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Research Article

Determinants of Release Rate of Tetanus Vaccine from Polyester Microspheres

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Controlled-release formulations based on poly(lactic) (PLA) and poly(lactic/glycolic) acid (PLGA) microspheres containing tetanus vaccine were designed. The polymers forming the microspheres were L-PLA of different molecular weights and DL-PLGA, 50:50. These microspheres were prepared by two solvent elimination procedures, both using a double emulsion, and were characterized for size, morphology, and toxoid release kinetics. The influence of formulation variables such as polymer type, vaccine composition, and vaccine/polymer ratio was also investigated. Both techniques yielded microspheres with similar size, morphology, and release properties. Microsphere size was dependent on the type of polymer and the presence of the surfactant L-α-phosphatidylcholine, which led to a reduction in microsphere size. On the other hand, the release kinetics of encapsulated protein were affected by the polymer properties (ratio lactic/glycolic acid and molecular weight) as well as by the vaccine composition, vaccine loading, and microsphere size. Moreover, for some formulations, a decrease in microsphere size occurred simultaneously, with an increase in porosity leading to an augmentation of release rate. The changes in the PLA molecular weight during *in vitro* release studies indicated that release profiles of tetanus toxoid from these microspheres were only marginally influenced by polymer degradation. A significant fraction of protein (between 15 and 35%) was initially released by diffusion through water-filled channels. In contrast, the decrease in the PLGA molecular weight over the first 10 days of incubation suggested that erosion of the polymer matrix substantially affects protein release from these microspheres. Among all formulations developed, two differing in microsphere size, polymer hydrophobicity, and release profile were selected for *in vivo* administration to mice. Administration of both formulations resulted in tetanus neutralizing antibody levels that were higher than those obtained after administration of the fluid toxoid.

KEY WORDS: vaccine delivery system; controlled release; poly(lactic/glycolic acid); biodegradable microspheres; tetanus.

INTRODUCTION

The controlled release of proteins from PLA and PLGA systems has become an important area of research (1–6). Specifically, protein-based vaccines represent a good example of bioactive materials which can potentially benefit from controlled-release technology since, in general, administration of several doses of the antigen, followed by a booster, are required to achieve an optimum immune response. This situation is clearly illustrated by tetanus toxoid. At present, it has been established that three consecutive injections of this antigen are essential to achieve suitable protection and, to prevent neonatal tetanus, women of childbearing age must receive at least two spaced doses of the vaccine. However, this schedule is rarely utilized by the people who require it. Indeed, in many countries the dropout rates from individuals receiving a first dose but not successive doses can reach 70% and the number of neonates who still die of tetanus every year is close to 700,000 (7). Consequently, for tetanus vaccine, the design of an injectable formulation which releases the antigen in a controlled manner for a time that is sufficient to induce long-lasting immunity may be a promising improvement in immunization coverage and individuals compliance.

We have previously demonstrated that continuous release of model antigens from polymers provided a prolonged immunological response while avoiding the need of multiple injections (8,9). These studies helped provide evidence for the value of controlled release technology for antigen delivery. Other investigators have recently shown that the encap-

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sulation of antigens (10–12) into polymers induces a higher immunological response to these specific vaccines than that achieved with the nonencapsulated vaccine. However, there are still very few data demonstrating the ability of polymers to encapsulate and release antigens efficiently. Moreover, the potential relation between the *in vitro* release behavior and the *in vivo* immunologic response has not yet been investigated.

In this paper, we report the design and characterization of degradable microspheres based on PLA and PLGA for the controlled release of tetanus toxoid vaccine. The experimental variables affecting the physical properties of these formulations and the release mechanism of this vaccine were investigated. The immunogenicity of the encapsulated protein was also examined.

MATERIALS AND METHODS

Materials

The polymer poly(L-lactic acid) (PLA) and the emulsifier poly(vinyl alcohol) (PVA) (MW 25,000, 88% mol hydrolyzed) were obtained from Polysciences, Inc., Warrington, PA. The copolymer poly(DL-lactic/glycolic acid), with a comonomer ratio of 50/50 and an inherent viscosity $\eta = 0.8$ (Resomer RG 506), was from Boehringer Ingelheim, Ingelheim, Germany. The surfactant L-α-phosphatidylcholine was obtained from Avanti Polar-Lipids, Inc, Alabaster, AL. Two lots of vaccine powder obtained from the same original batch of tetanus toxoid (Institute Pasteur Merieux, Serums and Vaccines, Paris, France) were provided by the World Health Organization (WHO). Both lots differ in the amount of salts (sodium phosphate and sodium chloride) incorporated to the tetanus toxoid solution prior to freeze-drying. The purity of each lot, expressed in terms of Limes flocculation units (Lf; the International Unit for vaccines) per milligram of powder, was 18.5 (lot 1) and 92 (lot 2). Purified tetanus toxoid was used as a control. Pure toxoid was prepared by detoxification of the pure toxin as described (13). Other chemicals were reagent grade.

Animals

Female CD-1 mice weighing about 20 g from Charles River Breeding Laboratory, Wilmington, MA, were used and maintained on a normal diet throughout the study.

