J. Pharm. Pharmacol. 1994, 47: 556–560
Received July 27, 1993
Accepted February 15, 1995

Particle Size Studies for Subcutaneous Delivery of Poly(lactide-co-glycolide) Microspheres Containing Ovalbumin as Vaccine Formulation

TAKAHIRO UCHIDA, SHIGERU GOTO AND TODD P. FOSTER*

*Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan and *Drug Delivery Research and Development, Upjohn Company, Kalamazoo, Michigan 49001, USA*

Abstract—The primary objectives of the present study were to produce poly(lactide-co-glycolide) (PLGA) microspheres with different diameters, to characterize these microspheres which were loaded with a model antigen, ovalbumin and to evaluate the effect of microsphere particle size on the serum antibody levels following administration to mice.

Four kinds of ovalbumin-loaded PLGA microspheres with different diameters (1·2, 3·5, 7·0 and 14·3 μm as mean volume diameter) were manufactured by a w/o/w emulsion/solvent evaporation method. Low loading percent (0·08%–0·25%w/w) and efficiencies (8–25% w/w) were observed. Examination using scanning electron photomicrographs showed smooth spherical particles. The in-vitro release of ovalbumin from microspheres showed an expected burst release with all batches and the extent of the burst release increased with decreasing diameters of spheres; PLGA microspheres with the smallest diameter (1·2 μm) showed an 80% burst release within one day. Approximately 10–60% of ovalbumin remained unreleased 30 days later.

The single subcutaneous administrations of ovalbumin-loaded PLGA microspheres with different diameters to mice induced good antibody responses above ovalbumin saline negative controls at 3, 6, 9, and 12 weeks after inoculation. Especially, 0·16% ovalbumin-loaded PLGA microspheres having mean volume diameter of 3·5 μm exhibited the best immune responses with values greater than those obtained after inoculation with adjuvants such as complete Freund's adjuvant or alum as positive control.

The strong adjuvant activity of PLGA microspheres as vaccine formulation was suggested.

The recent emergence of the acquired immunodeficiency syndrome (AIDS) has highlighted the need for innovative approaches in the production of the new generation of vaccines against infectious diseases. The major goal in the area of vaccine development is the production of a vaccine formulation that will have the desirable effects, and lack of deleterious side effects. There are considerable deficiencies in existing vaccines, including unwanted side effects and difficulties in manufacturing, storage, and delivery systems. Since live attenuated or newly available sub-unit vaccines of single antigens and those produced by means of recombinant DNA generally represent low immunogenecity, they will require appropriate manipulations, the so-called 'adjuvants', to enhance their immunogenic properties.

Recently, there have been several reports demonstrating the usefulness of sustained-antigen releasing microspheres with small particle size as a subcutaneous vaccine (Kreuter & Liehl 1981; Schroder & Stahl 1984; Artursson et al 1986). Kreuter et al (1988) incorporated bovine serum albumin into microspheres made of various hydrophobic polymers and reported their effectiveness as a polymeric adjuvant by the subcutaneous route. Eldridge et al (1991) reported that antigen-containing microspheres 1–10 μm in diameter, exhibited strong adjuvant activity in mice and suggested that particle size was the important factor for immune response. However, in-vitro characterization by size distribution or dissolution studies has not been reported.

In the present study, therefore, we selected poly(lactide-co-glycolide) (PLGA) as the biodegradable polymer because PLGA has excellent biocompatible properties and is already utilized as a biomaterial (Vert et al 1984; Strobel et al 1987) and as a carrier of unstable peptide by the subcutaneous route (Heya et al 1991; Okada et al 1991). The present study describes the preparation of ovalbumin-loaded PLGA microspheres with different diameters and their characterization.

Materials and Methods

Materials

Ovalbumin (Grade V) was purchased from Sigma Chemical Co. (St Louis, USA). Poly(lactide-co-glycolide) (grade 50/50; vis = 0·53 dL g^{-1}, mol.wt 53000) were from Medisorb Technologies (Cincinnati, OH, USA). Polyvinyl alcohol was purchased from Nakarai Co. (Kyoto, Japan). Goat serum, incomplete or complete Freund's adjuvant (ICFA or CFA), alum, and goat anti-mouse IgG were purchased from Gibco Co. (Grand Island, NY, USA). Other agents were all of special reagent grade.

