Oral Delivery of Poly(lactide-co-glycolide) Microspheres Containing Ovalbumin as Vaccine Formulation: Particle Size Study

Takahiro UCHIDA and Shigeru GOTO*

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan.

Received March 2, 1994; accepted May 11, 1994

The objective of the present study was to produce ovalbumin (OVA) loaded poly(lactide-co-glycolide) (PLGA) microspheres with different diameters and to evaluate their possibilities as vaccine formulation in mice following oral inoculation. Four kinds of OVA loaded PLGA microspheres with different mean average volume diameters (1.3, 4.0, 7.5, and 14.0 μm) were manufactured using a w/o/w emulsion/solvent evaporation method. Low loading efficiencies (8–20% w/w) were observed in all batches although smooth spherical particles were obtained. Single oral administrations of OVA loaded PLGA microspheres with different diameters to mice produced immune responses (serum IgG levels by ELISA) which were statistically higher than OVA solution as negative control (Fisher's paired *t*-test). A dose-response was observed, and single and double inoculation orally produced similar serum antibody levels.

The rank of immune response was as follows: 4.0 μm > 1.3 μm = 7.5 μm > 14.0 μm -microspheres. The oral inoculation with 0.13% OVA loaded PLGA microspheres having a mean volume diameter of 4.0 μm exhibited the best immune responses with values greater than those obtained after subcutaneous inoculation with complete Freund's adjuvant (CFA) as positive control, and not significantly different from those obtained after subcutaneous inoculation with the same microspheres.

Keywords microsphere; poly(lactide-co-glycolide); ovalbumin; vaccine; oral inoculation; adjuvant

Despite rapid progress in the fields of science and biotechnology, many infectious diseases still afflict hundreds of millions of persons throughout the world, mostly in developing countries, resulting in physical disabilities and death. For example, even for diseases such as hepatitis B, although a vaccine formulation has been developed, it is not available to the most of the patients those infected with the virus. For many other diseases, no appropriate vaccines exist. 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 two prime requirements of a vaccine are safety and efficacy. Classical vaccines generally consist of either inactivated or live attenuated microorganisms which are often not sufficiently immunogenic to induce potent immune responses without the use of an appropriate manipulation, the so-called "adjuvants." Since live attenuated or newly available sub-unit vaccines of single antigens produced by means of recombinant DNA generally represent low immunogenicity, they will require an appropriate adjuvant.

Controlled-release systems, already applied to deliver a whole array of drugs and hormones, both in man and in cattle, appear to be one way of accomplishing the above goal. There have been several works demonstrating the usefulness of sustained-antigen releasing microspheres with small particle size as subcutaneous vaccine.¹⁾

Whereas oral immunization is likely to offer a lot of inherent advantages over the parenteral route: it would be no pain with patient, easy, considerably free from side effects, would be more acceptable to the majority of patients. Recently, O'Hagan *et al.* reported the induction of secretory and serum immune response following oral administration of a polyacrylamide microsphere with 2.55 μm .²⁾ Nevertheless, in that study, no significant difference was observed between serum immune response

levels obtained after oral administration of microspheres and antigen solution as control. In addition, *in vitro* characterizations such as size distribution or morphology were not performed at all.

In the present study, we selected poly(lactide-co-glycolide) (PLGA) as wall material for the particle, a polymer which has excellent biocompatible property and has already been utilized in biomaterial applications³⁾ as well as a carrier of unstable peptide.⁴⁾ Small fluorescent particles under 10 μm were reported to be advantageous for uptake by Peyer's patches (PPs) that are known to play a critical role in the oral immunization process, and which finally transport into the draining lymph nodes⁵⁾ and its size dependency was observed.⁶⁾

Therefore, we loaded model antigen ovalbumin (OVA) into PLGA microspheres with different diameters (1.3, 4.0, 7.5 and 14.0 μm), and their *in vitro* characterization of size distribution and morphology were performed. These microspheres were administered to mice orally in varying doses, serum immune response was measured, and the possibility of PLGA microspheres for use as an oral vaccine was evaluated.