Microsphere Preparation

PLA and PLGA microspheres were prepared by using two procedures based on an in-water drying process in a complex emulsion system. These procedures differ by the way the solvent was eliminated: evaporation or extraction. The solvent evaporation technique using a double emulsion system was previously reported by our laboratory specifically for the encapsulation of proteins (3). In both procedures, a specific amount of vaccine powder was dissolved in 50 μL of water and then emulsified in 1 mL of methylene chloride containing variable amounts of polymer as indicated in Table I. For some formulations, 200 μL of the lipophilic surfactant L-α-phosphatidylcholine in chloroform (2 mg/mL) was added to the organic phase containing the polymer. The emulsification was carried out by sonication at output 4 (50 W) for 10 sec (ultrasonic probe, Sonic & Materials Inc.) or homogenization at 15,000 rpm for 10 sec (Omni 2000 homogenizer). In one of the formulations the protein was directly dispersed, as a powder, in the organic solution containing the polymer. The resulting emulsion or suspension was further emulsified in 1 mL of an aqueous solution of PVA (1%) by vortexing for 10 sec and then diluted in 100 mL of PVA aqueous solution (0.3%). Finally, the system was stirred magnetically for 3 hr to allow the evaporation of the solvent (solvent evaporation procedure) or for 5 min and then poured into 200 mL of an aqueous solution of isopropanol (2%) to extract the methylene chloride to the external aqueous phase (solvent extraction procedure). In the latter case the system was maintained under magnetic stirring for 30 min to assure the total extraction of the solvent to the external aqueous phase.

Table I. Physical Properties Determined by Scanning Electronic Microscopy of Several Formulations of Tetanus Toxoid (Batch 1)-Loaded Microspheres Prepared by the Solvent Extraction/Double-Emulsion Technique

	Formulation parameter		Physical characteristics	
Polymer	Conc. (mg/mL)[a]	Surfactant[b]	Mean size (μm)[c]	Appearance
PLA[d]	500	—	55 ± 12	Smooth
	500	Yes	30 ± 8	Porous
	200	Yes	18 ± 4	Highly porous
PLA[e]	500	—	20 ± 4	Smooth
	200	—	12 ± 3	Smooth
	200	Yes	9 ± 3	Smooth
PLGA[f]	200	—	60 ± 10	Smooth
	200	Yes	20 ± 6	Porous
	100	Yes	20 ± 7	Highly porous

[a] Polymer concentration in the organic phase.
[b] L-α-Phosphatidylcholine.
[c] Average ± standard deviation; three determinations.
[d] MW 50,000.
[e] MW 3000.
[f] 50/50, MW 100,000.

Microspheres were finally collected by centrifugation, washed three times with double-distilled water, and freeze-dried to obtain a free-flowing powder.

The total amount of the vaccine powder used to prepare the microspheres was different for the different vaccine batches, i.e., 5 mg for the vaccine of lot 1 and 1 mg for the vaccine of lot 2, in order to maintain the same dose in terms of activity units, i.e., 180 Lf. In all preparations the vaccine was dissolved in a constant volume of water (50 μL). The amount of polymer dissolved in 1 mL of methylene chloride was varied, as shown in Table I.

Tetanus Toxoid Characterization

The molecular weight profile of chromatographically purified tetanus toxoid and those corresponding to the two tetanus toxoid lots used to prepare the microspheres were determined by high-pressure size exclusion chromatography (Waters Millipore, Millford, MA). Samples of the protein powder dissolved in water were injected and eluted with

phosphate-buffered water, pH 7.4, through a gel column (TSKgel G3000PWx, TosoHaas, Philadelphia, PA).

Protein Release Studies

Samples of 30 mg of microspheres were placed in 5-mL tubes and incubated in 3 mL phosphate-buffered saline, pH 7.4, under agitation at 37°C. At desired times, the samples were collected and centrifuged for 20 min at 6000*g* (Sorvall RC centrifuge, Du Pont Instruments). Two milliliters of the release medium was assayed for tetanus toxoid release and replaced by 2 mL of a fresh buffer to maintain sink conditions. Tetanus toxoid concentration in the release medium was determined by a microBCA protein assay (Pierce, Rockford, IL). Release experiments were done independently in triplicate.

Polymer Degradation Studies

Molecular weight distributions of PLA and PLGA polymers before and after different incubation times were determined on a Perkin–Elmer GPC system with a refractive index detector. Samples of fresh microspheres and microspheres from the degradation experiments were freeze-dried, dissolved in chloroform, and filtered to eliminate undissolved protein. The samples were eluted with chloroform through a Phenogel column (linear 0–10,000 K, mixed bed) (Phenomenex) at a flow rate of 1 mL/min. The molecular weight was determined relative to polystyrene standards (Polysciences; molecular weight range of 1250–233,000).

Particle Size and Morphology

Size, morphology, and surface appearance of microspheres before and during degradation studies were examined by scanning electron microscopy (SEM) (Cambridge Instruments 250 MK or Amray AMR 1000A). Samples for SEM were freeze-dried, mounted on metal stubs with double-sided tape, and coated with gold to a thickness of 200–500 Å. Pictures were taken and the microsphere size distributions were determined according to a reference scale. Particle size distribution of PLA (MW 3000) microspheres was also analyzed by a Coulter counter (Coulter Electronics Inc., Hialeah, FL) and the mean area diameter distribution was determined.

