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# Modification of release rates of cyclosporin A from poly(L-lactic acid) microspheres by fatty acid esters and in-vivo evaluation of the microspheres

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## Abstract

Biodegradable microspheres containing cyclosporin A (CyA, cyclosporine) were prepared using poly(L-lactic acid) (PLA) and poly(lactide-co-glycolide)(PLGA) by a solvent evaporation method. CyA was efficiently entrapped in PLA, PLGA(50/50) and PLGA(75/25) microspheres in a range of 81–85%. CyA released constantly from PLA microspheres without any lag time, whereas the drug from PLGA(50/50) and PLGA(75/25) microspheres was released after lag time of about 1 and 3 weeks, respectively. Addition of fatty acid esters enhanced the release rates of CyA from PLA microspheres. In-vivo study was performed using rats with adjuvant-induced arthritis. PLA microspheres with ethyl myristate sustained high blood levels of CyA compared with the microspheres with no additives over 4 weeks. In addition, the PLA microspheres improved the symptoms such as the decrease in body weight and the increase in paw swelling occurred by adjuvant-induced arthritis in rats. Consequently, the release rate of CyA from PLA microspheres can be improved by adding fatty acid esters and PLA microspheres with fatty acid esters seem to be a useful dosage form for autoimmune disease therapy. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Microspheres; Cyclosporin A; Poly(L-lactic acid); Poly(lactide-co-glycolide); Fatty acid esters; Adjuvant

## 1. Introduction

Cyclosporin A (CyA), a potent immunosuppressive agent, is widely used for the inhibition of graft rejection in various organ transplantations. The drug is a highly specific inhibitor of T-lymphocyte function which plays an important role in the induction of immune responsiveness. Therefore, it has also been applied to the treatment of patients with selected autoimmune diseases such as rheumatoid arthritis [1]

and Behçet's disease [2]. However, there are some problems with the treatment with the available dosage forms of CyA in spite of the great therapeutic interest in the drug. CyA is currently marketed in Japan as either an oil-based oral solution, a soft gelatin capsule or an injection containing polyoxyethylated castor oil due to its lipophilic nature. When administered orally, the bioavailability of CyA in its conventional oral preparation displays considerable inter- and intra-patient variability presumably due to its poor and highly bile-dependent absorption as well as involvement of intestinal metabolism [3–5]. This low and variable bioavailability sometimes

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causes rejection after organ transplantation and serious side effects and makes it difficult to institute and monitor immunosuppressive therapy. On the other hand, since CyA injection has a risk of anaphylaxis by an additive, polyoxyethylated castor oil, its use is limited to patients who are unable to take the oral preparations. There is a great interest in the development of the alternative dosage forms to overcome these problems.

Various approaches to controlled release systems have been investigated to improve the therapeutic efficacy of CyA. One approach is the way which uses polymers in the form of biodegradable microspheres based on lactic or glycolic acids and its co-polymers. Recently, a great deal of attention has been focused on microsphere preparations for peptide delivery. Several formulations based on these polymers have been investigated for injectable microspheres and some of them are already in clinical use [6,7]. The study on CyA microspheres has also been reported using poly(DL-lactide-co-glycolide) (PLGA) which is considered to be suitable for this purpose [8]. However, the paper presented in-vitro results that cumulative amounts of CyA released during 4 weeks were 50% at the greatest in all formulations and their release patterns were biphasic and characterized by a rapid release referred to as an initial burst followed by a slower release thereafter. Further, the release of CyA was greater only in the initial burst even if PLGA nanospheres of smaller size were prepared. Accordingly, their formulations seem to be not optimal in view of an efficient and constant release of CyA.

We have also been investigating a possible use of poly(DL-lactic acid) (PLA) microspheres as the sustained release dosage forms of drugs by modifying microsphere size, drug contents and PLA molecular weight and by using additives [9–14]. The present study is an extension of previous works on the design of sustained release microspheres using PLA. The preparation and in-vitro release characteristics of PLA microspheres containing CyA were evaluated in the presence of fatty acid esters as additives. Moreover, in-vivo studies were performed to evaluate the effect of the microspheres in rats with adjuvant-induced arthritis, a model of autoimmune diseases.