Preparation of microspheres

A water-in-oil-in-water(w/o/w) emulsion/solvent evaporation method as reported by Ogawa et al (1988) was adopted and modified to prepare PLGA microspheres containing ovalbumin. Ovalbumin (20 mg) was dissolved in 800 μL water. The solution was emulsified with 40 mL methylene chloride containing 2·0 g PLGA 50/50 for 30 s using a sonicator homogenizer (UD-200, Tomy Seiko Co., Ltd, Tokyo). The emulsion (approximately 40·8 mL) was poured into a 2 L beaker containing 800 mL 0·3% PVA

solution. Emulsification was continued using a homogenizer (NS-60, Nichionrikakikai Co. Ltd, Tokyo) at appropriate stirring rates (see Table 1) for 4 min then stirred gently for 8 h using a stirring bar. The microspheres were collected by centrifugation at 7000 rev min^{-1} for 10 min. The obtained microspheres were washed with water and recentrifuged three times then dried for at least 24 h under reduced pressure at room temperature (21°C). Microspheres were stored in a desiccator at −30°C until formulated.

Loading test

About 10 mg of microspheres was precisely weighed and dissolved in 2 mL actetone in a glass vial. The polymer solution containing suspended ovalbumin was centrifuged 3000 rev/min^{-1} for 10 min. The acetone was decanted and replaced with fresh acetone. The procedure was repeated three times. The remaining pellet was dissolved in 4 mL purified water. The concentration of the solution was determined using HPLC. Twenty microlitres was injected onto the chloromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10AV), an integrator (Shimadzu C-R6A) and reversed phase C8 column (SG 300, 4·6 × 150 mm, Shiseido, Tokyo, Japan). The mobile phases employed were A (0·10% (w/v) trifluoroacetic acid in water) and B (0·10% (w/v) trifluoroacetic acid in acetonitrile); buffer B was linearly varied from 45 to 60% (v/v) over 10 min. The flow rate was 2·0 mL min^{-1}. The wavelength was set at 214 nm and the column was operated at 40°C. The loading was calculated from the weight of the initial microspheres and the amount of drug incorporated.

Microsphere size

A Microtrac Particle Size Analyzer (Model 7995-30, Leeds and Northrup, North Wales, PA USA) was utilized to determine the microspheres' sizes as average volume.

Morphology

Scanning electron microscopy (SEM) was utilized to examine the surface and morphology of the microspheres. The microspheres were coated with a gold and palladium alloy using a sputter coater (Technics Hummer X Sputter Coater, Cambridge, NY, USA) with the vaccuum set at 0·1torr and a current of 20 μA. Samples were coated twice for 2 min to achieve continuous coverage (Jeol JSM-T300, Tokyo, Japan) with the emission current at 20 mA and an acceleration voltage of 15 KeV.

In-vitro ovalbumin release test

The in-vitro release profile of ovalbumin from various microspheres was determined as follows. Microspheres corresponding to 100 μg were suspended in 2-mL PBS (pH 7·4)/well and shaken horizontally at 75 rev min^{-1} at 37°C. At predetermined time intervals, 150 μL of the suspension was centrifuged (12000 rev min^{-1}, 4 min) and the supernatant analysed by HPLC as described above. Experiments were performed four times.

Animals

Balb C female mice, aged up to 6–8 weeks, were used throughout the experiment. Mice were housed in groups of 10 and had free access to food and water. Mice were inoculated subcutaneously using 100 μL volumes. Animals were bled from the retro-orbital fossa and separated serum was stored in the freezer at −30°C until testing.