Immunization Protocol

Groups of female mice CD-1 were inoculated subcutaneously (22-gauge needle) on the left side of the abdomen, with a single dose (5 Lf) of tetanus toxoid-containing microspheres or fluid vaccine (vaccine powder dissolved in saline). For each injection 7 mg of microspheres was suspended in 0.5 mL of phosphate-buffered saline, pH 7.4. Blood was collected, by cardiac puncture, periodically and the sera were separated by centrifugation. Aliquots of the sera of mice from each group (six to nine per group) were pooled and evaluated for tetanus antitoxin by the toxin neutralization test (14,15). The toxin neutralization test was conducted at several antisera dilution levels and the AU/mL of serum samples was determined against US standard tetanus antitoxin (Lot No. E134). Individual samples of sera were also assayed for IgG antibodies to tetanus toxin by the enzyme-linked immunosorbent assay (ELISA). Briefly, high-binding easy-wash 96-well microtiter plates (Corning Glass Works, Corning, NY) were coated with 100 μL of purified tetanus toxin diluted to 5 μg/mL in phosphate-buffered saline (PBS), pH 7.2, at room temperature (RT) overnight. The plates were washed three times with PBS containing 0.05% Tween 20 between each step. Hyperimmune anti-tetanus toxoid mouse serum containing 225 AU/mL was used as a reference for ELISA and included in each plate. The samples and the reference serum were serially diluted at twofold dilution steps in the plates using PBS with 0.1% Brij 35 and 0.5 bovine serum albumin as a diluent (PBB). The plates were held at RT for 2 hr and washed. Goat anti-mouse IgG alkaline phosphatase conjugate (Fisher Biotechnology, Springfield, NJ) diluted 1:1000 in PBB was added to plates. The plates were again incubated at RT for 2 hr and washed. Finally, *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) diluted to 1 mg/mL in 1*M* diethanoleamine, 0.5 m*M* magnesium chloride buffer was added to plates. The plates were held at RT for 30 min and read at 405-nm wavelength on an ELISA reader. The unitage of the serum samples was calculated against hyperimmune mouse serum by extrapolation from a standard curve.

RESULTS

Polymer Characterization

The molecular weight distributions of the polymers and copolymers used in this study were determined by gel permeation chromatography. The polymers used to prepare the microspheres were PLA with two number-average molecular weights: 3000 and 50,000 (polydispersities, 1.4 and 1.3, respectively) and PLGA, 50/50, with a number-average molecular weight of 100,000 (polydispersity, 1.2).

Tetanus Toxoid Characterization

The HPLC chromatograms of two lots of different purities of tetanus vaccine provided by the WHO as a lyophilized powder are shown in Fig. 1. The profile corresponding to the chromatographically purified liquid tetanus toxoid is shown as a reference. The presence of a peak corresponding to aggregated protein is noticeable for both lots of freeze-dried vaccine, although its intensity is variable for each lot.

Characteristics of Tetanus Toxoid-Loaded Microspheres

Table I shows the influence of some formulation variables (polymer type and concentration in methylene chloride and the presence of the lipophilic surfactant L-α-phosphatidylcholine) on the physical characteristics of the microspheres, such as microsphere size and morphology. Results indicate that polymer type and concentration play an important role in controlling microsphere size (this is also illustrated in Fig. 2, which shows microspheres prepared under exactly the same conditions except for polymer type). In particular, the influence of the polymer concentration (in the formulation process) was statistically significant (ANOVA, $P < 0.5$) for PLA microspheres where the polymer concentrations investigated were 500 and 200 mg/mL. In addition, for PLA, MW 50,000 and PLGA, MW 100,000, the incorpora-

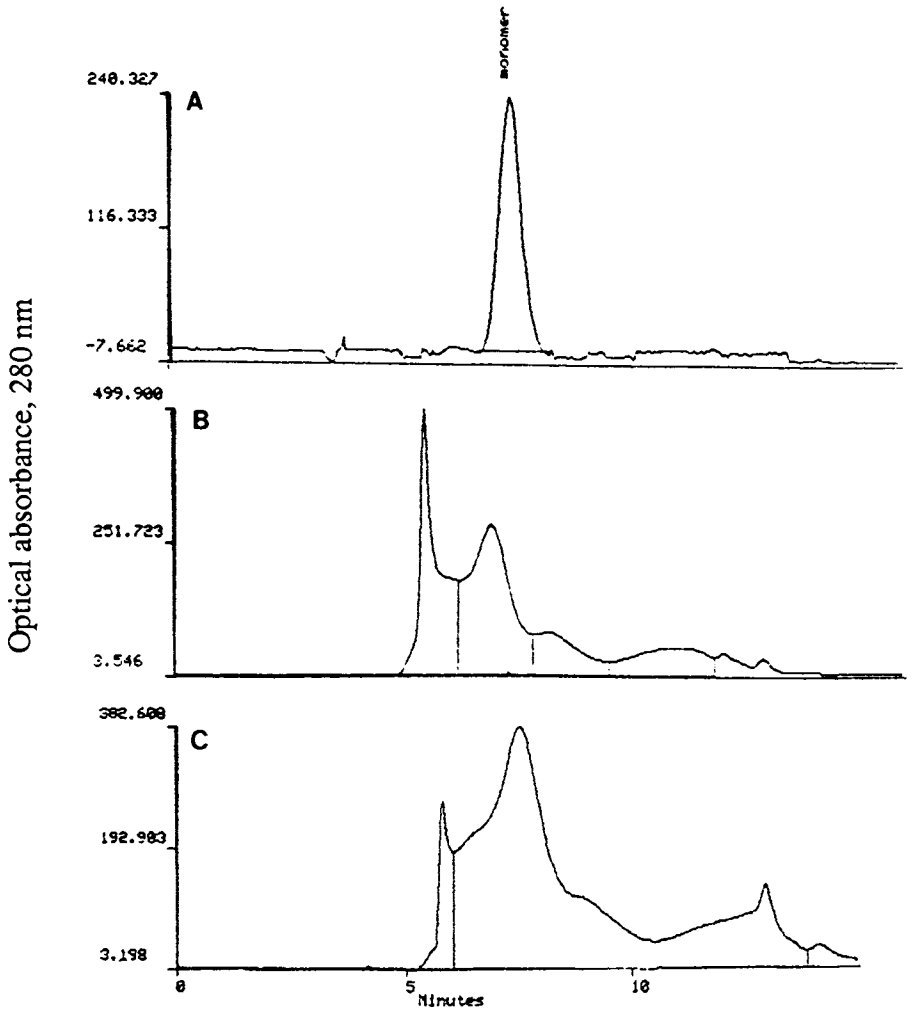

Fig. 1. HPLC chromatograms profiles of tetanus toxoid. (A) Chromatographically purified toxoid. (B) Tetanus vaccine Lot 1. (C) Tetanus vaccine Lot 2.

