acid/polyglycolic acid copolymer (15–16) has been applied. These systems are required to prolong their retaining time in the articular cavity and to improve more their biologic safety and stability in the joints after administration. Recently, a new clinical problem of "crystal-induced pain" caused by the substance remaining in the joint has begun to receive considerable attention (17). The mechanism by which crystal-induced pain is generated in the human joint cavity remains unknown. Since the pain has not arisen so often with aqueous drug preparations, it is thought that the bio-incompatibility and the physicochemical properties (i.e., diameter, shape) of the drug particles are closely related to the pain induction. Davis *et al.* (11–12) found that steroidal microspheres prepared with several polymeric materials were phagocytosed by the synovial activated cells depending on their particle size. They assessed that the irritancy with synovial tissues depended on the biocompatibility of the colloidal particles. Hincal et al. (15) concluded that biodegradable PLGA microspheres are an excellent drug carrier for arthritic lesions using the radiopharmaceutical scintigraphical study in rabbits.

In our previous study (18), it was found that PLGA nanospheres injected into the rat joint cavity were preferably phagocytosed by the macrophages in the synovium. PLGA nanospheres might be more suitable for drug delivery to inflamed synovial tissue than microspheres due to their ability to penetrate into the synovium. We assumed that crystal-induced pain might depend on the size and bioimcompatibility of the microparticles introduced in the joint tissue. In this paper, a water-soluble corticosteroid was encapsulated into the nanospheres with a biodegradable polymer such as DL-lactide/glycolide copolymer, and poly D,L-lactic acid. The function of the resultant nano-particulate system was evaluated by *in vitro* and *in vivo* studies to develop a new prolonged anti-inflammatory system based on the above assumptions.

EXPERIMENTAL

Materials and Methods

Materials

DL-lactide/glycolide copolymer (PLGA) with average molecular weights (MW) of 19,900, 9900, and 5900, whose

0724-8741/02/0400-0403/0 © 2002 Plenum Publishing Corporation

copolymer ratio of D,L-lactide to glycolide was 75:25, were supplied by Wako Pure Chemical Industries Ltd.(Osaka). PLGA with MW of 18,500, 6500, whose copolymer ratio of 50:50 and poly D,L-lactic acid (PLA) with MW of 19,700, 6100 (PLA-0020, PLA-0005) were also supplied by Wako. These MWs of PLGAs or PLAs were determined by gel-permeation chromatography calibrated with a refractive index detector (LC-10A, Shimadzu, Kyoto). As a standard, polystyrenes were used. A water-soluble corticosteroid: betamethasone sodium phosphate (BSP) was obtained from Roussel Uclaf (Roma, Inuille-France). PVA with 80%-hydrolyzation degree and 300-polymerization degree (PVA-403, Kuraray, Osaka) was used as a dispersing agent. All other chemicals and solvents were of reagent grade.

Preparation of BSP-Loaded Nanospheres

BSP-loaded nanospheres were prepared using a modified emulsion solvent diffusion method in oil (19–21). Ten mg of BSP and 100 mg of the polymer, were dissolved in 3 mL of a mixture of acetone and methanol dissolved with 100 mg of sorbitan monooleate (Span-80, WAKO). The resultant polymer-drug solutions were poured into 60 ml of caprylate and caprate triglyceride (Triester F-810, Nikko Chemical, Tokyo) containing 2% hexaglycerin condensed ricinoleate (Hexaglyn PR-15, Nikko) at 2 mL/minute, under stirring at 400 rpm (Heidon 600G, Shinto, Osaka). The entire dispersed system was then centrifuged (43,400 ×g for 10 min, Kubota, Osaka). The sediment was dispersed in aqueous PVA solution (10mL) and centrifuged under the same conditions as above. An additional dispersing in distilled water (5mL) was carried out. The redispersed suspension was dried using a freeze dryer (VD-60, TAITEC, Osaka).

Physicochemical Properties of BSP-Loaded Nanosphere

The average particle size of the nanospheres dispersed in the aqueous medium was measured by means of a laser (He-Ne) particle analyzer (LA-700, Horiba, Kyoto).

The BSP-loaded nanospheres were dissolved in 5mL of acetone and added 15mL of a mixture of 1/30M phosphate buffer (pH 7.2) and methanol dissolved with buthyl *p*-hydroxybenzoate (internal standard; 62.5μg/mL). The resultant BSP-polymer solutions were assayed using high-performance liquid chromatography (LC-10A, Shimadzu, Kyoto) with an ultraviolet detector equipped with a column, ODS (A-312, YMC, Osaka) ; mobile phase, a mixture of 20 mL acetonitrile and 300 mL of 1/30 M phosphate buffer; wavelength, 223 nm. The drug recovery and content in the nanospheres were calculated from equations (1) and (2), respectively.

Drug recovery (%)
= weight of drug in nanospheres/weight of drug fed into the system (1)

Drug content (%)
= weight of drug in nanospheres/weight of nanospheres recovered (2)

The theoretical drug content is 9.09%, as calculated from the amounts of drug and polymer loaded.

In Vitro Drug Release Study

The BSP-loaded nanospheres were dispersed in 10 mL of 1/30 M phosphate buffer saline (pH 7.2) in a sealed jacketed beaker at 37°C. The suspension was stirred continuously at a constant rate using a shaker (Yamato, Japan). Aliquots of 1 mL were taken at various times up to 21 days. The BSP content in the dissolution medium was determined using the method mentioned above after sedimenting the nanospheres by centrifugation (at 43,400 ×g for 10 min).

The degradation of the PLGA chain in the nanospheres during the release-test was detected by measuring the MW at suitable intervals using gel permeation chromatography (with a high-performance liquid chromatograph LC-10A system, Shimadzu, Kyoto).

