# Dose and Load Studies for Subcutaneous and Oral Delivery of Poly(lactide-co-glycolide) Microspheres Containing Ovalbumin

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*Received September 1, 1993; accepted January 25, 1994*

Poly(lactide-co-glycolide) microspheres containing different loads of OVA (0.05, 0.1, 0.5 and 1.0% w/w) were manufactured by a w/o/w emulsion/solvent evaporation method. Low load efficiencies of less than 20% were observed. Normal size distributions with mean volume diameters ranging from 3.7 to 4.7 μm were obtained for different batches. The *in vitro* release of OVA from different loaded microspheres showed an expected burst release with all batches. The *in vivo* dose study (1, 10, 25, 50 μg of OVA) was performed by subcutaneous and oral inoculation in mice by single (0 week) or double (0 and 3 weeks) administration of PLGA 50/50 microspheres containing 0.1% OVA. Subcutaneous administration showed an immune response (serum Ig levels by ELISA) statistically (Fisher's paired t-test; $P < 0.05$) above OVA saline negative controls at 3, 6 and 12 weeks after administration. Oral administration of microspheres produced statistically higher systemic immune responses at the higher doses. Single and double inoculation orally and subcutaneously produced similar serum antibody levels. The *in vivo* load study was performed by subcutaneous and oral administration to mice of 25 μg OVA contained in various loaded (0.05, 0.1, 0.5 and 1.0% w/w) microspheres. Serum immune responses at 3, 6, and 12 weeks after inoculation were statistically above OVA saline controls and were inversely proportional to the OVA load using either route. This observation suggested a relationship between the number of microspheres delivered and the *in vivo* serum response. Single subcutaneous administration of 0.05 or 0.1% OVA loaded PLGA 50/50 microspheres induced larger immune responses compared with complete Freund's adjuvant.

**KEY WORDS:** microspheres; antigen; poly(lactide-co-glycolide); immune response, vaccine; adjuvant.

## INTRODUCTION

Vaccines are often given as repeated doses or cause possible harmful residues. The currently available oil and mineral adjuvants like complete Freund's adjuvant (CFA) can cause adverse reactions such as local granulomas, pain, fever, and possibly malignancies (1,2). Therefore, a major goal in vaccine development is to design formulations that produce immune responses but lack deleterious side effects.

Recently, the usefulness of sustained-antigen releasing microspheres was demonstrated as potential subcutaneous or oral vaccines (3–6). However, few studies have examined the antigen dose-immune response relationships or *in vitro* release of antigens from microspheres.

In this study we selected poly(lactide-co-glycolide) (PLGA) as a biodegradable polymer because PLGA has excellent biocompatible properties. Poly(lactide-co-glycolide) is presently used in biomaterial applications (7,8) and is administered subcutaneously to deliver the peptide LHRH (9,10). We prepared PLGA microspheres containing different loading (0.05, 0.1, 0.5, 1.0% w/w) of a model antigen ovalbumin (OVA). The microsphere size distributions, morphology and *in vitro* release of antigen were characterized. These microspheres were administered to mice subcutaneously or orally, immune responses in the serum were measured, and the effects of OVA dose and percent load on the immune response in mice serum were evaluated. Important factors of the microspheres attributed to the immune response were examined.

## EXPERIMENTS

### Materials

Materials used included ovalbumin (OVA; grade V), Trizma Base and Trizma Hydrochloride all from Sigma Chemical Co., St. Louis, MO; Poly(lactide-co-glycolide) 50/50 (vis = 0.53 dl/g, MW = 53,000) (PLGA) were from Medisorb Technologies, Cincinnati, OH; Bicinchoninic acid (BCA) reagent from Pierce Co., Rockford, IL; polyvinyl alcohol (PVA) from Polysciences, Inc.; 0.9% Sodium Chloride Injection USP from Kendall-MacGaw Laboratories, Inc., Irvine, CA; and analytical grade methylene chloride from Baxter, MacGaw Park, IL.; goat serum was purchased from Gibco. Co., Grand Island, NY; affinity purified antibody peroxidase labeled goat antimouse IgA, IgG, IgM was purchased from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD; complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (ICFA) were purchased from Gibco, Grand Island, NY; Alum as alhydrogel was distributed by Accurate Chemicals, Westbury, NY; All other reagents were special reagent grade.