## 2. Materials and methods

### 2.1. Chemicals

CyA was kindly supplied by Sandoz Yakuhin K. K. (Novartis), Tokyo. Poly(lactic acid) (PLA; mean molecular weight of 26,000) was kindly supplied by Mitsui Toatsu Chemicals, Inc., Nagoya. PLGA (lactic/glycolic: 75/25 and 50/50; mean molecular weight of 20,000) was purchased from Wako Pure Chem. Ind., Osaka. All other chemicals used were of analytical grades.

### 2.2. Preparation of microspheres

PLA microspheres were prepared by a solvent-evaporation method as reported previously [15]. Briefly, about 10 mg of CyA, an additive, and 50 mg of a polymer (PLA or PLGA) were dissolved in 2 ml of methylene chloride. When one of three fatty acid esters was used as an additive, the loading level of the additive was in a range of 10–100% of the polymer. The solution was then dispersed in 0.1% (w/v) polyvinyl alcohol solution under stirring at a rate of 700 rpm by means of a magnetic stirrer. The stirring was continued for 2 h at room temperature (25°C) to evaporate methylene chloride off. The microspheres were collected by filtration, washed with distilled water, and dried under reduced pressure at room temperature for 2 days.

### 2.3. Determination of drug contents

Weighed amounts of microspheres were dissolved in 2 ml of methylene chloride in a screw-capped test tube. Then, 5 ml of hexane was added, and the mixture was shaken for 15 min to extract CyA. After centrifugation at 3000 rpm for 10 min, the supernatant was taken and dried, and after adding methanol the aliquot was injected into high performance liquid chromatograph (HPLC).

### 2.4. Sizes of microspheres

Microspheres were observed under an optical microscope (Olympus BH-2) to determine their size and shape.

### 2.5. Drug release in vitro

Weighed amounts of microspheres were put into a flask containing 20 ml of a normal saline containing 0.01% (w/v) polysorbate 80. The flask was immersed in a shaker bath maintained at  $37.0 \pm 0.1^\circ\text{C}$  and shaken horizontally. At predetermined intervals, an appropriate volume of the solution was sampled and the same volume of fresh medium was added. The amount of CyA released was determined by HPLC.

### 2.6. Animal study

Male Wistar rats, weighing 200–230 g was used in these experiments. The rats were randomly divided into four groups of 5–6 animals each. Arthritis was induced by a well-known method [16], i.e. intradermal injection of a 0.6 mg/0.1 ml suspension of dry heat-killed *Mycobacterium butyricum* (Difco), an adjuvant agent, in liquid paraffin into the root of the tail of rats. The microspheres containing CyA or CyA suspension were subcutaneously administered to nuchal regions of rats at a dose of 15 mg CyA/animal on the day treated with injection of the adjuvant. CyA-unloaded microspheres were used as a control in the same manner. In all four groups, CyA level in whole blood, the change in body weight, the volume of edema and the inflammation score were determined at the same time on 0, 2, 5, 7, 9, 11, 13, 16, 20, 24 and 28 day after the treatment of the adjuvant. The hind paw swelling was expressed as the percentage as compared with the initial hind paw volume. The inflammation score was estimated by scoring the extent of inflammation according to the method of Koga and Pearson [17].

### 2.7. CyA analysis

CyA in whole blood was determined by HPLC (Shimadzu LC-6A, Kyoto, Japan). Into a 10 ml screw cap tube were added 500  $\mu\text{l}$  of whole blood sample and 1,500  $\mu\text{l}$  of acetonitrile containing an internal standard (cyclosporin D 0.1  $\mu\text{g}/\text{ml}$ ). After centrifugation, the supernatant was transferred into another tube containing 6 ml of distilled water. This was applied to a C18 cartridge column (Adsorbex

CN, Merck, Darmstadt, Germany) and washed with 5 ml of water, subsequently 0.5 M acetic acid–acetonitrile (4:1) and 30% methanol, then eluted with 2 ml of acetonitrile. After evaporating the solvent, 100  $\mu\text{l}$  of the mobile phase (hexane/ethanol=85:15, v/v) was added and injected into HPLC. Separation was performed with a reversed phase-type column (LiChrospher Si 60, 5  $\mu\text{m}$ , 4.0 mm I.D.  $\times$  250 mm L). At a flow rate of 1.0 ml/min, the eluate was monitored for absorbance at 210 nm.

### 2.8. Statistical analysis

The results from the in-vivo study were expressed as the mean  $\pm$  standard error. Statistical analysis was performed using one-way analysis of variance ( $p < 0.05$  or  $p < 0.01$ ), followed by a Fisher's PLSD-test for differences.