In Vivo Drug Release Study

Eight male wistar rats (age; 6–7 W, weighing 190–230 g) were used in an *in vivo* release-study. Ten mL of air was injected subcutaneously into the backs of the rats. After 24 h, to accelerate exudation, a 2% carboxymethyl-cellulose sodium aqueous solution (6mL) containing penicillin/streptomycin (10,000 IU, 10mg) was injected subcutaneously. After 24 h, to accelerate exudative inflammation, 0.5 mL of lipopolysaccharide (10 ng/mL) was injected subcutaneously and then 1mL of the BSP-loaded nanospheres (prepared with PLGA-7520; containing 5mg of the drug) dispersed in physiological saline was injected. The pouch was opened at 24, 72, and 168 h after the administration, and the exudation was immediately washed with saline. The amounts of the drug released in the inside exudation and those remaining in the nanospheres in the pouch were determined separately by the HPLC method. The BSP aqueous solution containing 50 mg of the drug was administered into the pouch and the drug content in the exudation was monitored as a control.

In Vivo Studies with Antigen-Induced Arthritic Rabbit

The antigen-induced arthritis in rabbit joints was prepared using the modified ovalbumin method (22,23). Monoarticular arthritis (left joint) was induced in the knee joints of 12 male New Zealand White rabbits (weighing; 2.5–3.5 kg).

Induction of Arthritis. Rabbits were immunized by intradermal injections of Freund's Complete Adjuvant (:FCA, Gibco, 1 mL emulsion containing 5 mg of ovalbumin, Sigma). At three weeks after the immunization, the arthritis was induced in the left knees by injecting 1 mL of a sterile saline containing 5mg ovalbumin. Simultaneously with boosting, the suspension of the nanospheres containing 3mg of BSP was administered into the articular joint cavity. An aqueous BSP (3mg) solution and physiological saline were used as a control and a blank, respectively. The animals were sacrificed by an overdose of pentobarbitone at 42 days after the joint challenge.

Measurement of Joint Swelling and Joint Temperature. The outside diameter of the rabbit knee was measured with calipers, at intervals between 1 and 42 days after the joint challenge. The amount of joint swelling was assessed by the width of the joints before (initial) and after the ovalbumin challenge. The latter value was subtracted from the former to give the amount of joint swelling. The joint skin temperature was measured with a contact-type surface thermometer (DUAL THARMO, Anritsu, Tokyo).

Cellular Infiltration into Joint Cavity. The number of cells contained in the joint wash was counted by a Tatai haemocytometer after being sacrificed at 42 days after the joint challenge.

Titer of Serum Antibodies to Ovalbumin. The inhibition of the ovalbumin-antibody generated in the rabbit serum was measured using an enzyme-linked immunosorbent assay (ELISA). Blood was collected from rabbits at 2, 4, and 6 weeks after the challenge. The serum antibodies to immunization with ovalbumin were assessed spectrophotometrically. The diluted serum samples were incubated in 96-well ELISA plates coated with ovalbumin (10μg/mL) for 2 h at 4°C. The amounts of bound anti-ovalbumin antibody were determined by using a goat anti-rabbit IgG-peroxidase conjugate (NEOGEN, Tokyo). The absorbance of individual wells with diluted sample product of peroxidase at 450 nm was measured with a micro-plate reader (Thermo Max, Molecular Devices). The results were represented as 50% of the maximum value in the serum dilution curves.

Measurement of Cartilage Hydroxyproline (HYP) Content. Slices of the articular cartilage were removed from weight-bearing areas of the rabbit femoral condylar and tibial plateaus, and digested with papain (0.3 mg/mL) . The resulting cartilage was hydrolyzed in hydrochloric acid (6N, 0.2 mL) at 110°C for 18 h. The hydrolysates were dried, then dissolved in water (1mL) and incubated with chloramin-T reagent (50 mMole, 100 μL/well, Nacaraitesuque, Kyoto) in microtiter plates for 20 min, followed by addition of dimethylaminobenzaldehyde (DMAB; 1 Mole, 100 μL/well). The plates were heated for 20 min at 65°C, and the absorbance of individual wells was measured at 550 nm with a micro-plate reader. The hydroxyproline content (HYP; μg) was expressed as per mg of the wet weight cartilage.

Histological Observation of Synovial Tissue. At 6 weeks after the ovalbumin challenge, the infrapatellar folds containing the synovial membrane and the adipose tissue from both the joints were removed, and fixed in neutral buffered formalin. After decalcification, the tissues were embedded in paraffin wax, sectioned at 5 μm, and stained with Haemotoxylin and Eosin for the histologic microscopic observation.

RESULTS AND DISCUSSION

Physicochemical Properties of BSP-Loaded Nanospheres

Table I shows the physicochemical properties of the BSP-loaded nanospheres as a function of MW and the copolymer ratio of D,L-lactide to glycolide. The present nanospheres exhibited unimodall particle size distribution, with a mean diameter range from 300 to 490 nm, and the standard deviations were rather low. These values agreed with those observed by SEM in Fig. 1. The freeze-dried nanospheres were easily redispersed in saline by shaking manually. It was assumed that the reduced particle size of the W/O emulsion droplet in the presence of Span 80, and the PVA adsorbed on the surface of the nanospheres improved the wettability on redispersing (19,20). The recovery and the drug content of the BSP-loaded nanospheres were dependent on the molecular weight and the size of the polymer, which mainly determined the precipitation rate of the polymers in the oil phase; caprylate and caprate triglyceride. With increasing LA ratio and MW of the polymer, the hardening of emulsion droplets became faster because of rapid precipitation of the polymer. In these conditions, BSP was efficiently entrapped even in the lipophilic polymer, although it has hydrophilic properties. It was found that during the preparation of the BSP-loaded nanospheres, the MW of the polymer was not changed.