Immunization protocols and ELISA

The required dose of microspheres was weighed and resuspended in the appropriate volume of 0·9% NaCl. Groups of mice were inoculated with 1, 5, 25 μg ovalbumin formulated in saline, alum, or complete or incomplete Freund's adjuvant (CFA, ICFA). Alum was used at a dose of 1 mg/inoculation and oil adjuvant as a 1:1 emulsified mixture with ovalbumin. In addition, a further group remained as an uninoculated control. Groups of animals were inoculated with ovalbumin-loaded PLGA microspheres with different sizes subcutaneously at 0 weeks, or 0 and 3 weeks. All animals were bled at 3, 6, 9 and 12 weeks. Serum was tested using an indirect ELISA as follows. Corning plates were coated overnight with 100 μL/well of 150 μg mL^{-1} ovalbumin and were washed once in 100 μL purified water and blocked for 2 h at 37°C with Tris-buffered saline containing Tween 20 (TBST) and 5% goat serum (100 μL/well). After washing twice in purified water, serum test samples (100 μL) at dilutions from 1 to 50 in TBST plus 5% goat serum were added to the wells and incubated for 2 h at 37°C. The plates were washed five times in purified water and 100 μL HRPO labelled-goat anti-mouse IgG diluted (1:2000) was added to the wells and incubated at 37°C for 1 h. The plates were washed five times and 100 μL TBST and H_2O_2 were added to each well. The reaction was stopped after 20 min by the addition of 50 μL diluted hydrofluoric acid (1:400). ELISA plates were read in a plate reader and results were expressed as optical density at 415 nm for each serum sample.

Table. 1. Components and observed parameters of poly(lactide-co-glycolide) microspheres with different diameters.

Formulation	F1	F2	F3	F4
PLGA (g)	2·0	2·0	2·0	2·0
Ovalbumin (mg)	20·0	20·0	20·0	20·0
Drug content(%)				
Theoretical	1·0	1·0	1·0	1·0
Found*	0·08	0·16	0·19	0·25
Load efficiency(%)	7	14	16	22
Stirring rates (rev)	3000	1500	1200	800
Mean average diameter (μm)	1·2	3·5	7·0	14·3

*The values means the average of 4–5 experiments. CV was within 3% in every case.

Results and Discussion

Preparation of microspheres

Ovalbumin-loaded PLGA (50/50) microspheres with four different sizes were prepared by changing stirring rates for the second stage of emulsification (Fig. 1). The corresponding preparation conditions, and characterization for four batches (F1-F4) are summarized in Table 1. These actual drug loadings obtained were much lower than theoretical (1·0%) due to the loss of the water-soluble ovalbumin into the continuous outer aqueous phase during the formation of the w/o/w emulsion droplet. The extent of loss was proportional to the stirring rates used in the second emulsification process.

Ogawa et al (1988) demonstrated the addition of gelatin to the internal phase produced good entrapment efficiency; they also reported that the efficiency of proteins using w/o/w emulsion methods without using gelatin were between 1·9 and 6·7%. In thus study, additives such as gelatin or fatty acids (Yamakawa et al 1992) were not used, to prevent interference with factors which might affect immune response in-vivo.

Morphology

The photographs of ovalbumin-loaded PLGA microspheres with diameters of 1·2 μm (F1) and 3·5 μm (F2) are shown in Fig. 2. These photographs suggest that the microspheres were spherical and fairly nondisperse. The approximate size confirms observations during microsphere sizing.

In-vitro ovalbumin release test

The release profiles of ovalbumin from differently loaded microspheres show the so-called burst phenomena in all cases; the extent with decreasing diameters of spheres as shown in Fig. 3. In particular, PLGA 50/50 microspheres with the smallest diameter (1·2 μm) showed an 80% burst release within one day, whereas 14·3-μm microspheres produced only 30% burst release within a couple of days. No additional release following the burst release was observed in either case and approximately 10–60% of ovalbumin remained unreleased 30 days later.