tion of L-α-phosphatidylcholine in the organic solvent leads to a significant reduction (ANOVA, $P < 0.5$) in microsphere size; for these formulations the reduction in size appears simultaneously with an increase in porosity. The influence of some technological variables such as the way to disperse the protein in the polymer organic solution (as a powder or as an aqueous solution) and the solvent elimination procedure on microspheres morphology was also investigated. When the vaccine powder was dispersed directly in the organic phase, the microsphere population was irregular, showing some broken particles (results not shown), compared to that obtained when the vaccine was dissolved in water prior to dispersion into the polymer solution. No influence of the solvent elimination procedure (extraction and evaporation) on microsphere size and morphology was observed (results not shown).

Degradation Characteristics of Tetanus Toxoid-Loaded Microspheres

Figure 3 displays micrographs showing PLGA microspheres containing tetanus toxoid (Lot 1) during various stages of *in vitro* release studies. This formulation was prepared by using 200 mg polymer/mL of methylene chloride. Immediately after preparation, microspheres show an overall intact outer surface, with very tiny pores noticeable only at close examination (A). After 10 days of incubation the size of the pores increased notably (B), and 1 month later most of the microspheres appeared highly eroded and porous (C).

The decrease in the number-average molecular weight of the polymer forming the microspheres during incubation is shown in Fig. 4. A reduction in the number-average molecular weight from 105,680 to 24,760 daltons and an augmentation in the polydispersity from 1.2 to 1.6 was observed for PLGA after 10 days of degradation. In contrast, a slow decrease in the molecular weight was observed over the 30-day degradation experiment independent of the initial molecular weight (3000 and 50,000) of the PLA-loaded microspheres. The final number-average molecular weights for both microsphere formulations after 1 month degradation were, respectively, 2275 and 40,000 daltons.

Tetanus Toxoid Release

The influence of previously mentioned formulation and technological variables on the *in vitro* release behavior of

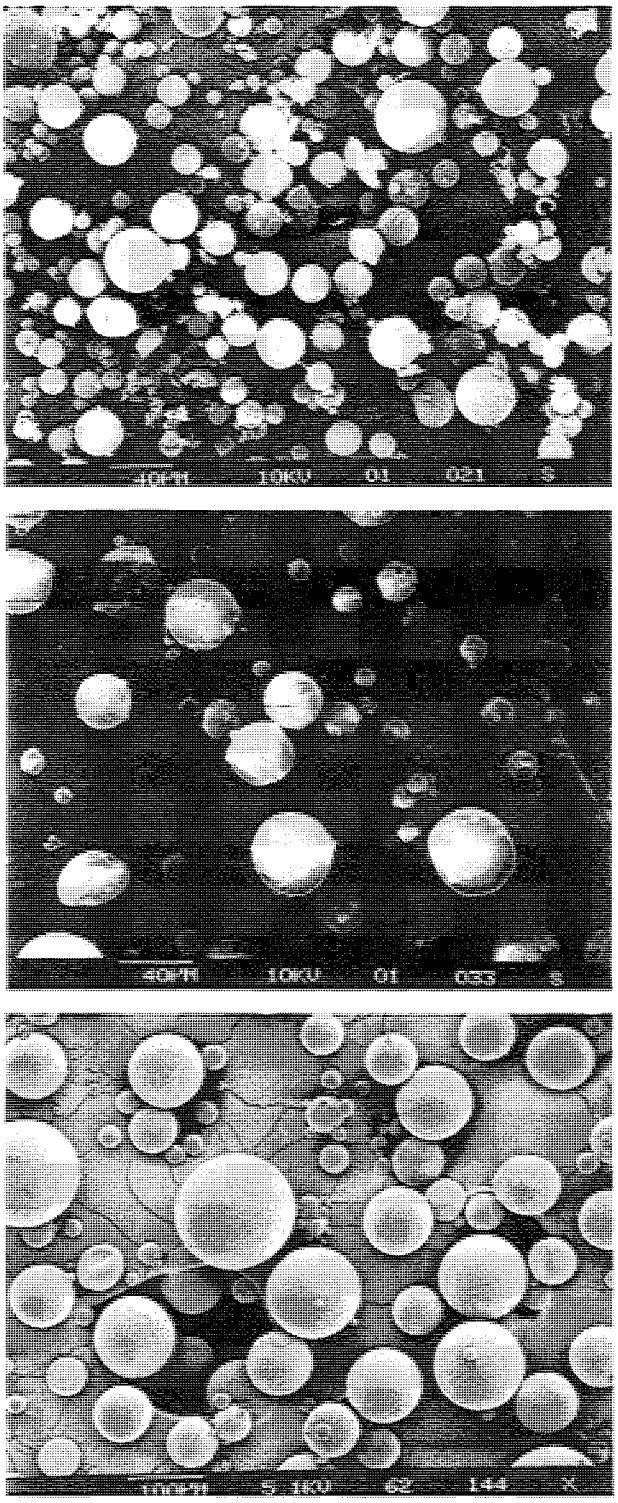

Fig. 2. SEM photographs of tetanus toxoid-loaded microspheres prepared under the same processing conditions but using different polymers. (A) PLA (MW 3000); (B) PLA (MW 50,000); (C) PLGA, 50:50 (MW 100,000).