### Preparation of Microspheres

Microspheres containing various loads of OVA (0.05, 0.1, 0.5 and 1.0% w/w) dispersed in PLGA were produced using a water-in-oil-in-water (W/O/W) emulsion/solvent evaporation method. Single batches were produced of the 0.5 and 1.0% loaded microspheres while multiple batches were needed to produce enough microspheres having 0.05 and 0.1% loadings. Various amounts of OVA (0, 10, 20, 100 and 800 mg) were dissolved in 600 μl of water. The solution was emulsified with 30 ml of methylene chloride containing 2.0 g PLGA 50/50 for 1 minute using a sonicator homogenizer (VirSonic, (½)" probe, Virtis Co., Gardiner, NY). The approximately 30.6 ml of w/o emulsion was transferred to a two L breaker containing 400 ml of a 0.5% PVA solution. Emulsification occurred using a homogenizer (VirTishear, Virtis Co., Gardiner, NY) at 2400 rpm for 3 minutes followed

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by gentle stirring for 6 hours. The microspheres were collected by centrifugation (B-20, International Equipment Co., Needham, MA) at 7000 rpm for 10 minutes. The obtained microspheres were rinsed with water and centrifuged three more times, then dried for at least 24 hours in a vacuum oven at room temperature. Microspheres were stored desiccated at −20°C until formulated.

### *In Vitro* Characterization of Microspheres

*Loading Test*— The actual load was determined by placing 20 mg of microspheres in 400 μl of methylene chloride and extracting the OVA twice with 800 μl of pH 7.4 Tris buffer. The extraction test solution was assayed using a Bicinchoninic Acid (BCA) Total Protein Assay (11). Triplicate samples were conducted. The actual loading was calculated from the weight of the initial microspheres and the amount of drug incorporated.

*Microsphere Sizes and Distributions*— A Microtrac Particle Size Analyzer (Model 7995-30, Leeds and Northrup, North Wales, PA) was utilized to determine the microspheres' average volume diameters and distributions.

*Morphology*— Scanning electron microscopy (SEM) was utilized to examine the surface and morphology of the microspheres, as described previously (12).

*In Vitro OVA Release Test*— The *in vitro* release of OVA from various microspheres was determined. Microspheres corresponding to 100 μg of OVA were suspended in 2 ml of Tris buffer (pH 7.4 and 37°C) in a 7 ml glass vial and shaken orbitally at 70 rpm. At predetermined time intervals, 150 μl of the suspension was removed, 100 μl of supernatant was obtained by centrifugation (12,000 rpm, 4 min) and the concentration of the supernatant was determined using a BCA Total Protein Assay. Experiments were performed in triplicate.

*Calculation of Particle Number of Microspheres Administered Per Mouse*— A value of 1.55 g/ml for the specific gravity of PLGA was used as the specific gravity for the PLGA microspheres since the antigen loading was low (<1.0%). The microspheres were assumed to be perfect spheres and the mean average volume diameters were used as the diameters.

### *In Vivo* Study

*Animals*— Twelve Balb C mice 6–8 weeks of age (obtained from HSD, Indianapolis, IN) were used in each inoculation group. Mice were given laboratory chow and water *ad libitum*. Mice were inoculated subcutaneously or orally using 100 μl. Animals were bled from the retro-orbital fossa and separated serum was stored at −20°C until assayed.

*Immunization protocols*— The required doses of microspheres were weighed and resuspended in the appropriate volume of 0.9% NaCl Injection USP. Groups of animals were inoculated subcutaneously or intragastrically with PLGA microspheres containing various OVA loading at 0 weeks, or 0 and 3 weeks using a 24-gauge needle (gavage needle for oral). Besides those mice receiving the microspheres, other groups were inoculated with OVA formulated in saline, alum, complete or incomplete Freund's adjuvant (CFA, ICFA). Alum as Alhydrogel was used at a dose of 1 mg/inoculation and an oil adjuvant at a 1:1 emulsified mixture with OVA. It was not emulsified with the antigen. In addition, a group remained as an uninoculated control. All animals were bled at 0, 3, 6, and 12 weeks. Serum was assayed using an indirect ELISA with OVA as the solid phase.

*Measurement of Serum Antibody Level by ELISA*— The ELISA was performed using Corning plates which were coated overnight with 100 μl per well of OVA 15 μl/ml dissolved in 0.034 M sodium bicarbonate, 0.106 M sodium carbonate buffer (pH 9.6). They were washed once in 100 μl of purified water and blocked for 2 hrs at 37°C with TBST (0.02M Tris buffered, 0.5M saline with 0.3% Tween 20 at pH 7.5) containing 5% goat serum in 100 μl per well. After washing three times in purified water, serum test samples (100 μl) at dilutions of 1/50 in TBST plus 5% goat serum were added to the wells and incubated overnight at 4°C. The plates were washed five times in purified water and 100 μl of HRPO (horseradish peroxidase) labelled-goat antimouse IgG, IgA and IgM conjugates diluted (1:3000) were added to the wells and incubated at 37°C for 2 hrs. The plates were then washed five times and 100 μl of 2,2-azino-di(3-ethyl-benzthiazoline sulfonate at a concentration of 0.3 g/l in a glycine/citric acid buffer containing 0.1% $H_2O_2$ were added to each well. The reaction was stopped after 20 min by the addition of 50 μl of diluted HF acid (1:400). ELISA plates were read in a plate reader and results were expressed as an optical density at 415 nm.