## 3. Results and discussion

### 3.1. In vitro release of CyA from PLA, PLGA(50/50) and PLGA(75/25) microspheres

CyA is a neutral and hydrophobic polypeptide and highly soluble in methylene chloride which is a solvent of polymers. Therefore, it was expected that CyA would be easily incorporated in microspheres by a solvent evaporation method. In fact, CyA was efficiently entrapped in PLA, PLGA(50/50) and PLGA(75/25) microspheres in a range of 81–85% (Table 1). The microspheres observed under an

Table 1  
Entrapping efficiency of PLA and PLGA microspheres prepared with a solvent evaporation method

Polymer	Drug content (%)		Trapping efficiency (%) <sup>c</sup>
	Det. <sup>a</sup>	Cal. <sup>b</sup>	
L-PLA	17.14	20.06	85.43
PLGA(50/50)	16.83	19.98	84.22
PLGA(75/25)	16.21	19.96	81.23

<sup>a</sup> Determined average drug content.

<sup>b</sup> Calculated drug content.

<sup>c</sup> Trapping efficiency is defined as the ratio of determined average drug content to calculated drug content.

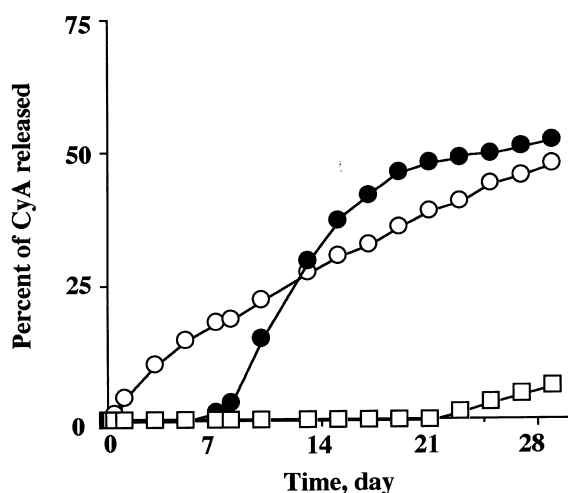


Fig. 1. Release profiles of CyA from PLA and PLGA microspheres without fatty acid esters in saline containing 0.01% polysorbate 80. ○: PLA microspheres, ●: PLGA (50/50) microspheres, □: PLGA(75/25) microspheres.

optical microscope were spherical and their diameters were mostly in a range of 50–60  $\mu\text{m}$  (data not shown). Fig. 1 shows the release patterns of CyA from PLA, PLGA(50/50) and PLGA(75/25) microspheres with no additives in normal saline containing 0.01% (w/v) polysorbate 80. CyA released constantly from PLA microspheres without lag time after starting the release test. On the other hand, the drug from PLGA(50/50) microspheres was rapidly released after a lag time of about 1 week and thereafter changed into a slow release from about 3 weeks. CyA from PLGA(75/25) microspheres also had a long lag time of 3 weeks and the amounts of CyA

released from the microspheres were too small throughout 4 weeks. The cumulative amounts of CyA released from PLA, PLGA(50/50) and PLGA(75/25) microspheres over 4 weeks were 47.9%, 52.5% and 6.28%, respectively. Although the difference in the release rates between PLA and PLGA microspheres is still unclear, a possible explanation may be due to the difference in crystallinity of polymers forming the microspheres. It is known that the crystallized polymers forming the microspheres increase the release rates of drug as compared with those of the amorphous polymers [18]. In fact, X-ray analysis showed that PLA was present in the crystalline state in the microspheres, while PLGA was in the amorphous state (data not shown). The crystallization of PLA may induce micro voids in the microspheres which functioned as channels for water penetration. Accordingly, if the microspheres using PLGA(50/50) and PLGA(75/25) microspheres are to be prepared by this method, a design which eliminates the lag time may be needed. Thus, it was shown that PLA was a suitable materials that could form a sustained release system of CyA.