MATERIALS AND METHODS

Materials OVA (grade V) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PLGA (grade 50/50; molecular weight (M.W.) = 48000) was from Medisorb Technologies (Cincinnati, OH, U.S.A.). Polyvinyl alcohol (grade 87–89% hydrolyzed; M.W. = 85000–146000) was purchased from Aldrich Chemical Co. (Milwaukee, WIS, U.S.A.). Goat serum, incomplete or complete Freund's adjuvant (ICFA or CFA), aluminum, and goat antimouse IgG were purchased from Gibco. Co. (Grand Island, NY, U.S.A.). Other agents were all of special reagent grade.

Preparation of Microspheres In our laboratory, a

water-in-oil (w/o) or an oil-in-oil (o/o) method has been utilized to incorporate various drugs into microspheres several hundred μm in size.⁷⁾ In this study, we adopted a water-in-oil-in-water (w/o/w) emulsion/solvent evaporation method in order to prepare a water soluble protein loaded with PLGA microspheres with even smaller diameter. The actual procedure was as follows: 20 mg of OVA was dissolved in 800 μl water. The solution was emulsified with 40 ml of methylene chloride containing 1.98 g PLGA 50/50 for 30 s (15000 rpm) using a microhomogenizer (Ultra-Turrax T25, Hansen Co., Kobe, Japan). The approximately 40.8 ml of this w/o emulsion was poured into a 2 l beaker containing 400 ml of 0.6% polyvinyl alcohol solution. Emulsification was continued using a homogenizer (PT 3000, Centralkagaku Co., Tokyo, Japan) at appropriate stirring rates (four batches were prepared; see Table I) for 3 min then stirred gently for 6 h using a stirring bar. The microspheres were collected by centrifugation at 8000 rpm for 10 min. The obtained microspheres were washed with water and recentrifuged three times and then dried for at least 24 h under reduced pressure at room temperature. Microspheres were stored desiccated at -30°C until formulated.

The actual load was determined by placing about 150 mg of microspheres in 400 μl of methylene chloride, extracting OVA twice with 800 μl of the purified water. Following the extraction, the concentration of the extracted OVA solution was determined using Bicinchoninic acid total protein assay.⁸⁾ The procedure was repeated three times. A particle size analyzer (Model PC-320, Particle Scientific Co., Elgin, IL, U.S.A.) was utilized to determine microsphere sizes as mean volume diameter.

Morphology Scanning electron microscopy (SEM) was utilized to examine the topography and morphology of the microspheres. The microspheres were coated with a gold and palladium alloy using a sputter coater (Eiko engineering coater, IB-3 type, Tokyo, Japan) with the vacuum set at 0.1 Torr 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 electron voltage of 15 keV.

Animals Balb C female mice, aged up to 6–8 weeks were used throughout the experiment. Mice were housed in groups of 8–10 animals and given food and water *ad libitum*. They were inoculated orally or subcutaneously with a volume of 100 μl . Animals were bled from the retro-orbital fossa and separated serum was stored in a freezer at -30°C until testing.

Immunization Protocols and ELISA The required doses of microspheres were weighed and resuspended in an appropriate volume of 0.9% NaCl solution. Groups of mice were inoculated intra-gastrically with 1, 10, 100 μg OVA formulated in saline and microspheres with different sizes at 0 week, or 0 and 3 weeks. Subcutaneous inoculation with alum, or CFA or ICFA was performed as a positive control in addition to inoculation with saline and microspheres. Alum was used at a dose of 1 mg/inoculation and oil adjuvant as a 1:1 emulsified mixture with OVA. A further group remained as an uninoculated control. All animals were bled at 3, 6, 9 and 12 weeks. Serum was tested on an indirect ELISA as described in the previous

paper,²⁾ and results were expressed as optical density at 415 nm for each serum sample.

Statistical Analysis The results from the *in vivo* mouse study were expressed as the mean $\pm$ standard error for 8–10 mice. Statistical analysis was performed using one-way analysis of variance ($p < 0.05 = a$), followed by Fisher's paired *t*-test for differences.