*Statistical analysis*— The results from the *in vivo* mouse study were expressed as the mean ± standard error for 12 mice. Statistical analysis was performed using one-way analysis of variance ($p < 0.05$ or $p < 0.01$), followed by Fisher's paired t-test for differences.

## RESULTS AND DISCUSSIONS

### *In Vitro* Study of PLGA Microspheres Containing OVA

*Preparation of Microspheres*— Microspheres containing various percents of OVA (0.05, 0.1, 0.5 and 1.0% (w/w)) dispersed in PLGA 50/50 were prepared. The corresponding preparation conditions, actual OVA loading %, yield and mean volume average diameters for various batches (F1–F4) are summarized in Table I. Actual drug loadings ranged be-

Table I. Components and Measured Parameters of Poly(ester) Microspheres

| | Formulation No. | | | |
|---|---|---|---|---|
| | F1 | F2 | F3 | F4 |
| PLGA (g) | 2.0 | 2.0 | 2.0 | 2.0 |
| OVA (mg) | 10.0 | 20.0 | 100.0 | 800.0 |
| Drug Content (%) | | | | |
| Theoretical | 0.50 | 0.99 | 4.76 | 28.6 |
| Found | 0.05 (0.003)* | 0.13 (0.004) | 0.56 (0.004) | 1.09 (0.021) |
| Load Efficiency (%) | 10.6 | 13.0 | 11.8 | 3.8 |
| Yield (%) | 78 | 75 | 73 | 67 |
| Average Mean Volume Diameter (μ) | 4.39 | 3.69 | 4.34 | 4.65 |

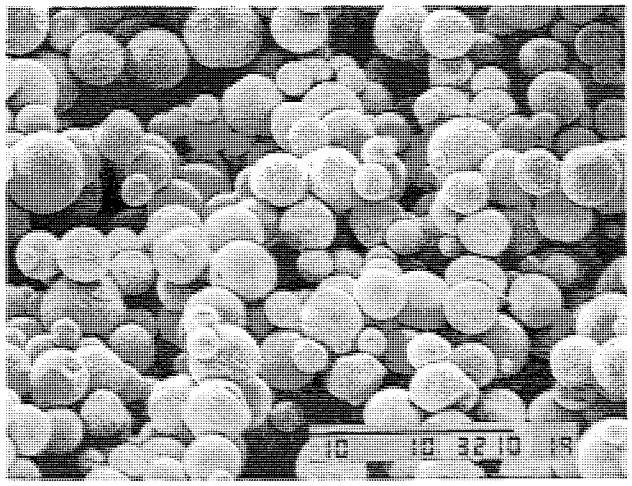
* The figures in parentheses represent ±S.E.M. of 4–5 studies.

tween 0.05 and 1.09% with load efficiencies ranging between 3.8 and 15.8%. Yields averaged 73%. Actual drug loadings obtained were much lower than theoretical due to loss of the water-soluble OVA in the continuous aqueous phase. This effect was caused by the exposure of the small w/o emulsion droplets to the aqueous continuous phase during formation of the w/o/w emulsion. The lowest drug load efficiency (3.8%) and yield (67%) were obtained with the PLGA 50/50 microspheres containing 1% OVA (F4). Ogawa *et al.* (13) has developed a w/o/w solvent evaporation method to entrap a water-soluble peptide into PLGA microspheres of 20–125 μm. They demonstrated the addition of gelatin to the internal phase produced good entrapment efficiency. They stated entrapment efficiencies of proteins in microspheres using w/o/w emulsion methods without using gelatin were between 1.9–6.7%. In this study, additives like gelatin or fatty acid (14) were not used in order to prevent interference with the immune responses in mice.

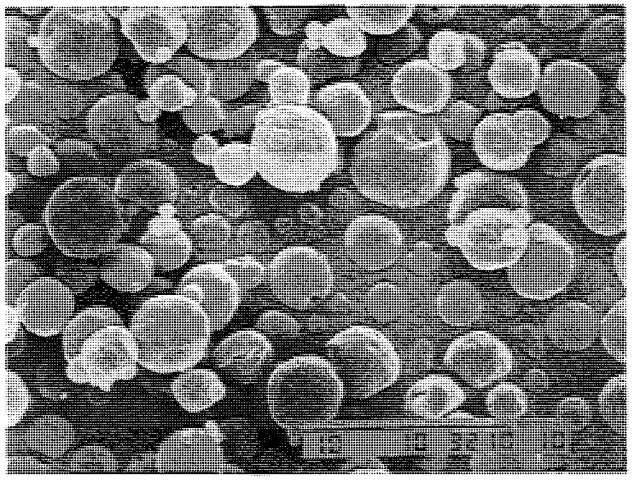
Poly(lactide-co-glycolide) microspheres with different loadings (F1–F4) had similar size distributions as shown in Figure 1. The average mean volume diameters were also similar ranging from 3.69 to 4.65 μm for the four different OVA loaded formulations.