Drug Release Properties of BSP-Loaded Nanospheres *in Vitro* and *in Vivo*

Figure 2 shows the *in vitro* releasing profiles of the BSP-loaded nanospheres as a function of molecular weight and copolymer ratio of the polymer. The nanospheres prepared with lower molecular weight polymers (PLGA-7505, PLGA-5005, PLA-0005:MW = 5900–6500) released the drug immediately within a few days. When the molecular weight of the polymer was increased up to 19,900 (PLGA-7520), the initial burst of the drug release significantly decreased. The nanospheres prepared with higher molecular weight polymers (PLGA-7520, PLGA-5020, PLA-0020:MW = 18,500–19,900) prolonged the drug release for over three weeks at a lower concentration ([0.016 $\mu g/mL \cdot h^{-1}$]) . The degradation of the polymer was proven by the decrease in the molecular weight of the nanospheres monitored using gel-permeation chromatography. The molecular weight of the BSP-loaded nanospheres prepared with PLGA-7520 decreased gradually (initial MW = 18,900, MW after 7 day s = 16,800, MW after 14 days = 12,700, MW after 21days = 8900). Thus, MW was the main factor in prolonging the drug release and the drug release kinetics were determined by the polymer degradation rate. The drug release was also prolonged with the increasing

Table I. Physicochemical Properties of Betamethasone Sodium Phosphate in Various Polymeric Nanospheres Prepared by Modified Emulsion Solvent Diffusion Method in Oil

Polymer grade	Average molecular weight[a]	Mean diameter ± s.d. (nm)	Recovery of nanospheres[b] (%)	Drug recovery in nanospheres (%)	Drug content in nanospheres (%)
LA/GA = 75/25					
PLGA-7520	19,900	302 ± 43	75.3	60.0	5.45
PLGA-7510	9,900	379 ± 64	35.8	31.5	2.86
PLGA-7505	5,900	463 ± 74	25.6	6.8	0.62
LA/GA = 50/50					
PLGA-5020	18,500	349 ± 42	56.2	29.2	2.65
PLGA-5005	6,500	482 ± 89	22.7	7.4	0.67
LA/GA 100/0					
PLA-0020	19,700	375 ± 61	76.1	50.6	4.59
PLA-0005	6,100	375 ± 63	31.1	13.0	1.18

[a] weight-based average molecular weight measured by GPC.
[b] defined as the weight ratio of the freeze-dried nanospheres.

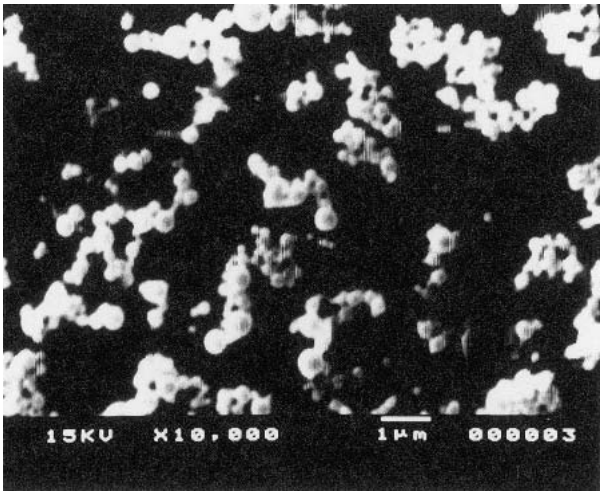

Fig. 1. Scanning electron micrograph of BSP-PLGA nanospheres. Magnification; ×10,000.

LA ratio in the polymer with slow degradation due to lower hydrophillicity (3). In the present system, the drug release-rate from the nanosphere prepared with copolymer LA/GA ratio of 75/25 or 50/50 was slightly faster than that of 100/0.

The method of drug release test *in vivo* was developed using the rat air-pouch model in which the biologic exudation generated was similar to those of acute inflammatory response (24). The drug releasing profiles of the BSP-loaded nanosphere prepared with PLGA-7520 and the BSP aqueous solution in the rat air-pouch model are shown in Fig. 3, exhibiting the BSP dissolved in the inside exudation and the

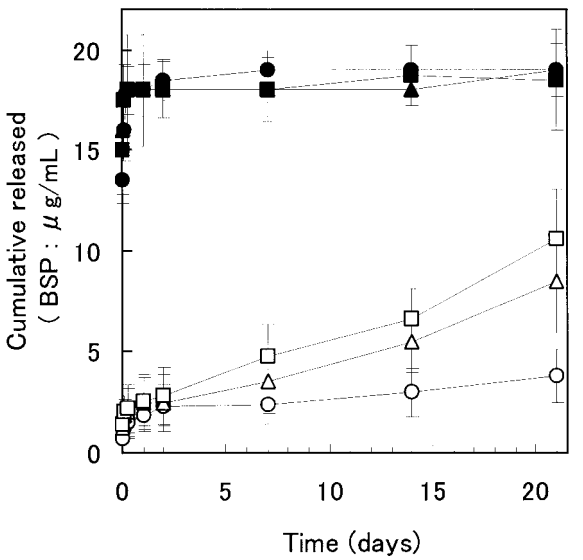

Fig. 2. The BSP releasing profiles of the PLGA nanospheres prepared by the emulsion solvent diffusion method. The equilibrium concentration of BSP was 20 μg/mL.