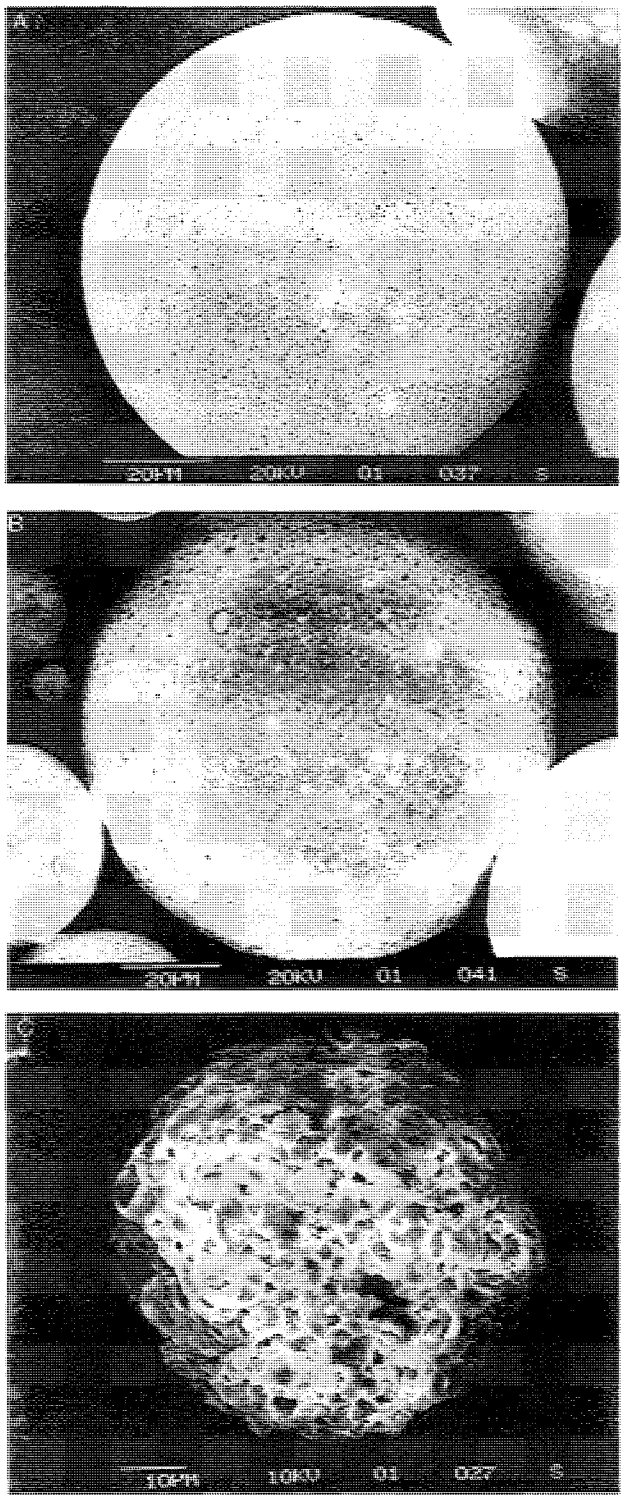

Fig. 3. SEM photographs of tetanus toxoid-loaded microspheres at different degradation stages. Immediately after preparation (A); after 10 days (B) and 30 (C) days in release medium at 37°C.

tetanus toxoid-loaded microspheres was also investigated. The effect of the vaccine loading and vaccine purity (Lot 1 vs Lot 2) was also analyzed. Among the technological variables, the solvent elimination procedure used to prepare the microspheres (solvent extraction or solvent evaporation) did not affect the release behavior (results not shown). However, a significant effect of the type of protein dispersion was noted in particular for PLA (MW 3000) microspheres. A faster and less reproducible release from PLA microspheres

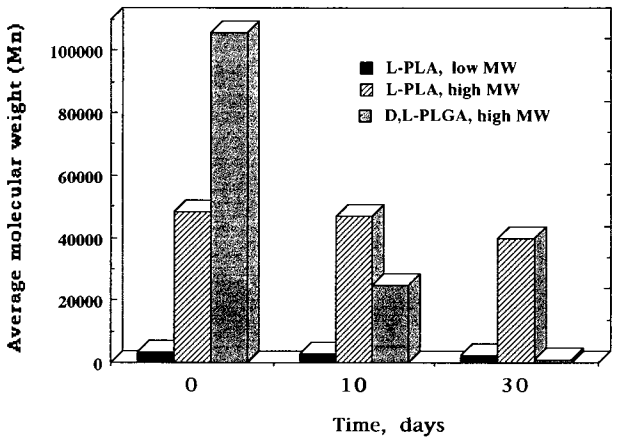

Fig. 4. Degradation profiles of different polymer types of tetanus toxoid-loaded microspheres. (□) PLA (MW 3000); (▨) PLA (MW 50,000); (▩) PLGA (MW 100,000).

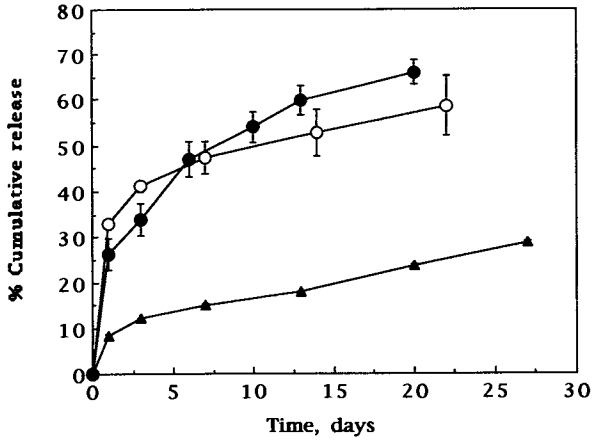

Fig. 6. Cumulative release of tetanus toxoid from different types of polymer microspheres, prepared under the same processing conditions, in PBS at 37°C: (○) PLA (MW 50,000); (▲) PLGA (MW 100,000); (●) PLA (MW 3000). Polymer concentration in the organic phase: 200 mg/mL. Vaccine Lot 1.

was observed when the protein was dispersed as a lyophilized powder into the organic polymer solution as seen by the high standard deviation values (Fig. 5). Taking into account this information, the solvent extraction technique carried out in a double-emulsion system was selected as the shortest and most adequate technique for further experiments dealing with the encapsulation of tetanus toxoid.