*Morphology*— Scanning electron photomicrographs of the microspheres containing 0.1% OVA (F2) are shown in Figure 2a. Microspheres were spherical and fairly monodispersed. A few holes were observed on the surfaces of the microspheres as shown in the 1.0% OVA microspheres (F4) in Figure 2b. The pores were most likely caused by organic solvent diffusing from the formed microspheres during curing.

*In Vitro OVA Release Test*— The release of OVA from different OVA loaded microspheres showed a burst release in all batches as shown in Figure 3. Over 60% of the total OVA was released from the 1% loaded microspheres within a day. About 20% of OVA remained unreleased at 3 weeks (after dissolution test, remaining OVA in PLGA matrix was extracted using same method as described in loading test and



a.



b.

Fig. 2. Scanning electron micrographs of PLGA 50/50 microspheres containing (a) 0.1% and (b) 1.0% OVA produced using a Virtishear homogenizer.

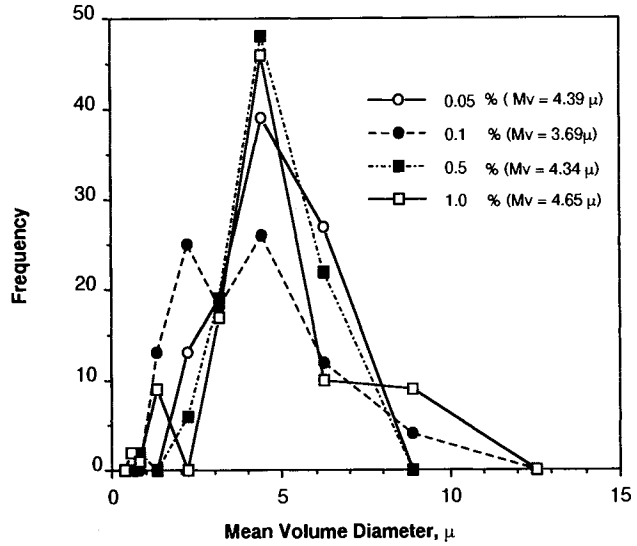



Fig. 1. Size-frequency distributions for various batches of PLGA 50/50 microspheres.

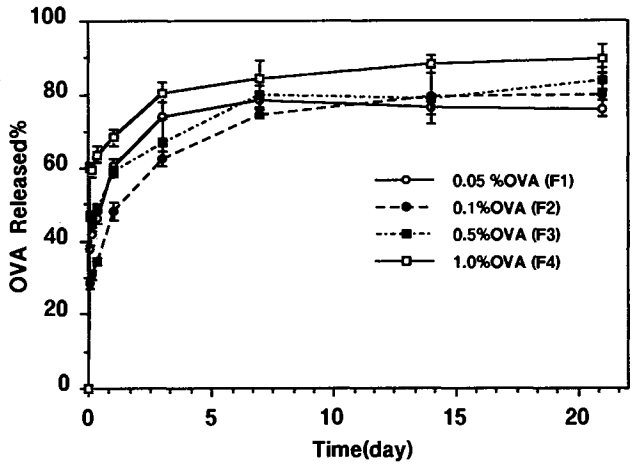



Fig. 3. Release of OVA from PLGA 50/50 microspheres with different OVA loading.

Table II. Serum antibody responses to ovalbumin (OVA) after subcutaneous and oral inoculation of PLGA microspheres or saline OVA solution in various doses