There have been several reports demonstrating in-vitro sustained-release of protein or peptide release from PLGA microspheres (Benita et al 1984; Cohen et al 1991; Furr & Hutchinson 1992). Particle size of those PLGA microspheres was approximately 100 μm, or much greater than those reported here. Tabata & Ikada (1987) examined the release profile of antitumour agent from PLGA microspheres which had diameter of several μm, and reported about 60% of the drug was released within one day, and

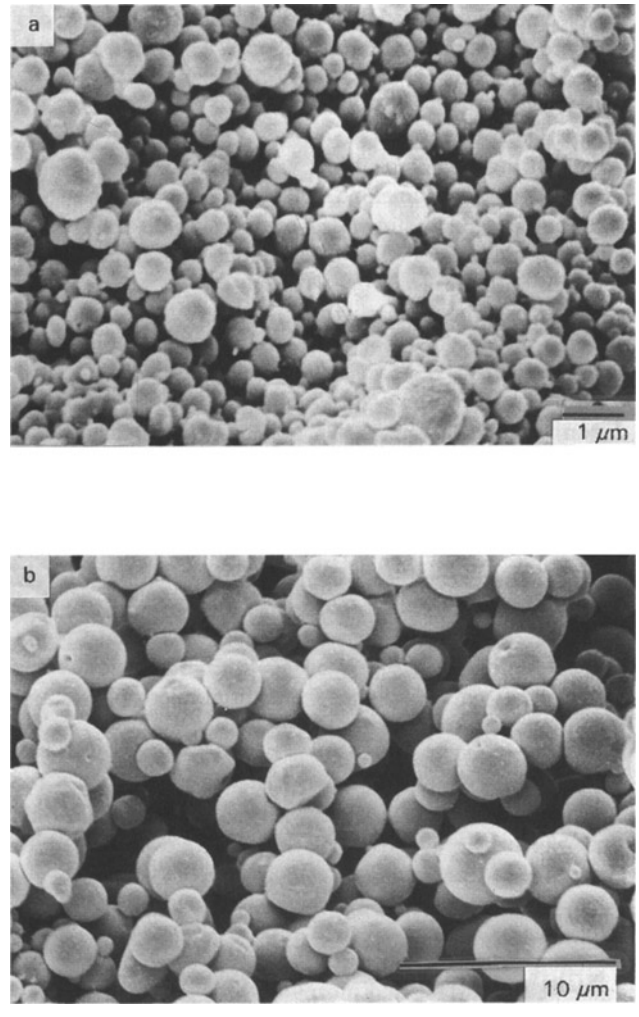

FIG. 2. Scanning electron micrographs of s. 0·08% ovalbumin-loaded PLGA 50/50 microspheres with 1·2 μm diameter and b. 0·16% ovalbumin-loaded PLGA 50/50 microspheres with 3·5 μm diameter.

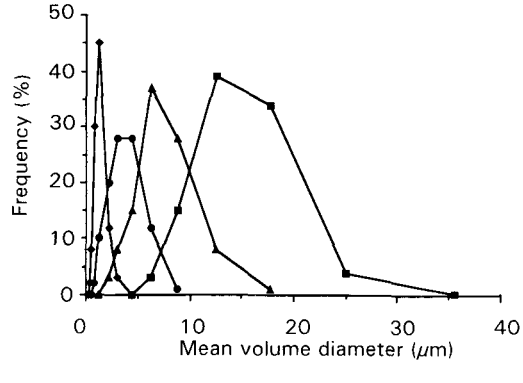

FIG. 1. Size-frequency distributions for various batches of PLGA 50/50 microspheres with different diameters used in the present study. ◆ 1·2 μm, ● 3·5 μm, ▲ 7·0 μm, ■ 14·3 μm.

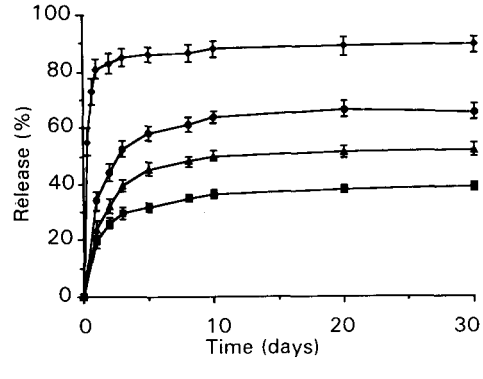

FIG. 3. Release of ovalbumin from PLGA 50/50 microspheres with different diameters. ◆ 1·2 μm, ● 3·5 μm, ▲ 7·0 μm, ■ 14·3 μm. The dissolution medium was PBS (pH 7·4) kept at 37°C. Each point represents the mean ± s.d. of four experiments.