—○— BSP-loaded nanosphere (LA/GA=100/0, PLA-0020)
—△— BSP-loaded nanosphere (LA/GA=75/25, PLGA-7520)
—□— BSP-loaded nanosphere (LA/GA=50/50, PLGA-5020)
—●— BSP-loaded nanosphere (LA/GA=100/0, PLA-0005)
—■— BSP-loaded nanosphere (LA/GA=75/25, PLGA-7505)
—▲— BSP-loaded nanosphere (LA/GA=50/50, PLGA-5005)

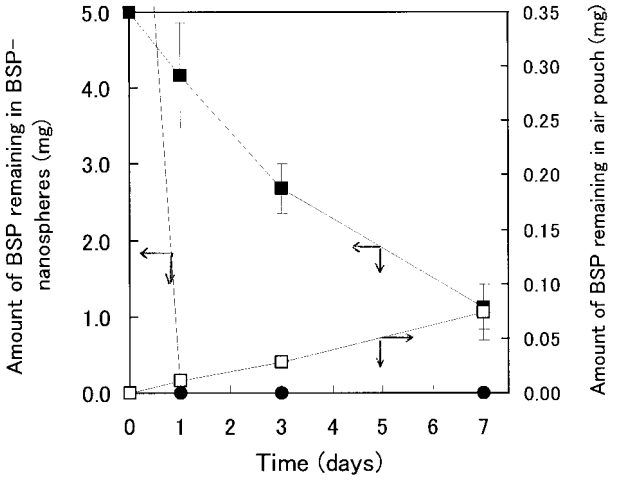

Fig. 3. Release profiles of loaded BSP in the exudation and amount remaining in the air pouch induced by subcutaneous injection.

—■— BSP-loaded nanosphere (LA/GA=75/25, PLGA-7520)
—●— BSP aqueous solution
—□— BSP-loaded nanosphere (LA/GA=75/25, PLGA-7520)

BSP remaining in the nanosphere in the pouch. The BSP (50 mg)-aqueous solution administered in the pouch was immediately absorbed into the subcutaneous tissues and the drug in the pouch was immediately removed within 24 h. However, the BSP (5 mg)-loaded nanosphere administered in the pouch permeated into the subcutaneous tissues. Therefore, the amount of BSP in the pouch increased gradually as shown in Fig. 3, due to the prolonged release of the drug from the nanospheres deposited in the tissue. The amount of BSP contained in the pouch-exudation was inconsistent with that of BSP released from the nanospheres as shown in Fig. 3. This finding was described in terms of the two compartment model determining the residual amount of BSP (X_1) in the nanospheres and the BSP released (X_2) in the pouch,

$$d X_1/d t = -K_R \cdot X_1$$

$$d X_2/d t = K_R \cdot X_1 - Ke \cdot K_2$$

where K_R is the drug release rate constant in the pouch from nanospheres and K_R and Ke were found to be 22.7μg /mL · h^{-1} and 23.1μg /mL · h^{-1}, respectively, from the drug concentration profiles in Fig. 3. The concentration of the BSP introduced as an aqueous solution in the pouch rapidly diminished, however the drug concentration in the pouch for the nanospheres administered was gradually increased (Fig. 3). These findings suggested that the release of BSP from the nanospheres was comparably high and sustained in the pouch. It was found that K_R was markedly higher than Kr [0.016μg /mL · h^{-1}] as found in Fig. 2. The molecular weight of the BSP nanospheres residing in the pouch was decreased as initial MW = 18,900 and MW = 14,200 after 7days. In contrast with the *in vitro* drug release-trial, the molecular weight of the BSP-loaded nanospheres in the exudation significantly decreased over a period of 7days. It was predicted that the biologic enzymes contained in the pouch accelerated the degradation of the nanospheres in the tissue leading to increased drug release. Davis *et al.* (13) demonstrated that *in vivo* re-

lease-rates of the microspheres were not faster but were proportionally related to *in vitro* release-rates, because the polymer degradation was limited. However, our previous study demonstrated that the PLGA nanospheres injected into rat knee joints were preferably phagocytosed by the macrophages infiltrated through the synovial tissues, which depended strongly on the size of the particulate system (18). These findings suggested that the BSP-loaded nanospheres introduced in the pouch were phagocytosed and degradated by the macrophages in the pouch. Then the BSP released into the pouch was delivered to the underlying tissue where they exert biologic effects. After 7 days, there was no inflammatory response in the tissues of the rat air-pouch, nor did any physiological change occur.

Reduction in Joint Swelling and Surface Skin Temperature of Antigen-Induced Arthritic Rabbit with BSP-Loaded Nanospheres

The intra-articular injection of ovalbumin into the knee joints of rabbits produced chronic arthritis, which closely resembled rheumatoid arthritis (22). It was found that the swelling of the knee joint reached a maximum at 24 h after the ovalbumin challenge and was accompanied with an acute inflammatory fever. In our study, there was a mean joint swelling of 7.35 ± 0.65mm (mean ± s.e.m.) at 24 h after the intra-articular challenge in the control. The swelling was effectively suppressed for one day after administration of the BSP aqueous solution (Fig. 4). Thereafter, the swelling markedly increased to a degree similar to the swelling in the control. The BSP-loaded nanosphere prepared with PLGA-7520 significantly depressed the joint swelling even for 21 days, but then gradually swelled. This sustained anti-inflammatory effect with the nanospheres in the RA rabbit-model was caused by the prolonged drug release behavior as suggested in Fig. 3. As described by Ratcliffe (11), the particles administered in the joint were diffused into the underlying synovial site via the synovial site's biologic action. In our previous study (18), it was found that the fluoresceinamine (FA) bound PLGA nanospheres administered into the rat joint cavity were readily phagocytosed by the macrophages in the synovial membrane. In addition, the phagocytic-uptake by macrophages continued and the phagocytosed nanospheres were transferred into the submembranous adipose tissue through the cell junction. These results suggested that drug release was sustained from the BSP-loaded nanospheres phagocytosed by macrophages infiltrating through the underlying tissue in the synovium after administration.

The BSP-loaded nanospheres significantly reduced the surface skin temperature of the knee joint, compared with the control, whereas the biologic response of the BSP solution was not different from that of the control. With the BSP aqueous solution as the control, the drug pharmacological suppression for acute inflammatory 'flare' activation might be superimposed on underlying chronic arthritis.