| Route Formulation | OVA dose (μg) | Immune responses (OD415) after single inoculation | | | Immune responses (OD415) after double inoculations | | |
|---|---|---|---|---|---|---|---|
| | | 3 week | 6 week | 12 week | 3 week | 6 week | 12 week |
| Subcutaneous | | | | | | | |
| Saline | 1 | 0.10 (0.02) | 0.15 (0.02) | 0.17 (0.02) | 0.20 (0.02) | 0.85 (0.05) | 0.77 (0.11) |
| | 10 | 0.37 (0.06) | 0.45 (0.04) | 0.35 (0.03) | 0.28 (0.03) | 1.20 (0.03) | 1.23 (0.05) |
| | 25 | 0.10 (0.02) | 0.26 (0.03) | 0.26 (0.03) | 0.37 (0.03) | 1.11 (0.03) | 1.07 (0.10) |
| | 50 | 0.40 (0.06) | 0.56 (0.06) | 0.52 (0.07) | 0.41 (0.05) | 0.92 (0.03) | 1.19 (0.05) |
| Microsphere | 1 | 0.28 (0.06)** | 0.48 (0.09)** | 0.88 (0.11)** | 0.20 (0.03) | 0.84 (0.08) | 1.09 (0.06)* |
| | 10 | 1.30 (0.02)** | 1.42 (0.04)** | 1.68 (0.03)** | 1.14 (0.04)** | 1.61 (0.03)** | 1 77 (0.03)** |
| | 25 | 1.45 (0.05)** | 1.57 (0.05)** | 1.81 (0.03)** | 1.34 (0.04)** | 1.72 (0.04)** | 1.82 (0.02)** |
| | 50 | 1.50 (0.04)** | 1.77 (0.02)** | 1.91 (0.03)** | 1.45 (0.07)** | 1.69 (0.14)** | 1.85 (0.02)** |
| Oral | | | | | | | |
| Saline | 1 | 0.10 (0.01) | 0.12 (0.01) | 0.10 (0.01) | 0.15 (0.01) | 0.16 (0.01) | 0.18 (0.02) |
| | 10 | 0.12 (0.02) | 0.14 (0.02) | 0.16 (0.01) | 0.21 (0.04) | 0.60 (0.13) | 0.48 (0.11) |
| | 25 | 0.10 (0.06) | 0.12 (0.02) | 0.13 (0.02) | 0.31 (0.05) | 0.62 (0.08) | 0.63 (0.09) |
| | 50 | 0.17 (0.01) | 0.20 (0.02) | 0.24 (0.02) | 0.19 (0.04) | 0.28 (0.06) | 0.38 (0.08) |
| Microsphere | 1 | 0.21 (0.03)** | 0.37 (0.07)* | 0.51 (0.07)** | 0.32 (0.08)* | 0.64 (0.09)** | 0.89 (0.15)** |
| | 10 | 0.26 (0.09) | 0.35 (0.13) | 0.35 (0.15) | 0.28 (0.07) | 0.61 (0.14) | 0.85 (0.18)* |
| | 25 | 1.13 (0.13)** | 1.27 (0.09)** | 1.39 (0.12)** | 1.27 (0.14)** | 1.51 (0.05)** | 1.53 (0.13)** |
| | 50 | 1.32 (0.15)** | 1.38 (0.16)** | 1.55 (0.15)** | 1.34 (0.07)** | 1.48 (0.17)** | 1.62 (0.10)** |

The immune responses were expressed as an optical density at 415 nm (OD415), and the figures in parentheses represent S.E.M. of 12 mice. The serum immune response level of non-inoculated mice group was 0.07 (0.01) as OD415. * and ** means the statistical differences ($p < 0.05$, $p < 0.01$, respectively) compared with the control OVA saline of the same dose at the same time.

good mass balance was ascertained (data not shown). Previous research has demonstrated long sustained-release of proteins or peptides from PLGA microspheres *in vitro* (15–17). But, the sizes of PLGA microspheres used in those studies were larger than in this study. Tabata and Ikada (18) examined release of an antitumor agent from PLGA microspheres several microns in diameter and reported about 60% of drug was released within a day. Our initial release patterns

Table III. Serum antibody responses to ovalbumin (OVA) after subcutaneous and oral inoculation of PLGA microspheres with various OVA loading, saline OVA solution and other adjuvants

| Route Formulation | Immune responses (OD415) after single inoculation | | | Immune responses (OD415) after double inoculations | | |
|---|---|---|---|---|---|---|
| | 3 week | 6 week | 12 week | 3 week | 6 week | 12 week |
| Subcutaneous | | | | | | |
| Saline | 0.19 (0.02) | 0.26 (0.03) | 0.26 (0.03) | 0.37 (0.03) | 1.11 (0.02) | 1.07 (0.10) |
| Alhydrogel | 0.48 (0.06) | 0.64 (0.03) | 0.74 (0.03) | 0.55 (0.05) | 0.72 (0.13) | 0.96 (0.12) |
| CFA | 0.92 (0.05) | 1.19 (0.03) | 1.32 (0.03) | 1.01 (0.05) | 1.18 (0.09) | 1.57 (0.03) |
| ICFA | 0.58 (0.05) | 0.80 (0.08) | 0.90 (0.04) | 0.65 (0.10) | 0.90 (0.07) | 1.17 (0.12) |
| Microsphere | | | | | | |
| 0.05% | 1.32 (0.21)[a)] | 1.67 (0.07)[b)] | 1.87 (0.03)[b)] | 1.35 (0.07)[b)] | 1.89 (0.03)[b)] | 1.87 (0.08)[b)] |
| 0.1% | 1.40 (0.02)[b)] | 1.47 (0.02)[b)] | 1.85 (0.03)[b)] | 1.40 (0.07)[b)] | 1.69 (0.12)[b)] | 1.85 (0.02)[b)] |
| 0.5% | 1.09 (0.05)[a)] | 1.28 (0.03)[a)] | 1.60 (0.07)[b)] | 0.96 (0.04) | 1.53 (0.05)[b)] | 1.53 (0.03) |
| 1.0% | 0.90 (0.04) | 1.08 (0.03) | 1.22 (0.05) | 0.94 (0.04) | 1.19 (0.12) | 1.44 (0.05) |
| Oral | | | | | | |
| Saline | 0.10 (0.01) | 0.12 (0.02) | 0.13 (0.02) | 0.31 (0.05) | 0.62 (0.08) | 0.63 (0.09) |
| Microsphere | | | | | | |
| 0.05% | 1.35 (0.05)** | 1.40 (0.06)** | 1.50 (0.10)** | 1.14 (0.15)* | 1.47 (0.16)** | 1.60 (0.13)** |
| 0.1% | 1.13 (0.13)** | 1.27 (0.09)** | 1.39 (0.12)** | 1.34 (0.07)** | 1.51 (0.05)** | 1.53 (0.13)** |
| 0.5% | 0.35 (0.09)* | 0.41 (0.11)** | 0.49 (0.14)* | 0.32 (0.09) | 0.70 (0.09) | 0.83 (0.15) |
| 1.0% | 0.62 (0.12)** | 0.67 (0.13)** | 0.82 (0.15)** | 0.60 (0.12)* | 0.86 (0.14) | 0.82 (0.12) |