most of the drug was released over 7 days. Recently, Lai et al (1993) also reported 70% of isoprenaline release within a few hours. The burst releases reported in the literature are in agreement with our result. Furthermore, Ogawa et al (1988) reported no additional release following an initial 25% release of peptide from PLGA microspheres with diameters of the order of 100 μm. The lack of additional release in the present study may be due to low diffusion caused by low antigen loading or interraction of the PLGA polymer with ovalbumin. The large burst release from 1·2 μm microspheres is probably due to its large surface area.

In-vivo immune response in mice

The antibody levels of mice inoculated with different size PLGA microspheres with various doses of antigen (1, 5 and 25 μg ovalbumin) are shown in Table 2 and compared with mice given saline/ovalbumin, alum, ICFA and CFA (Freund 1951) as controls. Single (0 weeks) and double inoculation (0 and 3 weeks) data are shown in Table 2. Ovalbumin-loaded PLGA microspheres of four different sizes gave good immune responses which were statistically higher than the saline/ovalbumin negative control ($P < 0·05$) at all doses.

The antibody response increased as the ovalbumin dose increased. No advantage in administering additional microspheres at 3 weeks was observed at 5 and 25 μg ovalbumin doses as shown in Table 2. In contrast, an effect of a second inoculation was observed to some extent at a low dose of 1 μg ovalbumin (Table 2). In that study, the number of microsphere particles administered may not be sufficient for update by macrophages and thereby cannot induce immune response. Not needing a second inoculation is one advance of sustained-antigen release using microspheres. For ovalbumin solutions, the second inoculation effect was observed even though the immune response was not strong. In fact, most commercial vaccines require two or three inoculations.

The rank of immune response was as follows: 3·5 μm > 1·2 μm = 7·0 μm > 14·3 μm-microspheres. 1·2- and 7·0 μm-microspheres exhibited almost the same or a higher level of antibody than alum or ICFA and were not significantly different from CFA at 5 and 25 μg of ovalbumin doses (see Table 2). For 14·3 μm-microspheres, antibody levels were smaller than positive controls at 3 and 6 weeks, but immune response increased with time and finally the immune response became almost the same or larger than alum or ICFA at 9 and 12 weeks (see Table 2). This may be due to the largest particle size of 14·3 μm-microspheres, since degradation of PLGA 50/50 polymer in-vivo is expected to be an important factor for the immunization process using this particle system, and erosion of a larger particle may take several weeks. In addition, particles < 10 μm seemed to not be advantageous for uptake by macrophages. Comparatively small burst release with 14·3 μm-microspheres might not be advantageous for inducing immune response in the initial stage.

Immunologic adjuvants generally act through the direct activation of lymphoid cells or through a depot. A previous report (Eldridge et al 1991) demonstrated that fluorescent

Table 2. Serum antibody responses to ovalbumin after subcutaneous inoculation with different diameter microspheres.