Depression of Cellular Infiltration in the Synovial Fluid with BSP-Loaded Nanospheres

The infiltration of leukocytes from the synovial tissue was investigated by counting the number of cells presented in the synovial fluid. Figure 5 shows the cellular infiltration in the synovial fluid at 42 days after the intra-articular injection of ovalbumin. The inflammatory leukocytes were not detected in the synovial fluid of the right knees. The control in the left-joint was swollen and the synovium contained a large number of inflammatory leukocytes ($161.0 \pm 132.3 \times 10^6$ cells/mL in joint wash), which revealed that the substantial inflammatory cell infiltration into the cavity showed continuous synovitis during the period. In both the groups treated with the BSP aqueous solution ($75.3 \pm 49.5 \times 10^6$ cells/mL) and the BSP-loaded nanospheres ($72.1 \pm 29.3 \times 10^6$ cells/mL), the cellular infiltration was significantly suppressed compared to the control. It was reported by Pettipher (22a) that the accumulation of inflammatory leukocytes in the synovial fluid

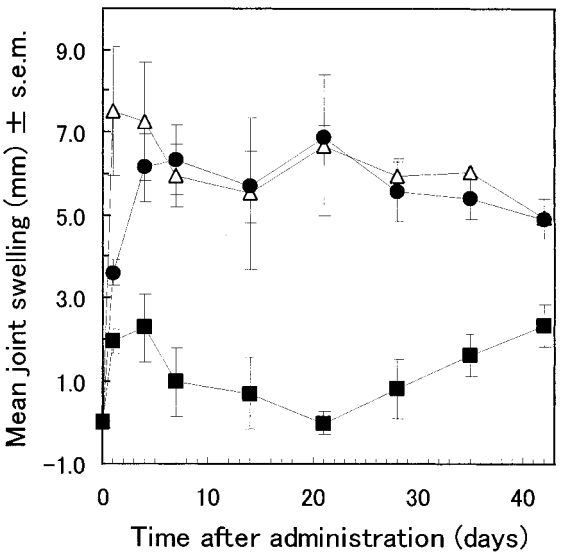

Fig. 4. Joint swelling in FCA-immunized rabbits at various times after the intra-articular challenge.

△— Physiological saline
■— BSP-loaded nanosphere (LA/GA=75/25, PLGA-7520)
●— BSP aqueous solution

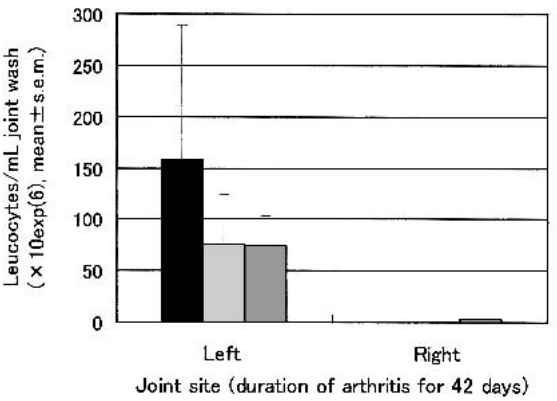

Fig. 5. Accumulation of inflammatory leukocytes in the synovial fluid of FCA-immunized rabbits at 6 weeks after the challenge.

■ Physiological saline
□ BSP aqueous solution
▩ BSP-loaded nanosphere (LA/GA=75/25,PLGA7520)

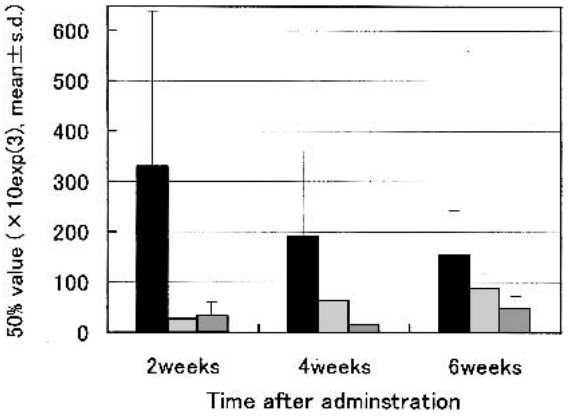

Fig. 6. 50% value of the maximum in titration curves as a function of time after administration.

■ Physiological saline
□ BSP aqueous solution
▩ BSP-loaded nanosphere (LA/GA=75/25,PLGA7520)

reached a maximum 24 h after the challenge, and persisted up to 42 days. We speculated that both the BSP aqueous solution and the BSP nanospheres reduced cellular infiltration in the early inflamed-stage. The type of infiltrated-leukocytes in the synovial fluid was assumed to be different because of the difference in the immune responses depending on the BSP aqueous solution or the BSP-loaded nanospheres in the period, although the microscopic measurement used in this study could not discriminate the type of the cellular infiltration such as lymphocyte, monocyte or macrophages.

Reduced Circulating Antibody Titer to Ovalbumin after Administrating BSP-Loaded Nanospheres

To verify the steroidal pharmacology, ovalbumin antibody generated in the rabbit serum was measured by the ELISA method. Figure 6 shows a 50% (±s.d.) value of the maximum in the titration curves, representing ovalbumin IgM-antibody generated in the immunized rabbit (25). The mean (50% value) in the groups administered the BSP-loaded nanospheres and the BSP aqueous solution were significantly lower than that of the control, although the difference between these groups was gradually decreased over time. The 50% value of the BSP nanospheres was lower than that of the BSP aqueous solution in 4 weeks and 6 weeks after the administration. These immunoreactions indicated that the inflammatory suppression was induced.