The immune responses were expressed as an optical density at 415 nm (OD415), and the figures in parentheses represent S.E.M. of 12 mice. The serum immune response level of non-inoculated mice group was 0.07 (0.01) as OD415. 0.05% in above Table means PLGA microspheres loaded 0.05% (w/w) OVA.

* or ** means the statistical differences ($p < 0.05$, $p < 0.01$, respectively) compared with the control OVA saline of the same dose at the same time. a) or b) represents the statistical differences ($p < 0.05$, $p < 0.01$, respectively) compared with CFA of the same dose at the same time. All studies were performed at dose of 25μg OVA.

were essentially the same as their results. Ogawa *et al.* (13) has reported about a 30% burst release and no additional release of a peptide from PLA microspheres 20 μm in diameter. Considering our small particle sizes of the PLGA microspheres, large initial burst releases were expected.

### *In Vivo* Immune Response in Mice

*Dose Study*— The administration of different antigen doses by varying the amount of microspheres delivered has not been previously reported. Therefore, we changed doses by administration of 1, 10, 25, and 50 μg OVA dispersed in 0.129% OVA loaded PLGA 50/50 microspheres (F2) which were suspended in normal saline. These microspheres had a small mean volume diameter (3.69 μm) which, according to previous reports, was critical to produce optimal immune responses (6,19).

The antibody levels of mice inoculated with microspheres subcutaneously at 0 or 0 and 3 weeks compared to mice given saline/OVA are shown in the top column of Table II. Microspheres preparations gave immune responses which were statistically higher than negative controls (shown in Table II as * = $p < 0.05$ or ** = $p < 0.01$ by Fisher's paired t-test). For example, administration of PLGA 50/50 microspheres at a dose of 10 μg OVA produced an immune response two times larger than the 50 μg dose of OVA solution which had five times more antigen. The antibody response increased as the OVA dose increased. No advantage in administering additional microspheres at 3 weeks was observed; obviating the need for a second injection highlights one advantage of sustained-antigen release using microspheres, as some commercial vaccines are administered two or three times.

Antibody titers in mice given intragastrically microspheres at 0 or 0 and 3 weeks are shown in the bottom column of Table II. PLGA 50/50 microspheres produced good immune responses at the high doses of 25 or 50 μg OVA. These responses were smaller than those observed subcutaneously at the same doses and had greater variation in the mean resulting from more nonresponders. The use of a booster at 3 weeks was not necessary at the high dose of 25 and 50 μg OVA. However, the second administration at 3 weeks was useful at the low doses of 1 or 10 μg. This observation was in contrast to subcutaneous administration which showed no need for boosting at any dose. This discrepancy seemed to be caused by differences in immunization mechanism between subcutaneous and oral inoculation using microparticle systems.

The higher immune response obtained when using the PLGA microspheres subcutaneously or orally could be attributed to the polymer itself acting as an adjuvant instead of a slow release of the antigen. However, we confirmed that inoculation with physical mixture of OVA solution and blank PLGA microspheres did not increase immune response compared with inoculation using OVA solution in advance (data not shown). Eldridge et. al. demonstrated the same result (19).

*OVA Load Study*— The development of a sustained antigen-releasing microsphere vaccine would require determining the antigen load giving optimal performance. Differences of OVA loading in PLGA polymer matrix may result in different *in vivo* antigen rates of release. Previous research has not examined the effect of loading on the serum immune response when using microspheres with diameters less than 10 μm. Therefore, PLGA 50/50 microspheres with different OVA loads (0.05, 0.1, 0.5 and 1.0%; at a 25 μg OVA dose) were administered to mice subcutaneously and orally, and serum immune responses at 3, 6, and 12 weeks after inoculation were examined. Subcutaneous results are shown in the top column of Table III compared with the results from