Ovalbumin (μg)	Treatment	Immune responses after single inoculation (optical density at 415 nm)				Immune responses after double inoculation (optical density at 415 nm)			
		3 week	6 week	9 week	12 week	3 week	6 week	9 week	12 week
1 μg	Microsphere								
	1·2 μm	0·17 (0·01)	0·18 (0·01)	0·28 (0·02)	0·26 (0·02)	0·19 (0·01)	0·29 (0·03)	0·36 (0·03)	0·36 (0·03)
	3·5 μm	0·28 (0·03)*	0·34 (0·03)*	0·40 (0·04)	0·44 (0·03)*	0·29 (0·02)	0·46 (0·02)*	0·54 (0·04)*	0·57 (0·04)*
	7·0 μm	0·11 (0·01)	0·16 (0·01)	0·25 (0·01)	0·25 (0·02)	0·19 (0·01)	0·24 (0·03)	0·24 (0·02)	0·36 (0·03)
	14·3 μm	0·03 (0·01)	0·11 (0·01)	0·17 (0·01)	0·21 (0·02)	0·09 (0·01)	0·15 (0·01)	0·25 (0·03)	0·29 (0·04)
	Saline	0·02 (0·00)	0·03 (0·00)	0·03 (0·01)	0·04 (0·01)	0·02 (0·00)	0·08 (0·01)	0·10 (0·01)	0·12 (0·04)
	Alum	0·10 (0·01)	0·14 (0·01)	0·16 (0·01)	0·17 (0·02)	0·14 (0·01)	0·17 (0·02)	0·18 (0·01)	0·21 (0·01)
	ICFA	0·16 (0·01)	0·18 (0·02)	0·20 (0·02)	0·20 (0·02)	0·14 (0·01)	0·22 (0·02)	0·32 (0·04)	0·31 (0·03)
	CFA	0·22 (0·03)	0·28 (0·02)	0·34 (0·03)	0·35 (0·03)	0·27 (0·02)	0·41 (0·02)	0·45 (0·03)	0·45 (0·03)
5 μg	Microsphere								
	1·2 μm	0·30 (0·03)	0·33 (0·04)	0·53 (0·04)	0·52 (0·03)	0·29 (0·03)	0·42 (0·02)	0·51 (0·05)	0·53 (0·04)
	3·5 μm	0·52 (0·05)*	0·63 (0·08)*	0·65 (0·06)*	0·71 (0·07)*	0·55 (0·04)*	0·62 (0·04)*	0·70 (0·07)*	0·77 (0·06)*
	7·0 μm	0·24 (0·02)	0·42 (0·05)	0·47 (0·05)	0·49 (0·08)	0·26 (0·03)	0·39 (0·04)	0·42 (0·03)	0·53 (0·05)
	14·3 μm	0·10 (0·01)	0·19 (0·02)	0·34 (0·03)	0·40 (0·04)	0·09 (0·04)	0·20 (0·03)	0·37 (0·04)	0·43 (0·03)
	Saline	0·06 (0·01)	0·06 (0·01)	0·80 (0·01)	0·08 (0·01)	0·06 (0·01)	0·11 (0·02)	0·18 (0·03)	0·16 (0·02)
	Alum	0·14 (0·02)	0·19 (0·02)	0·24 (0·03)	0·24 (0·03)	0·16 (0·01)	0·22 (0·03)	0·26 (0·02)	0·30 (0·02)
	ICFA	0·17 (0·02)	0·20 (0·03)	0·28 (0·03)	0·29 (0·03)	0·17 (0·01)	0·25 (0·02)	0·30 (0·03)	0·33 (0·03)
	CFA	0·36 (0·04)	0·38 (0·03)	0·44 (0·05)	0·49 (0·04)	0·34 (0·05)	0·42 (0·05)	0·50 (0·05)	0·57 (0·04)
25 μg	Microsphere								
	1·2 μm	0·52 (0·06)	0·70 (0·11)	0·72 (0·08)	0·75 (0·07)	0·54 (0·04)	0·65 (0·05)	0·73 (0·02)	0·77 (0·03)
	3·5 μm	0·85 (0·09)*	0·95 (0·10)*	0·97 (0·05)*	1·05 (0·07)*	0·77 (0·08)*	0·94 (0·09)*	0·95 (0·05)*	1·08 (0·05)*
	7·0 μm	0·34 (0·06)	0·68 (0·10)	0·68 (0·04)	0·70 (0·05)	0·34 (0·04)	0·60 (0·05)	0·66 (0·06)	0·71 (0·06)
	14·3 μm	0·12 (0·01)	0·28 (0·03)	0·48 (0·05)	0·58 (0·06)	0·15 (0·01)	0·23 (0·03)	0·53 (0·04)	0·56 (0·05)
	Saline	0·08 (0·01)	0·08 (0·01)	0·11 (0·02)	0·12 (0·01)	0·11 (0·02)	0·15 (0·01)	0·20 (0·02)	0·21 (0·03)
	Alum	0·23 (0·02)	0·30 (0·03)	0·34 (0·03)	0·35 (0·02)	0·26 (0·04)	0·37 (0·03)	0·40 (0·03)	0·42 (0·05)
	ICFA	0·28 (0·03)	0·35 (0·05)	0·39 (0·05)	0·40 (0·05)	0·25 (0·03	0·42 (0·04	0·45 (0·03)	0·46 (0·06)
	CFA	0·52 (0·07)	0·68 (0·06)	0·68 (0·10)	0·70 (0·07)	0·54 (0·07)	0·60 (0·06)	0·77 (0·07)	0·82 (0·08)