Figure 6 compares the release profiles of tetanus toxoid (Lot 1) from microspheres prepared with different polymers under the same processing conditions (polymer concentration, 200 mg of polymer/mL; theoretical loading, 5 mg vaccine powder/100 mg of polymer). A nearly constant release rate was observed after the third day of incubation from microspheres prepared with high molecular weight polymers (PLA and PLGA). However, the amount released during the first 3 days (burst effect) was 33% for PLA (MW 50,000) and only 8% for PLGA microspheres. In contrast, a parabolic release profile was observed for PLA (MW 3000) microspheres.

The influence of vaccine loading was investigated for PLA microspheres with different molecular weights (Figs. 7A and B). A more significant burst effect and higher release rate over the first week of the release study were observed for the high-loaded microspheres (5 mg of vaccine powder per 100 mg of polymer) (Fig. 7A) prepared with PLA (MW 3,000). The release rate from high molecular weight PLA (50,000) microspheres (Fig. 7B) was similar but the burst effect was doubled as a consequence of the higher amount of encapsulated material.

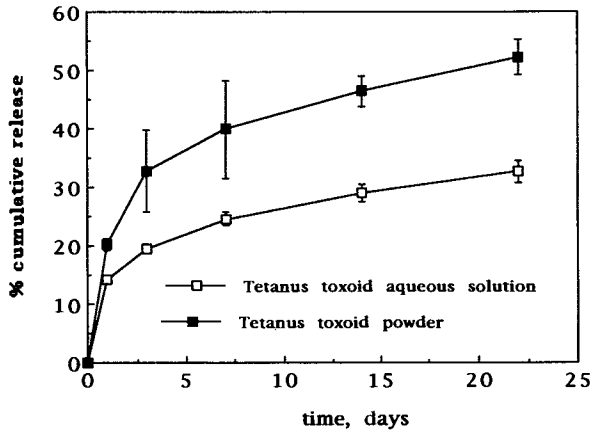

Fig. 5. Influence of the protein dispersion manner on the release profile of tetanus toxoid (batch 1; 2% loading) from oligomeric PLA microspheres in PBS at 37°C.

In order to determine the importance of the purity of tetanus vaccine on the design of controlled-release microspheres, the *in vitro* release behavior of some formulations prepared under the same conditions but using different tetanus toxoid lots was compared. Figures 8A and B compare the release profile of tetanus toxoid from PLA (MW 3000) and PLGA (MW 100,000), respectively, prepared with vaccine powders of different purities (Lot 1, 18.5 Lf per mg of powder; Lot 2, 92 Lf per mg of powder). The release rate of tetanus toxoid from PLA microspheres (Fig. 8A) was considerably faster for the formulation containing the less pure vaccine (Lot 1). A completely different profile was achieved after encapsulation of these vaccine lots in PLGA microspheres (Fig. 8B). Followed by a small initial burst (10% released the first day), a nearly constant release was achieved for the formulation prepared with the low-purity vaccine (Lot 1), whereas a sigmoidal profile characterized the release of the high-purity vaccine.

Immunization Study with Vaccine-Containing Microspheres

The systemic immune responses induced in mice following subcutaneous immunization with two formulations of tetanus toxoid-containing microspheres and fluid toxoid are displayed in Fig. 9 and Table II. The two formulations selected for *in vivo* studies were prepared with vaccine batch 1 and they displayed different microsphere size and different release profiles. One formulation, based on PLA (MW 3000) with a small particle size (Fig. 2A), released 50% of the protein within 10 days (Fig. 6) and the second formulation, based on PLGA (MW 100,000) with a larger size (Fig. 2C), released approximately 30% of the toxoid in 30 days (Fig. 6). The results of antibody levels over 6 months showed higher

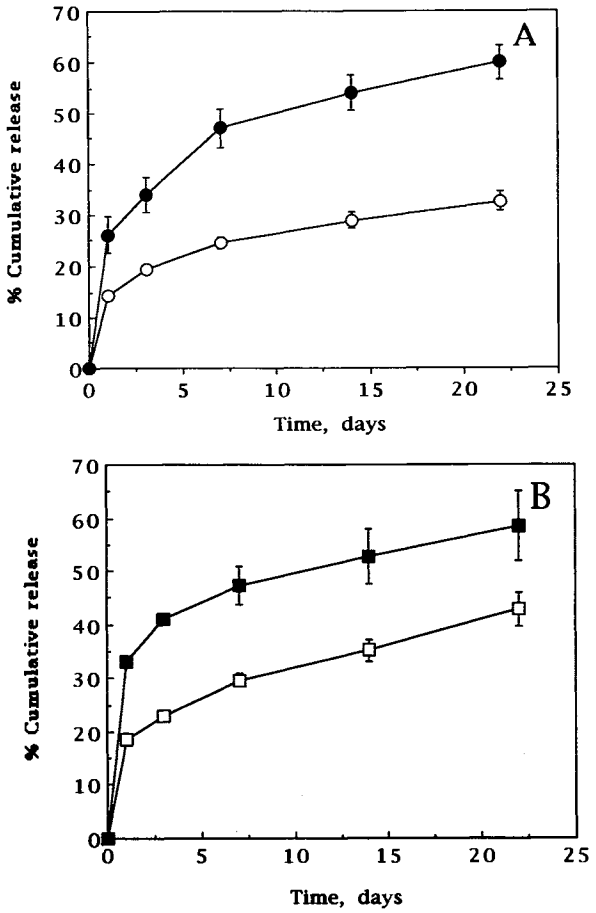

Fig. 7. Cumulative release of tetanus toxoid from PLA microspheres of different molecular weights and different protein loadings in PBS at 37°C. (A) PLA (MW 3000); (B) PLA (MW 50,000). Open symbols, 2% loading; filled symbols, 5% loading. Vaccine Lot 1.