RESULTS AND DISCUSSION

Preparation of Microspheres OVA loaded PLGA (50/50) microspheres with four different diameters were prepared by changing stirring rates for the second emulsification. The corresponding preparation conditions, theoretical or actual OVA loading %, yield and mean volume diameters for four batches (F1–4) are summarized in Table I. These actual drug loadings ranged between 0.08 and 0.20% (w/w) with loading efficiencies ranging between 8 and 20%. They were much lower than theoretical (1.0% w/w) due to the loss of the water soluble OVA into the continuous outer aqueous phase during the formation of the w/o/w emulsion droplet. The extent of loss seemed to be proportional to the stirring rates used in the second emulsification process. A previous report⁹⁾ demonstrated that the addition of gelatin to the internal phase produced good entrapment efficiency, but entrapment efficiencies of proteins using w/o/w emulsion method without using gelatin were poor (between 1.9 and 6.7% in the literature). In this study, additives such as gelatin or fatty acids¹⁰⁾ were not utilized to prevent interference with factors which might affect immune response *in vivo*. Size distributions for PLGA microsphere batches (F1–4) having mean volume diameters of 1.3, 4.0, 7.5 and 14.0 μm were obtained, respectively. Standard deviations for the diameter of each batch were comparatively small as shown in Table I.

Morphology The photographs of OVA loaded PLGA microspheres with a diameter of 1.3 μm (F1) and 14.0 μm (F4) are shown in Figs. 1A and B, respectively, and suggest that microspheres were also spherical and fairly monodisperse batches. The approximate size confirms the observations during microsphere sizing.

In Vivo Study The oral administration of different antigen doses by varying the amount of microspheres delivered has not been reported. Therefore, mice were inoculated orally with OVA loaded PLGA microspheres

TABLE I. Components and Observed Parameters of PLGA Microspheres with Different Diameters

Formulation	F1	F2	F3	F4
PLGA (g)	1.98	1.98	1.98	1.98
OVA (mg)	20.0	20.0	20.0	20.0
Drug content (%)				
Theoretical	1.0	1.0	1.0	1.0
Found ^{a)}	0.08	0.11	0.15	0.20
Load efficiency (%)	8	11	15	20
Stirring rates (rpm)	9000	7000	5000	3000
Mean volume diameter (μm)	1.3 (0.2) ^{b)}	4.0 (0.6)	7.5 (1.1)	14.0 (2.8)

a) The values mean the average of 5–6 experiments. b) The figures in parentheses means the standard deviations.

Fig. 1. Scanning Electron Micrographs of OVA Loaded PLGA 50/50 Microspheres with 1.3 μm Diameter (A) and 14.0 μm Diameter (B)

Fig. 2. Serum Antibody Responses at 3, 6, 9, and 12 Weeks to OVA Following the Oral Inoculation of PLGA 50/50 Microspheres with Different Diameters (●, 1.3 μm -; □, 4.0 μm -; ▲, 7.5 μm -; ■, 14.0 μm -) and OVA Solution (○)

A, B and C show the results following a single inoculation (0 week) with microspheres corresponding to 1, 10 and 100 μg of OVA respectively. Error bars represent S.E. of 8–10 mice. *a*) Means statistically different ($p < 0.05$) compared with other microspheres of the same dose at the same time.

with different diameters at various antigen doses (1, 10 and 100 μg OVA), and the induced serum immune responses were compared to animals given saline/OVA as control.

Figures 2A, B and C represent the serum immune responses following a single oral inoculation (0 week) of microspheres corresponding to 1, 10 and 100 μg of OVA, respectively. PLGA microspheres with mean volume average diameters of 1.3, 4.0, 7.5 and 14.0 μm gave good immune responses which were statistically higher than saline/OVA as negative control ($p < 0.05$ by Fisher's paired *t*-test) at all doses. The antibody response increased as the OVA dose increased. The rank of immune response was as follows: 4.0 μm - > 1.3 μm - = 7.5 μm - > 14.0 μm - microspheres. With 4.0 μm - microspheres induced the highest level of serum IgG titer. Even 3 weeks after inoculation, the IgG level was considerably high and increased gradually up to 12 weeks. The levels were statistically higher than other microspheres (shown as *a*) in Fig. 2). 1.3- and 7.5 μm - microspheres was likely to exhibit considerable high level of antibody, while 14.0 μm - microspheres produced the most smallest IgG levels among those of various diameters.

Several studies have reported the uptake of small particles ($< 10 \mu\text{m}$) across the gastrointestinal tract.¹¹⁾ The PPs rather than ordinary enterocytes are more likely to be the site of uptake for such particles. Jani *et al.*¹²⁾ demonstrated the uptake and transport of fluorescent latex particles across the gastrointestinal tract in rat. They reported that the advantages of particle less than 1 μm which were identified under a fluorescent microscope in the PPs, and migrated into mesenteric lymph node. In another study using mice, Le Fevre *et al.*¹³⁾ described the intestinal uptake of fluorescent microparticles (1.8 μm) in young and aged mice. Another report¹⁴⁾ also illustrated that the number of microspheres phagocytosed per macrophage is a maximum at a diameter between 1.0 and 2.0 μm . Therefore, we expected that 1.3 μm - microspheres would induce the highest serum IgG level, this was actually not the case.