The immune responses were expressed as an optical density at 415 nm, and the figures in parentheses represent s.d. of 10 mice. *$P < 0·05$ compared with the complete Freund's adjuvant of same dose at the same time.

microspheres with diameters less than 10 μm are phagocytized and transported by macrophages into the draining lymph nodes. If two different batches of microspheres differ two- or threefold in diameter, 10- to 30-fold more microspheres will be administered for the same dose. Tabata & Ikada (1991) also showed that the number of microspheres phagocytosed per macrophage has a maximum at a diameter between 1·0 and 2·0 μm, and we expected that particles with the smallest diameter (1·2 μm) would give rise to the highest immune response. However, even with oral inoculation with microspheres, 1·2 μm-microspheres were inferior to 3·5 μm in the inability to induce a serum immune response in our pilot study (data not shown) even though the previous study showed that polystyrene particles with smaller diameters were reported to be advantageous for uptake by Peyer's Patch tissue and migration to mesenteric lymph nodes (Jani et al 1989). This may be due to its large burst in-vitro effect observed with PLGA microspheres of 1·2 μm in diameter. In general, small microspheres (< 1 μm) have the possibility of a large burst release of antigen. If the diameter is very small there will be a rapid release of 100% of the antigen. The previous study using nanoparticles as adjuvant did not induce so high an immune response (Kreuter et al 1986). On the other hand, the mechanism for immunization would suggest some burst release will be needed to present the antigen as the immune response was reported to be determined in the initial stage after inoculation (Ada 1992).

Differences in immune responses induced following inoculation of microspheres with different size may also be due to differences in % ovalbumin-loading in batches of microspheres. Nevertheless, our pilot study suggested that in the range of 0·05–0·2% ovalbumin-loaded PLGA microspheres, the same level of high immune response, with values which were statistically higher than those from 1% or 1·5% ovalbumin-loaded PLGA microspheres (data not shown), even though over 1% antigen-loaded microparticles were used previously (O'Hagan et al 1991; Eldridge et al 1991). This suggested the possibility of decreasing the antigen dose if enough PLGA microspheres were administered.

In conclusion, we have demonstrated some advantages of ovalbumin-loaded PLGA microspheres with small particle size. The characterization of size distributions and in-vitro release seems to be essential to design the appropriate small-particle vaccine formulations. The antigen dose, % loading and size of particle must be optimized to produce a quality vaccine formulation. The possibility of single-step immunization with PLGA 50/50 microspheres of small size encourages further research using this system for application in man or animals.

References

Ada, G. L. (1992) Vaccine efficacy and the immune response. Vaccine Res. 1: 17–23

Artursson, P., Martensson, I.-L., Sjoholm, I. (1986) Biodegradable microspheres III: some immunological properties of polyacryl starch microspheres. J. Pharm. Sci. 75: 697–701

Benita, S., Benoit, J. P., Puisieux, F., Thies, C. (1984) Characterization of drug loaded poly(dl-lactide)microspheres. J. Pharm. Sci. 73: 1721–1724

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L. H., Langer, R. (1991) Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm. Res. 8: 713–720

Ebel, J. D. (1990) A method for quantifying particle absorption from the mouse small intestine. Pharm. Res. 7: 848–851

Eldridge, J. H., Hammond, C. J., Meulbroek, J. A., Staas, J. K., Gilley, R. M., Tice, T. R. (1990) Controlled vaccine release in the gut-associated lymphoid tissues 1. Orally administered biodegradable microspheres target in the Peyer's patches J. Contr. Rel. 11: 205–214