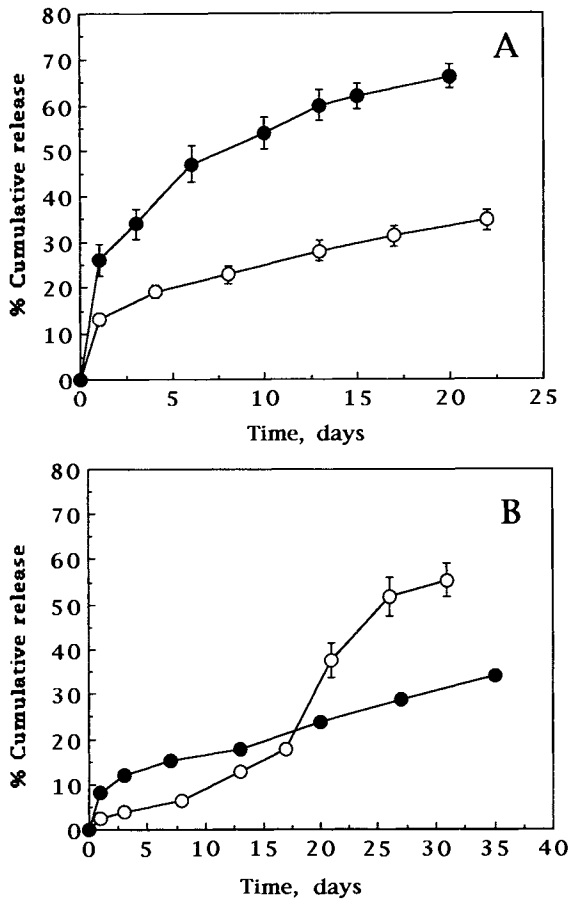

Fig. 8. Cumulative release of tetanus toxoid from microspheres prepared with different polymer and different vaccine purities. (A) PLA (MW 3000); (B) PLGA (MW 100,000). Filled symbols, Lot 1; open symbols, Lot 2.

values for the encapsulated antigen. These differences are statistically significant at any time for PLA microspheres and only at 13 weeks for PLGA microspheres (Dunnett's multiple-range test, $P < 0.05$), a fact that may be attributed to the large variation in the values obtained for this formulation. Nevertheless, increasing and similar titers of neutralizing antibodies against tetanus toxin were observed for both microsphere formulations. In addition, the antibody responses elicited by the encapsulated antigen were higher than those observed for the free antigen.

DISCUSSION

In the present study we investigated the suitability of PLA and PLGA microspheres for the encapsulation and slow release of tetanus toxoid vaccine. The polymers were selected based on such properties as degradability, tissue compatibility, and easy processing (16).

Two main ways to encapsulate hydrophilic material in hydrophobic polymers have been described. These processes are based on the dispersion of the material as a powder or as an aqueous solution into the organic solution containing the polymer (2,3). We compared both methods and showed that the double-emulsion method permitted the formation of more regular microspheres with a better capacity to control the release. As shown in Fig. 6 the percentages of protein released are higher and less consistent for microspheres prepared by the powder dispersion procedure. On the other hand, a way to accelerate the solvent elimination during the microencapsulation process was also investigated. This approach was based on increasing the solubility of the organic solvent (methylene chloride) in the external aqueous phase by adding an alcohol. The physical characteristics and controlled release properties are similar when comparing formulations prepared according to both techniques. Consequently, the solvent extraction technique was considered the most convenient way to encapsulate tetanus toxoid since microsphere formation occurred in only 30 min.

It has been reported that when microspheres are administered subcutaneously, depending on their size, they can either remain in the subcutaneous tissue or be phagocytized by macrophages (17). More specifically it has been indicated that the encapsulation of antigens in microspheres less than 10 μm may be of interest in order to target them to the macrophages (11). Therefore, we decided to investigate formulation parameters that influence microsphere size. Micrographs (Fig. 2) indicate that microsphere size is dependent on the polymer molecular weight; i.e., the mean diameter of PLA (MW 3000) microspheres is approximately 9 μm. However, the mean size of microspheres prepared with high mo-

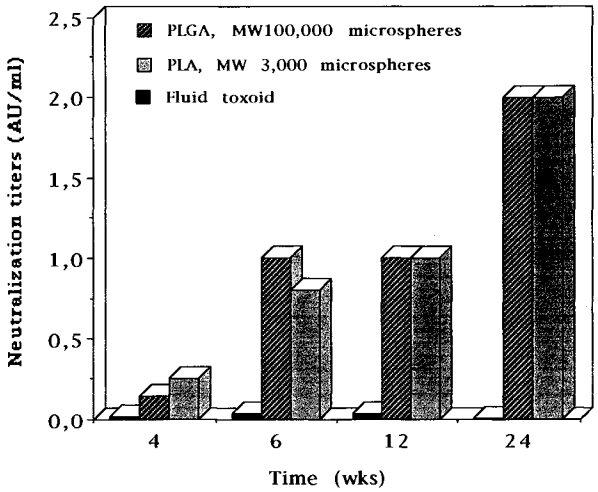

Fig. 9. *In vivo* neutralizing antibody response (IU/mL serum) to tetanus toxoid in mice following immunization with a single dose (5 Lf) of fluid vaccine (■) and two different formulations of microencapsulated tetanus toxoid: (▨) PLA (MW 3000) microspheres; (▩) PLGA (MW 100,000) microspheres.