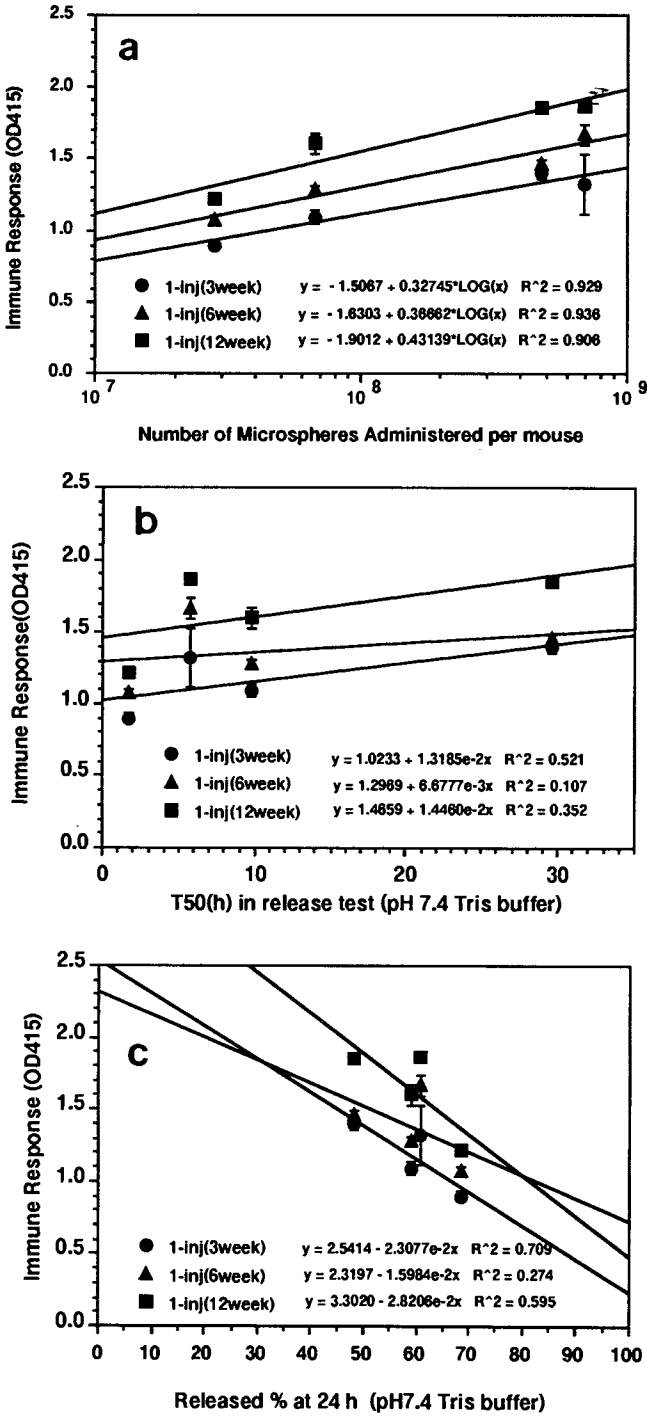



Fig. 4. Relationship between (a) *in vivo* immune responses (OD415) and particle number administered per mouse, (b) T50 of release test in Tris buffer and (c) release % at 24 h in Tris buffer. Subcutaneous loading data after single inoculation were used for the calculation.

administering complete Freund's adjuvant, incomplete Freund's adjuvant, alum as Alhydrogel. The resulting immune response with various OVA loaded microspheres was different although the total amount of OVA administered was kept constant. The immune response was inversely proportionally to the OVA load. The PLGA microspheres containing 0.05 or 0.1% OVA loads and administered subcutaneously induced good immune responses which were statistically larger than CFA ($p < 0.01$). With oral inoculation, 0.05 or 0.1% OVA loaded PLGA 50/50 microspheres gave good immune responses. Even with oral inoculation, no advantage of double inoculation was observed. This load study also suggested the possibility of decreasing the antigen dose if PLGA microspheres with load antigen loading will be administered.

*In Vitro-In Vivo Correlation*— To help explain the differences in immune responses observed in the loading study, two correlations were examined using the non-boosting subcutaneous data. First, the relationship between the microsphere number and immune responses was assessed. A correlation between the number of microspheres administered per mouse and the immune response was observed as shown in Figure 4a. Good correlation coefficients of 0.929, 0.936 and 0.906 were obtained for the data at 3, 6, and 12 weeks, respectively. The second relationship examined was between the *in vitro* and the *in vivo* immune response. Presentation of a foreign antigen in the body caused either development of antibodies or sensitized lymphocytes. An attempt to correlate this initial presentation of antigen to the immune responses measured at 21, 42, and 84 days was not successful. These correlations are shown in Figures 4b and 4c. The time to release 50% of the total OVA or the amount of OVA released at 24 hours did not predict what would happen *in vivo* weeks later.

*Effect of Particle Number*— The effect of the number of microsphere administered per mouse on the immune response was examined using all the *in vivo* data. The subcutaneous and oral results are shown in Figure 5a and b, respectively. In the case of subcutaneous inoculation with microspheres, immune responses increased from a minimum at $1.9 \times 10^7$ microspheres to a maximum when administering $9.5 \times 10^8$ microspheres.