### 3.2. Effect of contents of fatty acid esters on *in-vitro* release of CyA from PLA microspheres

In order to modify the release pattern of CyA, fatty acid esters were added in the preparation of PLA microspheres. Table 2 shows the trapping efficiency in the case of which fatty acid esters were added to PLA microspheres. When each fatty acid

Table 2  
Effect of kinds and contents of fatty acid esters on trapping efficiency of PLA microspheres containing CyA

Esters	Amounts of fatty acid esters added (% of polymer)											
	10%			30%			50%			100%		
	Drug content (%)		Trapping efficiency <sup>c</sup> (%)	Drug content (%)		Trapping efficiency (%)	Drug content (%)		Trapping efficiency (%)	Drug content (%)		Trapping efficiency (%)
	Det. <sup>a</sup>	Cal. <sup>b</sup>		Det.	Cal.		Det.	Cal.		Det.	Cal.	
Ethyl caprate	17.3	18.1	95.7	15.8	16.1	98.0	12.2	14.2	86.0	6.09	11.3	54.1
Ethyl myristate	16.5	18.7	88.5	13.7	16.9	81.0	12.4	14.5	85.7	6.36	11.1	57.1
Ethyl stearate	15.7	18.7	84.4	15.6	16.1	96.8	13.6	14.7	92.6	6.97	11.2	62.2

<sup>a</sup>Determined average drug content.

<sup>b</sup>Calculated drug content.

<sup>c</sup>Trapping efficiency is defined as the ratio of determined average drug content to calculated drug content.

ester was added in a range of 10–50%, CyA was greatly trapped in the microspheres and its trapping efficiency was more than 80%. However, in the case of 100% addition, the trapping efficiency lowered to approximately 60%.

Fig. 2 shows the release patterns of CyA from PLA microspheres containing various kinds and contents of fatty acid esters. Ethyl caprate slightly increased the release rate of CyA from the microspheres and their release patterns with 10–50% additives were all similar. Ethyl stearate increased the release rate of CyA with an increase in content of additives and the percentage of CyA released was more than 70% in 4 weeks in all cases with additives. When ethyl stearate was added, however, CyA was released in a biphasic manner, i.e. a rapid release with a subsequent slow release. On the other

hand, PLA microspheres with 30% and 50% ethyl myristate efficiently released approximately 90% of CyA over 4 weeks in an apparent zero-order manner. There was little difference in the release patterns between both preparations with 30% and 50% additives. Thus, it is suggested that ethyl myristate is more suitable for improving the release of CyA from PLA microspheres.

We previously reported that various fatty acid esters increased the release rates of drugs such as bleomycin [10], aclarubicin [19], and cisplatin [14]. From these results, the release rates of drugs could be controlled by adjusting the amount of fatty acid esters and/or by selecting the proper esters. Although the reason why ethyl myristate is more suitable for modifying a release profile remains to be elucidated, a possible explanation may be due to the