On the contrary, Eldridge *et al.*¹⁵⁾ demonstrated that smaller microspheres under 5 μm in diameter were transported through the efferent lymphatics within macrophages, while the majority of microspheres with 5–10 μm in diameter remained in the PPs. This phenomenon may suggest that small microspheres 1.3 μm

Fig. 3. Serum Antibody Responses to OVA at 3, 6, 9, and 12 Weeks Following the Oral Inoculation of PLGA 50/50 Microspheres with Different Diameters (●, 1.3 μm -; □, 4.0 μm -; ▲, 7.5 μm -; ■, 14.0 μm -) and OVA Solution (○)

A, B and C show the results following double inoculations (0 and 3 weeks) with microspheres corresponding to 1, 10 and 100 μg of OVA, respectively. Error bars represent S.E. of 8–10 mice. *a)* Means statistically different ($p < 0.05$) compared with other microspheres of the same dose at the same time.

in diameter are not necessarily advantageous for induction of high immune response, and that particles remaining in the PPs for a long time may be beneficial in prolonging the immune response. Another explanation for differences in immune responses induced following inoculation of microspheres with different diameters in the present study may be due to the differences of OVA loading % in the four batches of microspheres. Nevertheless, our pilot study suggested that from 0.05 to 0.2% OVA loaded PLGA microspheres exhibited almost the same level of high immune response, and that their values were statistically higher than 1% or 1.5% OVA loaded PLGA microspheres (data not shown); this was true even though over 1% antigen loaded microparticles had been used in previous work by O'Hagan *et al.*¹⁶⁾ and Eldridge *et al.*¹⁷⁾ This fact also suggested that the antigen dose could be decreased if PLGA microspheres are administered.

Secondly, the boosting effect was examined since many commercial vaccines must be administered two or three times. Figures 3A, B and C represent the serum antibody response following double inoculations (0 and 3 weeks) of microspheres corresponding to 1, 10 and 100 μg of OVA, respectively. The effect of the second inoculation was observed to some extent at a low dose of 1 μg of OVA (Figs. 2A, 3A). In this case, the number of microsphere particles administered per mouse may not have been enough for uptake by macrophages and may therefore induce an immune response since the uptake ratio of small particles was reported to be very low ($< 1.0\%$)¹⁸⁾; no advantage was observed by administering additional microspheres at 3 weeks at doses of 10 μg (Figs. 2B, 3B) and 100 μg in the OVA study (Figs. 2C, 3C). This observation of not needing a second inoculation is one advantage of the sustained-antigen release using microspheres. In the case of OVA solution, the second inoculation effect was observed even though the immune response was not strong.

Finally, the difference in inoculation routes was examined. Figure 4 shows the serum IgG titer following single subcutaneous inoculation (Subcu) with OVA saline, 4.0 μm -microspheres. Aluminum, ICFA, and CFA as a positive control were also utilized as adjuvants. The study

Fig. 4. Serum Antibody Responses to OVA at 3, 6, 9, and 12 Weeks Following a Single Subcutaneous Inoculation with Saline OVA Solution (○), Aluminum (●), ICFA (△), CFA (▲), 4.0 μm -Microspheres (■) and a Single Oral Inoculation with 4.0 μm -Microspheres (□)

All studies were performed using 100 μg of OVA. Error bars represent S.E. of 8–10 mice. *a)* Means statistically different ($p < 0.05$) compared with the CFA of the same dose at the same time.

was undertaken at a dose of 100 μg OVA, and single oral inoculation data (Oral) following 4.0 μm -microspheres (4.0 μm -MS) is also shown for comparison. The rank of immune responses was as follows: 4.0 μm -MS (Subcu) > 4.0 μm -MS (Oral) > CFA (Subcu) > ICFA (Subcu) > aluminum (Subcu) > saline (Subcu). CFA and ICFA are known as strong immune adjuvants even though they are approved for only animals. Aluminum is the only adjuvant approved for use in humans. The single oral inoculation with 4.0 μm -microspheres induced considerably strong immune responses with values greater than those obtained after subcutaneous inoculation with CFA (shown as *a)* in Fig. 4) and not significantly different from those obtained after subcutaneous inoculation with the same microspheres.