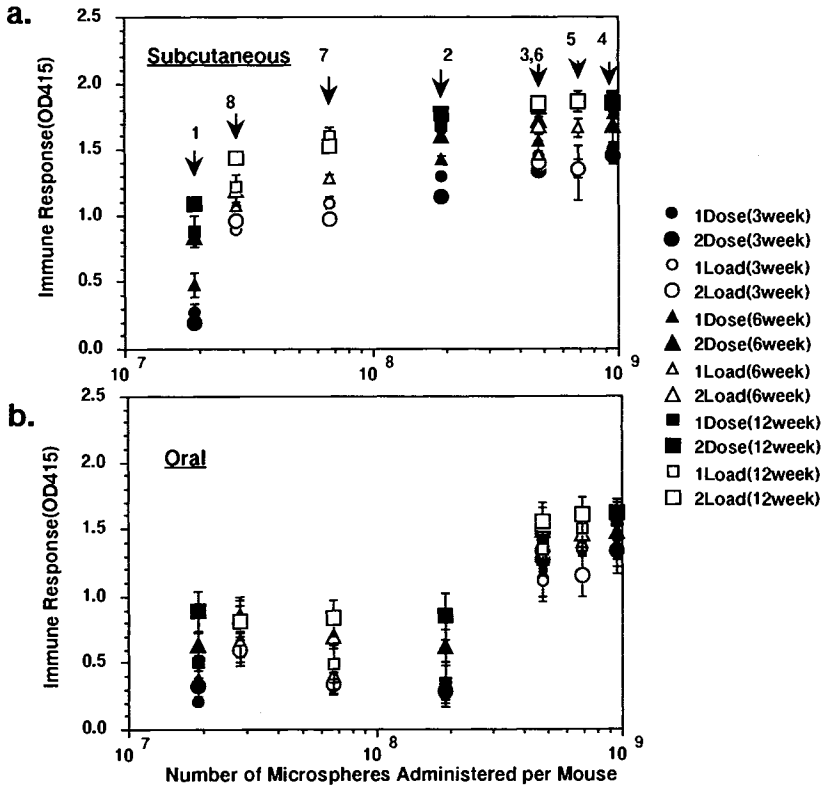



Fig. 5. Relationship between particle number administered per mouse and *in vivo* serum immune responses (OD415) after (a) subcutaneous and (b) oral inoculation. Each point represented mean ± S.E.M. of 12 mice. 1, 10, 25 and 50 mg of OVA from dose study was shown as number 1 to 4 in the figure and corresponding particle number was calculated as $1.9 \times 10^7$, $1.9 \times 10^8$, $4.75 \times 10^8$, and $9.5 \times 10^8$, respectively. Loading study containing 0.05, 0.1, 0.5 and 1.0% OVA was shown as number 5 to 8 in the above figure and corresponding particle number was calculated to be $6.88 \times 10^8$, $4.75 \times 10^8$, $6.70 \times 10^7$, and $2.81 \times 10^7$, respectively. Circle, triangle and square symbol represents the data of 3, 6, and 12 weeks after inoculation, respectively. 1Dose in the figure means the dose study data after 1 inoculation (0 week). 2Dose in the figure means the dose study data after 2 inoculations (0 and 3 weeks). 1Load in the figure means the loading study data after 1 inoculation (0 week). 2Load in the figure means the loading study data after 2 inoculations (0 and 3 weeks).

One advantage of subcutaneous administration compared to oral delivery of the same antigen loaded microspheres was the greater efficiency in producing an immune response upon subcutaneous administration. As the number of microspheres administered subcutaneously increased the immune response increased up to administering $4.8 \times 10^8$ microspheres. Above this number the immune response did not increase much further, possibly because of saturation of uptake by the macrophages. On the other hand, with oral inoculation, the immune responses were similar when administering $10^7$ to $10^8$ microspheres. However, the immune response increased suddenly when $4.8 \times 10^8$ microspheres were administered. Considering the poor efficiency in uptake of orally administered microspheres (20,21), oral inoculation with microspheres might be highly potent in inducing an immune response. This may explain the boosting effect of oral inoculation with microspheres at a low dose of OVA in the dose study.

The number of OVA loaded PLGA microspheres administered was suggested as a critical factor in causing an immune response in mice. If two different batches of microspheres differ two- or three-fold in diameter, 10- to 30-fold more microspheres will be administered if the dose remained constant. Particles with small diameters were reported to be advantageous for uptake by macrophages (22) or uptake by Peyer's Patch tissue and migration to mesenteric lymph nodes (23). Small microspheres less than one μm have the possibility of a large burst release of antigen. Therefore, characterization of size distributions and *in vitro* release seems to be essential to design the appropriate small particle vaccine formulations. The antigen dose, release, and size must be optimized to produce a quality vaccine formulation.

The possibility of single immunization with PLGA 50/50 microspheres using both routes encourages further studies using this biodegradable microsphere system for application to vaccination in humans or animals.

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