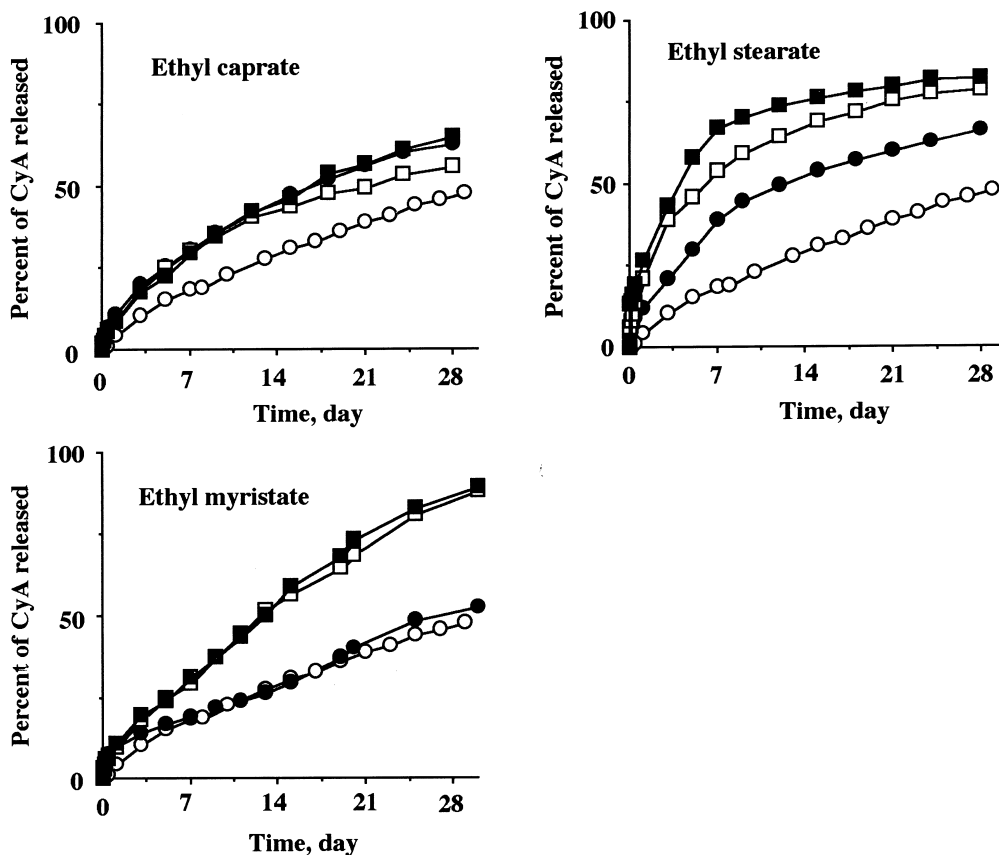


Fig. 2. Effect of content of fatty acid esters on the release rates of CyA from PLA microspheres in saline containing 0.01% polysorbate 80. ○: without additive, ●: with 10% additive, □: with 30% additive, ■: with 50% additive.

difference in the solubility of CyA in the fatty acid esters and the difference in the volatility of esters as reported on aclarubicin [19]. As reported previously, PLA microspheres containing a fatty acid ester are regarded as a composite matrix where the ester is dispersed in the PLA matrix and drugs can dissolve in both PLA matrix and ester portion [10]. A probable mechanism of the release is considered to be due to the diffusion through channels which are formed by esters [19]. Lipophilic CyA is considered to be mainly solubilized in the fatty acid esters forming the channels. We reported that the longer was carbon chain of the ester, the greater was the amount of the ester incorporated but that esters with a shorter acyl chain length might have dissolved in the dispersion phase during preparation of the microspheres since they have some aqueous solubility [19]. In addition, we suggested that the volatility of esters should be considered. Esters with shorter carbon chains would have evaporated during drying of the microspheres under vacuum. Due to the resulting low contents of esters with shorter carbon chains in the microspheres, their promoting effects on the release rate of the drug were small. Thus, the promoting effects of ethyl stearate with longer carbon chains for the release of CyA from PLA microspheres are expected to be greater than those of ethyl caprate with shorter carbon chains. Furthermore, we observed with a scanning electron microscope that the surface of PLA microsphere and the form of channels formed in the matrix were different depending on the contents and kinds of fatty acid esters in the microspheres [14]. The drug would be released through the channels and/or the micro voids formed by fatty acid esters. Thus, drug release from PLA microspheres would be greatly influenced by the physicochemical natures of fatty acid esters and drugs.

### 3.3. In vivo evaluation in rats with adjuvant-induced arthritis

#### 3.3.1. Blood concentration profile of CyA

It is well known that CyA concentrations correlate with the therapeutic effect and the risk of therapeutic failure. Thus, in order to evaluate the release profiles of CyA, in-vivo study was performed using rats with adjuvant-induced arthritis. Fig. 3 shows the blood

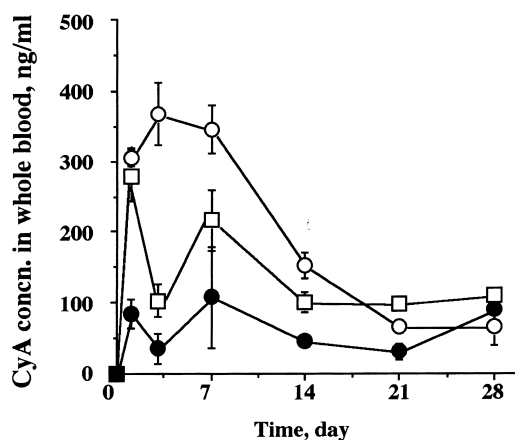


Fig. 3. The blood concentration-time profiles of CyA after subcutaneous injection of PLA microspheres and CyA suspension in rats with adjuvant-induced arthritis. ○: CyA suspension, ●: PLA microspheres without additive, □: PLA microspheres with 30% ethyl myristate. Each point represents the mean  $\pm$  SEM of 1–3 animals.

concentrations of CyA after subcutaneous injection of CyA-loaded microspheres prepared with PLA and suspension of CyA (15 mg as CyA). The suspension rapidly elevated blood levels of CyA and its blood levels attained 300–400 ng/ml within seventh day after the treatment and thereafter decreased to approximately 50 ng/ml within the 21st day. The microspheres with 30% ethyl myristate showed high blood levels of CyA at the 1st day after the treatment and thereafter sustained constant levels, probably because of continuous release of the drug. In the case of the microspheres with no additives, their blood level-time concentration profiles of CyA were similar to those with 30% ethyl myristate. However, the levels were markedly lower than those with ethyl myristate additive. These blood concentration profiles roughly reflected the results of in-vitro release profiles.