In conclusion, we have demonstrated some advantages of oral inoculation with OVA loaded PLGA microspheres with small diameters. The OVA dose and particle size seem to be important factors. The possibility of single step immunization with PLGA 50/50 microspheres with smaller diameter encourages our further research using this system

for application to vaccination in human or animals.

REFERENCES

- 1) a) P. Artursson, I.-L. Martensson, I. Sjöholm, *J. Pharm. Sci.*, **75**, 697 (1986); b) J. Kreuter, U. Berg, E. Liehl, M. Soliva, P. P. Speiser, *Vaccine*, **4**, 125 (1986).
- 2) D. T. O'Hagan, K. Palin, S. S. Davis, P. Artursson, I. Sjöholm, *Vaccine*, **7**, 421 (1989).
- 3) a) M. Vert, P. Cristel, F. Chabot, "Macromolecular Biomaterials," CRC Press, Boca Raton, 1984, p. 120; b) J. D. Strobel, T. J. Laughin, F. Ostroy, M. D. Lilly, B. H. Perkins, R. L. Dunn, *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, **14**, 261 (1987).
- 4) a) H. Okada, T. Heya, Y. Ogawa, H. Toguchi, T. Shimamoto, *Pharm. Res.*, **8**, 584 (1991); b) T. Heya, H. Okada, Y. Tanigawara, Y. Ogawa, H. Toguchi, *Int. J. Pharmaceut.*, **69**, 69 (1991).
- 5) a) M. E. Le Fevre, J. B. Warren, D. D. Joel, *Expl. Cell Biol.*, **53**, 121 (1985); b) J. D. Ebel, *Pharm. Res.*, **7**, 848 (1990).
- 6) M. E. Le Fevre, D. D. Joel, "Intestinal Toxicology," Raven Press, New York, 1984, p. 45.
- 7) a) S. Goto, T. Uchida, T. Aoyama, *J. Pharmacobio-Dyn.*, **8**, 270 (1985); b) T. Uchida, S. Goto, *Chem. Pharm. Bull.*, **36**, 2135 (1988); c) T. Uchida, T. Yasutake, S. Goto, *ibid.*, **40**, 463 (1992).
- 8) P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, *Anal. Biochem.*, **159**, 76 (1985).
- 9) Y. Ogawa, M. Yamamoto, S. Takada, H. Okada, T. Shimamoto, *Chem. Pharm. Bull.*, **36**, 1095 (1988).
- 10) I. Yamakawa, Y. Tsushima, R. Machida, S. Watanabe, *J. Pharm. Sci.*, **81**, 899 (1992).
- 11) a) J. M. Payne, B. F. Sansam, R. J. Garner, A. R. Thomson, B. J. Miles, *Nature (London)*, **188**, 586 (1960); b) H. O. Alpar, W. N. Field, R. Hyde, D. A. Lewis, *J. Pharm. Pharmacol.*, **41**, 194 (1989).
- 12) P. G. Jani, W. Halbert, J. Langridge, A. T. Florence, *J. Pharm. Pharmacol.*, **41**, 809 (1989).
- 13) M. E. Le Fevre, A. M. Boccio, D. D. Joel, *Proc. Soc. Exp. Biol. Med.*, **190**, 23 (1989).
- 14) Y. Tabata, Y. Ikada, *High Perf. Biomat.*, **1**, 621 (1991).
- 15) J. H. Eldridge, C. J. Hammond, J. A. Meulbroek, J. K. Staas, R. M. Gilley, T. R. Tice, *J. Contr. Rel.*, **11**, 205 (1990).
- 16) D. T. O'Hagan, H. Jeffery, M. J. J. Roberts, J. P. McGee, S. S. Davis, *Vaccine*, **9**, 768 (1991).
- 17) J. H. Eldridge, J. K. Staas, J. A. Meulbroek, T. R. Tice, R. C. Gilley, *Infect. Immun.*, **59**, 2978 (1991).
- 18) M. E. Le Fevre, D. D. Joel, G. Schidlovsky, *Proc. Soc. Exp. Biol. Med.*, **179**, 522 (1985).