Reduced Loss of HYP from the Cartilage after Administrating BSP-Loaded Nanospheres

In rheumatoid arthritis, the chronic inflammation in the synovium coexists with the destruction of articular cartilage (22a). In antigen-induced arthritis, the progressive loss of the extra-cellular matrix in the articular cartilage was reported previously (5). The destruction of articular cartilage occurs in the lesion by the action of pannus-cells, and furthermore the perpetuation of this chronic destructive synovitis leads to the loss of joint function (26). To confirm the safety of the present BSP-loaded nanosphere system in the joints, the change in the cartilage component (hydroxyproline, collagen, glycosaminoglycan) was investigated as a pharmacological side-effect.

Figure 7 shows the HYP contents of the articular cartilage (femoral condylar and tibial plateaus) in the immunized rabbits at 6 weeks after the challenge. The HYP of the femoral condylar after the administration of the BSP-loaded nanospheres was lower (1.17 ± 0.06 μg/mg, mean ± s.e.m.) than that of the control (2.53 ± 0.36μg/mg) and the BSP aqueous solution (1.93 ± 0.49 μg/mg). The HYP of the tibial plateau for the BSP-loaded nanospheres (1.96 ± 1.14μg/mg) and the control (1.28 ± 0.21μg/mg) were lower than that of the BSP

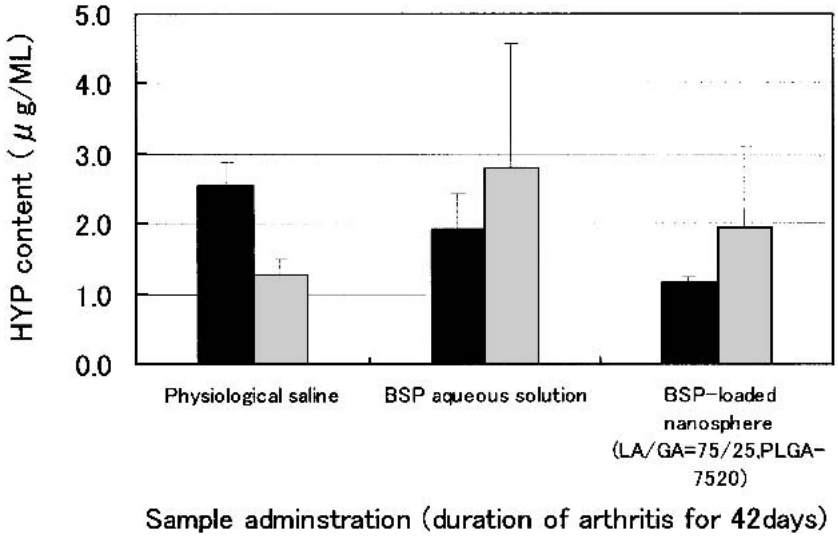

Fig. 7. Hydroxyproline (HYP) contents of articular cartilage in FCA-immunized rabbits at 6 weeks after the challenge.

■ Femoral condylar □ Tibial plateaus

aqueous solution (2.80 ± 1.78 μg/mg). Although the BSP-loaded nanospheres remained at sustained levels in the joint synovium, the cartilage degradation caused by the administration of the nanospheres was not significant compared to the control.

In another experiment, the amount of proteoglycan contained in the matrix of the articular cartilage with the administration of the BSP-loaded nanospheres slightly decreased compared to that in the control and the BSP aqueous solution. Therefore, cartilage degradation in the inflamed joints was not promoted because the drug release was prolonged at a lower level compared to that in steroid administration induced surface pathogenesis.

Histological Change after Administration of BSP-Loaded Nanospheres

A histologic study for the generalized inflammatory response was carried out to assess the biocompatibility of the nanospheres injected into the joint tissue. The insides of the articular cavities and surrounding tissues were morphologically observed and the histologic changes were detected as shown in Fig. 8. The synovial lining of the control joint was normal (Fig. 8a) and after the administration of BSP aqueous solution no distinguishable change was found as shown in Fig. 8b. With the administration of the BSP-loaded nanospheres, slight hyperplasia of the synovial membrane and widespread infiltration of cells throughout the linings were observed in Fig. 8c. The infiltration by macrophages and polymorphs was discriminated by H.E. staining and their phagocytosis was exhibited in the synovial membrane and the underlying tissue. It has been reported that polymerized albumin microspheres for intra-articular injection were phagocytosed by macrophages residing in the epithelium linings (11) and phagocytosis was an active process initiated by the binding to surface receptors (27). D'Souza (16,28–29) demonstrated that macrophages engulfed the foreign particles and the matrix of the microparticles was degraded by the lysosomal enzyme in the macrophages. The resultant biodegraded microparticles allowed the drug release into the lining cells.

In our previous study, a colloidal suspension of the FA-PLGA nanospheres (containing no-drug) was injected into the rat synovium. The nanospheres were phagocytosed by the macrophages and delivered to the deep underlying tissues with biocompatibility (18). This delivery system was safe and may be more suitable for drug delivery to inflamed synovial tissue than microspheres due to their ability of nanospheres to penetrate the synovium. In this study, even after the BSP-loaded nanospheres were phagocytosed by the macrophages in the synovial lining and slight changes in the histologic appearance were found for maximal phagocytosis (Fig. 8c). These findings suggested that the histologic changes were affected by BSP prolonged released from the nanospheres at low levels after administration. Thus, we considered that the administration of the BSP-loaded nanospheres are histologically safe.