#### 3.3.2. Evaluation of PLA microspheres for adjuvant-induced arthritis

We investigated the effect of CyA on body weight, paw swelling and inflammation score after subcutaneous administration of the microspheres and the CyA suspension in rats with adjuvant-induced arthritis. Fig. 4 shows the change in body weights after the subcutaneous administration of four preparations.

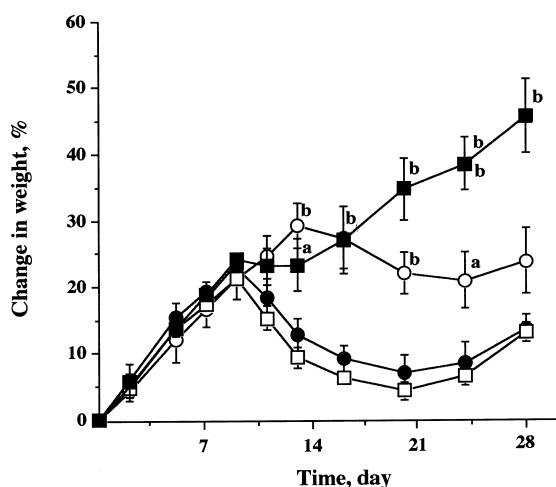


Fig. 4. Change in body weights after subcutaneous injection of PLA microspheres and CyA suspension in rats with adjuvant-induced arthritis. ●: CyA-unloaded PLA microspheres, ○: CyA suspension, □: PLA microspheres without additive, ■: PLA microspheres with 30% ethyl myristate. a, b: Significantly different from CyA-unloaded microspheres injection at  $p < 0.05$  and  $p < 0.01$ , respectively. Each point represents the mean  $\pm$  SEM of 5–6 animals.

Although CyA-unloaded microspheres continued to increase body weight until ninth day after the treatment with adjuvant, thereafter the body weight was markedly reduced. The microspheres with no additives also increased the body weight until ninth day and their behaviors were similar to those of CyA-unloaded microspheres. The microspheres with ethyl myristate and the suspension containing CyA were significantly different from CyA-unloaded microspheres for the change in body weight. The suspension continued to increase body weight until thirteenth day after the treatment but thereafter tended to reduce it. On the other hand, the microspheres with ethyl myristate constantly increased the body weight over 28 days except for a period from ninth day after the treatment.

Fig. 5 shows the change in the paw swelling of the left and right hind legs. In rats with CyA-unloaded microspheres, the edema was observed in both legs from ninth day and developed most greatly within 28th day after the treatment with adjuvant. The microspheres with no additives also had little inhibitory effect on the development of edema.