Eldridge, J. H., Staas, J. K., Meulbroek, J. A., Tice, T. R., Gilley, R. M. (1991) Biodegradeable and biocompatible poly(dl-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. Infect. Immunol. 59: 2978–2986

Freund, J. (1951) The effect of paraffin oil and mycobacteria on antibody formation and sensitization. Am. J. Clin. Pathol. 21: 645–656

Furr, B. J. A., Hutchinson, F. G. (1992) A biodegradable delivery system for peptides: preclinical experience with the gonadotrophin-releasing hormone agonist Zoladex. J. Contr. Rel. 21: 117–128

Heya, T., Okada, H., Tanigawara, Y., Ogawa, Y., Toguchi, H. (1991) Effects of counteranion of TRH and loading amount on control of TRH release from copoly(dl-lactic/glycolic acid) microspheres prepared by an in-water drying method. Int. J. Pharm. 69: 69–75

Jani, P., Halbert, G. W., Langridge, J., Florence, A. T. (1989) The uptake and translocation of latex nanospheres and microspheres after oral administration to rats. J. Pharm. Pharmacol. 41: 809–812

Kreuter, J., Liehl, E. (1981) Long-term studies of microencapsulated and absorbed influenza vaccine nanoparticles. J. Pharm. Sci. 70: 367–371

Kreuter, J., Berg, U., Liehl, E., Soliva, M., Speiser, P. P. (1986) Influence of the particle size on the adjuvant effect of particulate polymeric adjuvants. Vaccine 4: 125–129

Kreuter, J., Liehl, E., Berg, U., Soliva, M., Speiser, P. P. (1988) Influence of hydrophobicity on the adjuvant effect of particulate polymeric adjuvants. Vaccine 4: 253–256

Lai, Y-L., Mehta, R. C., Thaker, A. A., Yoo, S.-D., McNamara, P. J., DeLuca, P. P. (1993) Sustained bronchodilation with isoproterenol poly (glycolide-co-lacitide) microspheres. Pharm. Res. 10: 119–125

Ogawa, Y., Yamamoto, M., Takada, S., Okada, H., Shimamoto, T. (1988) Controlled-release of leuprolide acetate from polylactic acid or copoly(lactic/glycolic) acid microcapsules: influence of molecular weight and copolymer ratio of polymer. Chem. Pharm. Bull. 36: 1095–1103

O'Hagan, D., Jeffery, H., Roberts, M. J. J., McGee, J. P., Davis, S. S. (1991) Controlled release microparticles for vaccine development. Vaccine 7: 421–424

Okada, H., Heya, T., Ogawa, Y., Toguchi, H., Shimamoto, T. (1991) Sustained pharmacological activities in rats following single and repeated administration of once-a-month injectable microspheres of leuprolide acetate. Pharm. Res. 8: 584–587

Schroder, U., Stahl, A. (1984) Crystallized dextran nanospheres with entrapped antigen and their use as adjuvants. J. Immunol. Methods 70: 127–132

Strobel, J. D., Laughin, T. J., Ostroy, F., Lilly, M. D., Perkins, B. H., Dunn, R. L. (1987) Controlled-release systems for anticancer agents. Proc. Int. Symp. Contr. Rel. Bioact. Mater. 14: 261–262

Tabata, Y., Ikada, Y. (1987) Activation of macrophage in-vitro to acquire antitumor activity by a muramyl dipeptide derivative encapsulated in microspheres composed of lactide copolymer. J. Contr. Rel. 6: 189–204

Tabata, Y., Ikada, Y. (1991) Phagocytosis of polymeric microspheres. High Perf. Biomat. 621–646

Vert, M., Cristel, P., Chabot., F. (1984) Bioerodible plastic materials for bone surgery in macromolecular biomaterials. CRC Press, Boca Raton, pp 120–142

Yamakawa, I., Tsushima, Y., Machida, R., Watanabe, S. (1992) Preparation of eurotensin analogue-containing poly(dl-lactic acid) microspheres formed by oil-in-water solvent evaporation. J. Pharm. Sci. 81: 899–903