lecular weight polymers is larger. Low molecular weight polymers give low-density solutions when dissolved in methylene chloride. Accordingly, their dispersion in an external aqueous phase results in a smaller emulsion droplet size. Similarly, the reduction of the polymer concentration leads to a lower-density polymer solution and thus to a smaller microsphere size. On the other hand, the incorporation of a lipophilic surfactant, i.e., L-α-phosphatidylcholine, into the organic polymer solution led to a reduction in microsphere size; this may be attributed to a better stabilization of the initial emulsion. Despite the ability to reduce microsphere size using these approaches, with the exception of oligomeric PLA microspheres, the reduction in size occurs simultaneously with an increase in the porosity.

It is known that systems based on PLA and PLGA, in an aqueous environment, undergo hydration followed by bulk erosion (18,19). During erosion, the porosity of the matrix increases and the release of the protein by diffusion is facilitated. Assuming this behavior, not only the degradation rate of the polymer but also the initial inner structure of the microspheres should be considered as relevant factors controlling the release process.

By comparing the polymer degradation profiles (Fig. 4) and protein release profiles (Fig. 6), a minor contribution of the polymer degradation rate to the release of protein from formulations prepared with the low-purity vaccine (Lot 1) was noted. In fact, microspheres prepared with polymers which degrade slowly (PLA) displayed a fast release, suggesting that a large amount of the vaccine is released by diffusion through channels before significant degradation of the polymer occurs. In contrast, a good correlation was found between the polymer degradation and the protein release profiles for microspheres prepared with the high-purity vaccine (Lot 2) (Figs. 8A and B). Actually, the S-type shape of the release profile of the protein from PLGA microspheres (Fig. 8B) may be explained by the profile of decrease in polymer molecular weight. During the initial release period the hydrated polymer undergoes hydrolysis, but the reduction in the molecular weight has not reached a point at which the degradation products are sufficiently small to be soluble. Consequently during this initial period no significant release of tetanus toxoid was observed. When the reduction of the molecular weight became significant (at 10 days, 80% reduction; Fig. 4), a critical increase in the porosity of the matrix was achieved and the higher release rate began. This type of release profile is typical for bulk-eroding controlled-release systems based on high molecular weight polymers (20). In the case of oligomer PLA microspheres (Fig. 8A), due to their small size and water uptake capacity, an initial fast release occurs, yielding a parabolic-type release pattern. Microspheres containing the high-purity vaccine show a low burst effect (13%) followed by the release of 20% of their load in approximately 20 days; the last part of this profile could be related to the polymer degradation rate. Similar behavior has been observed for insulin-loaded oligomer microspheres (21).

The influence of vaccine loading on the release pattern was observed for PLA microspheres of different polymer molecular weights (3000 and 50,000). In both cases (Figs. 7A and B), by doubling the vaccine loading, the burst effect was doubled. This fact may be attributed to the larger amount of protein near the microsphere surface and, consequently, available for initial release. In addition, microspheres with a larger theoretical loading were prepared by reducing the polymer concentration in the organic phase, a circumstance that causes a reduction in microsphere size. Accordingly, the faster vaccine release from higher-loaded microspheres can be explained not only by the greater vaccine content but also by their smaller size.

Results from the *in vivo* study indicate that the immunogenic response, expressed as ELISA IgG titers, was significantly higher for antigen loaded-PLA microspheres than for the free antigen. The ELISA titers corresponding to PLGA microspheres were significantly higher than those observed for the fluid vaccine only at 13 weeks. The absence of statistical significant differences at any other time during the study may be due to the large variation of the antibody titers obtained for this formulation. Nevertheless, when the high-affinity neutralizing antibodies were determined, both microencapsulated antigen formulations displayed an increasing antibody response, which was substantially higher than that observed for the fluid vaccine. The difference in the *in vivo* responses obtained by the ELISA and neutralization tests is related to the type of antibodies determined by each

Table II. IgG Antibody Response by ELISA to a Single Dose (5 Lf) of Fluid and Microencapsulated (PLA, MW 3000, Microspheres[a] and PLGA, MW 100,000, Microspheres[b]) Toxoid Administered Subcutaneously to Mice

	Geometric IgG (AU/ml) in sera of mice after			
Formulation	4 weeks	8 weeks	13 weeks	26 weeks
Fluid toxoid	0.12	0.09	0.07	0.04
PLA MS[a]	1.73*	2.48*	1.28*	1.13*
PLGA MS[b]	0.38	0.50	0.43*	0.18

* $P < 0.05$ by Dunnett's multiple-range test comparing the microencapsulated vaccine to the corresponding fluid control.

technique; although both techniques determine specific antibodies, the neutralization test refers only to high-affinity antibodies. Therefore, the increasing neutralization titers may be explained by progressive affinity maturation of tetanus antibodies. More formulations in a longer-term immunization study should be investigated to obtain more definitive conclusions. In addition, formulations containing purified tetanus toxoid are being investigated (13). Nevertheless, the results presented here indicate that an increase in the immunogenic response of tetanus toxoid can be achieved from injectable controlled-release microspheres.

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