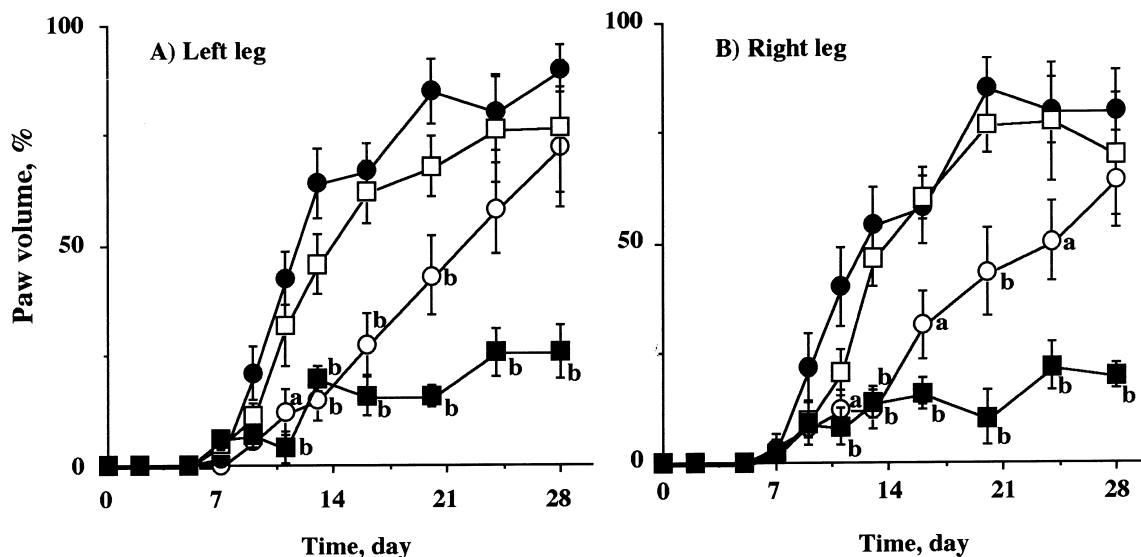


Fig. 5. Paw swelling of hind legs after subcutaneous injection of PLA microspheres and CyA suspension in rats with adjuvant-induced arthritis. ●: CyA-unloaded PLA microspheres, ○: CyA suspension, □: PLA microspheres without additive, ■: PLA microspheres with 30% ethyl myristate. a, b: Significantly different from CyA-unloaded microspheres injection at  $p < 0.05$  and  $p < 0.01$ , respectively. Each point represents the mean  $\pm$  SEM of 5–6 animals.

Although the CyA suspension significantly inhibited development of the initial edema compared with that of CyA-unloaded microspheres, the swelling was increased thereafter by degrees. On the other hand, the microspheres with 30% ethyl myristate showed a significant inhibition on the edema throughout 4 weeks. This suggests that the microspheres with ethyl myristate kept releasing CyA in the body for over 4 weeks as well as in-vitro.

Fig. 6 shows the inflammation scores of four paws after subcutaneous administration of four preparations in rats with adjuvant-induced arthritis. There was little difference in the scores between CyA-unloaded microspheres and the microspheres with no additives. Although the suspension completely inhibited the score until thirteenth day after the treatment, the inflammation score was gradually increased thereafter. On the other hand, sustained inhibition of inflammation was attained by a single injection of PLA microspheres with 30% ethyl myristate. The microspheres with ethyl myristate significantly lowered the score compared to CyA-unloaded microspheres throughout 28 days. These results may reflect the observed blood level profiles of CyA (Fig. 3). Thus, it may be confirmed that the

present in-vivo studies using rats with adjuvant-induced arthritis reflected the in-vitro release profile.

#### 4. Conclusions

In the present study, we evaluated application of PLA microspheres as an injectable sustained-release dosage form of CyA using rats with adjuvant-induced arthritis. It was shown that CyA was efficiently incorporated in microspheres because of its lipophilic nature by a solvent evaporation method. In addition, the release rate of CyA could be controlled by using fatty acid esters with different alkyl chains. From the results of the in-vitro release studies and in-vivo studies in rats with adjuvant-induced arthritis, it was confirmed that PLA microspheres were successful sustained depot formulations of CyA. The microspheres could be a useful dosage form for various autoimmune disease therapy without affecting inter- and intra-patient variability due to poor absorption from the gastrointestinal tract and variability in metabolism.

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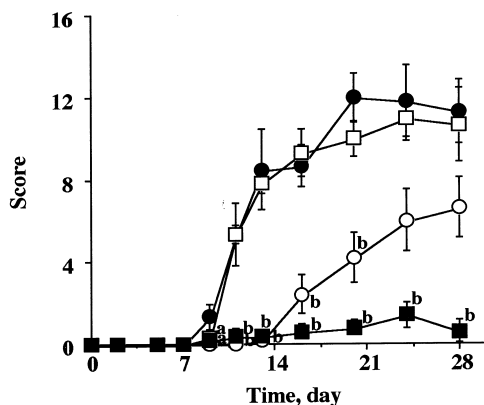


Fig. 6. Inflammation scores of four paws after subcutaneous injection of PLA microspheres and CyA suspension in rats with adjuvant-induced arthritis. ●: CyA-unloaded PLA microspheres, ○: CyA suspension, □: PLA microspheres without additive, ■: PLA microspheres with 30% ethyl myristate. a, b: Significantly different from CyA-unloaded microspheres injection at  $p < 0.05$  and  $p < 0.01$ , respectively. Each point represents the mean  $\pm$  SEM of 5–6 animals.



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