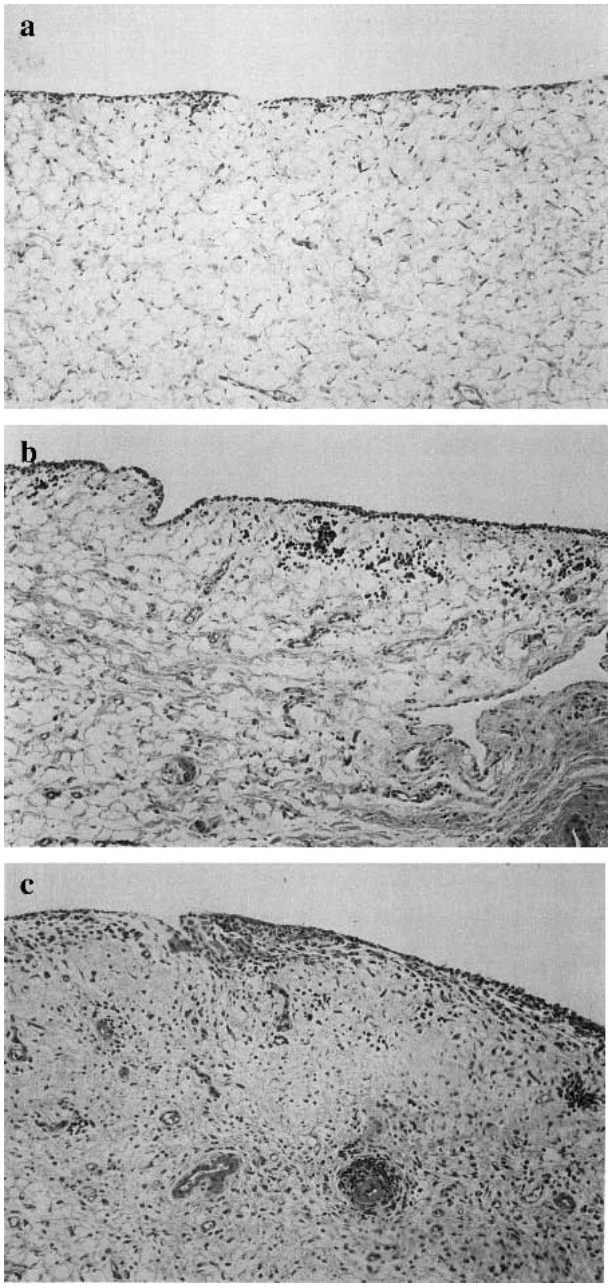

Fig. 8. Synovial membrane section at 6 weeks after the challenge in FCA-immunized rabbits. (magnification: ×25) a) Injection of physiological saline; b) Injection of BSP aqueous solution; c) Injection of BSP-loaded nanosphere suspension.

CONCLUSION

The PLGA nanospheres with a water-soluble steroid provided a sustained drug release in phosphate buffer saline and in the rat air-pouch. Direct intra-articular injection of the present PLGA nanosphere system provided a prolonged pharmacological efficacy in the joints of arthritic rabbits. The histologic safety of nanospheres administration to inflamed synovial tissue was confirmed. The PLGA particulate system can provide prolonged local- antiinflammatory action in joint diseases without biologic damage.

ACKNOWLEDGMENTS

The authors would like to acknowledge many valuable discussions with Dr. Y. Imasato and Mr. A. Komura during the preparation of this manuscript. This study was supported

in part by a Grant-in-Aid for Scientific Research (A-13309013) from Japan Society for the Promotion Science.

REFERENCES

1. J. L. Hollander, E. M. Brown, R. A. Jessar, and C. Y. Brown. Hydrocortisone and cortisone injected into arthritic joints. *J. Am. Med. Assoc.* **147**:1629–1635 (1951).
2. E. J. Goetzl, N. E. Bianco, J. S. Alpert, C. B. Sledge, and P. H. Schur. Effects of intra-articular corticosteroids *in vivo* on synovial fluid variables in rheumatoid synovitis. *Ann. Rheum. Di.* **33S**:62–66 (1974).
3. D. D. Friedman and M. E. Moore. The efficacy of intraarticular steroids in osteoarthritis:a double-blind study. *J. Rheumatology* **7:**850–856 (1980).
4. a) Y. Mizushima, H. Miyake, K. Fujikawa, N. Ono, and K. Takayama. A highly topically active corticosteroid. *Arzneim.-Forsch/Drug Res.(1)*, **30**:274–275 (1980); b) K. Hoshi. Clinical application of DDS, steroid therapy and NSAIDs. *Nippon Rinsho.* **47**:1302–1307 (1989).
5. H. Derendorf, H. Mollmann, A. Gruner, D. Haack, and G. Gyselby. Pharmacokinetics and pharmacodynamics of glucocorticoid suspensions after intra-articular administration. *Clin. Pharmacol. Ther.* **39**:313–317 (1986).
6. I. H. Shaw, C. G. Knight, and J. T. Dingle. Liposomal retention of a modified anti-inflammatory steroid. *Biochem. J.* **158**:473–476 (1976).
7. J. T. Dingle, J. L. Gordon, B. L. Hazleman, C. G. Knight, D. P. Page-Thomas, N. C. Phillips, I. H. Shaw, F. J. T. Fildes, J. E. Oliver, G. Jones, E. H. Turner, and J. S. Lowe. Novel treatment for joint inflammation. *Nature* **271**:372–373 (1978).
8. N. C. Phillips, P. Page-Thomas, C. G. Knight, and J. T. Dingle. Liposome-incorporated corticosteroids. II.therapeutic activity in experimental arthritis. *Ann. Rheum. Dis.* **38**:553–557 (1979).
9. W. C. Foong and K. L. Green. The retention and distribution of dual-labeled loposomes injected into arthritic joints. *Br. J. Pharmaco.* **80**:522 (1983).
10. L. G. Francisco, M. V. A. Jose, G. Francisco, L. Rafael, M. Francisco V. Jose, and C. G. F. Juan. Intra-articular therapy of experimental arthritis with a derivative of triamcinolone acetonide incorporated in liposomes. *J. Pharm. Pharmaco.* **45**:576–578 (1993).
11. J. H. Ratcliffe, I. M. Hunneyball, A. Smith, C. G. Wilson, and S. S. Davis. Preparation and evaluation of biodegradable polymeric systems for the intra-articular delivery of drugs. *J. Pharm. Pharmacol.* **36**:431–436 (1984).
12. J. H. Ratcliffe, I. M. Hunneyball, C. G. Wilson, A. Smith, and S. S. Davis. Albumin microspheres for intra-articular drug delivery: investigation of their retention in normal and arthritic knee joints of rabbits. *J. Pharm. Pharmacol.* **28**:290–295 (1987).
13. a) D. J. Burgess, S. S. Davis, and E. Tomlinson. Potential use of albumin microspheres as a drug delivery system. I. preparation and in vitro release of steroids. *Int. J. Pharm.* **39**:129-136 (1987); b) D. J. Burgess and S. S. Davis. Potential use of albumin microspheres as a drug delivery system. II. *in vivo* deposition and release of steroids. **46**:69–76 (1988).
14. K. E. Brown, K. Leong, C-H. Huang, R. Dalal, G. D. Green, H. B. Haimes, P. A. Jimenez, and J. Bathon. Gelatin/chondroitin 6-sulfate microspheres for the delivery of therapeutic proteins to the joint. *Arthritis Rhem.* **41**:2185–2195 (1998).
15. a) M. Tuncay, S. Calis, H. S. Kas, M. T. Ercan, H. Erturk, and A. A. Hincal. Dicrofenac sodium incorporated biodegradable microspheres. II:antiinflammatory activity in the knee joints of rabbits. "Challenges for drug delivery and pharmaceutical technology" Tokyo:188 (1998): b) *idem,* Dicrofenac sodium incorporated PLGA (50:50) microspheres:formulation considerations and *in vitro/in vivo* evaluation. *Int. J. Pharm.* **195**:179–188 (2000); c) M. Tuncay, S. Calis, H. S. Kas, M. T. Ercan, I. Peksoy, and A. A. Hincal. *In vitro* and *in vivo* evaluation of dicrofenac sodium loaded albumin microspheres. *J. Microencupsulation* **17**:145–155 (2000).
16. M. J. D'Souza and P. DeSouza. Preparation and testing of cyclosporine microsphere and solution formulations in the treatment of polyarthritis in rats. *Drug Dev.Ind.Pharm.* **24**:841–852 (1998).
17. T. Tosu. Steroid induced arthropathy. *J. Joint Surgery* **11**:87–95 (1992).
18. E. Horisawa. K. Kubota, I. Tuboi, K. Sato, H. Yamamoto, H. Takeuchi, and Y. Kawashima. Size-dependency of DL-lactide/glycolide copolymer particulates for intra-articular delivery system on phagocytosis in rat synovium. "Annual meeting of the academy of pharmaceutical science and technology" Tokyo:168 (2001).
19. T. Niwa, H. Takeuchi, T. Hino, M. Nohara, and Y. Kawashima. Biodegradable submicron carriers for peptide drugs:preparation of DL-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate by a novel emulsion-phase separation method in an oil system. *Int. J. Pharm.* **121**:45–54 (1995).
20. Y. Kawashima, H. Yamamoto, H. Takeuchi, T. Hino, and T. Niwa. Properties of a peptide containing DL-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion method. *Eur. J. Pharm. Bio.* **45**:41–48 (1998).
21. a) H. Murakami, Y. Kawashima, T. Niwa, T. Hino, H. Takeuchi, and M. Kobayashi. Influence of the degrees of hydrolyzation and polymerization of poly (vinylalchol) on the preparation and properties of poly (DL-lactide-co-glycolide) nanoparticle. *Int. J. Pharm.* **149**:43–49 (1997); b) H. Murakami, M. Kobayashi, H. Takeuchi, and Y. Kawashima. Preparation of poly (DL-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method. *Int. J. Pharm.* **187**:143–152 (1999).
22. a) E. R. Pettipher, G. A. Higgs, and B. Henderson. PAF-acether in chronic arthritis. *Agents Action* **21**:98-103 (1987); b) E. R. Pettipher and B. Henderson. The relationship between cell-mediated immunity and cartilage degradation in antigen-induced arthritis in the rabbit. *Br. J. Exp. Path.* **69**:113–122 (1988)
23. A. Blackham and R. I. Griffiths. The effect of FK506 and cyclosporin A on antigen-induced arthritis. *Clin. Exp. Immunol.* **86**: 224–228 (1991).
24. K. Ohuchi and N. Hirasawa. Method for the evaluation of anti-inflammatory and allergic drugs using experimental animal models. "*Experimental manuals for bio-pharmaneutical sciences*." **12**: 65–75 (1993).
25. A. Fox and L. E. Glynn. Persistence of antigen in nonarthritic joints. *Ann. Rheum. Dis.* **34**:431–437 (1975).
26. D. C. Dumonde and L. E. Glynn. The production of arthritis in rabbits by an immunologic reaction to fibrin. *Br. J. Exp. Path.* **43**:373–383 (1962).
27. A. S. Shanbhag, J. J. Jacobs, J. Black, J. O. Galante, and T. T. Glant. Macrophage/particle interactions:effect of size, composition and surface area. *J. Bio. Material Res.* **28**:81–90 (1994).
28. M. J. D'Souza, C. W. Oettinger, A. S. Shah, P. G. Tipping, X. Ru Huang, and G. V. Milton. Macrophage depletion by albumin microencapsulated clodronate:attenuation of cytokine release in macrophage-dependent glomerulonephritis. *Drug Dev. Ind. Pharm.* **25**:591–596 (1999).
29. C. W. Oettinger, M. J. D'Souza ,and G. V. Milton. Targeting macrophages with microspheres containing cytokine-neutralizing antibodies prevents lethality in gram-negative peritonitis. *J. Interferon Cytokine Res.* **19**:33–40 (1999).