A Novel System Based on a Poloxamer/PLGA Blend as a Tetanus Toxoid Delivery Vehicle

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Purpose. Previous work on the encapsulation of proteins and antigens in poly(lactic-co-glycolic acid) (PLGA) microspheres has led to the conclusion that microencapsulated antigens are frequently inactivated due to their interaction with the polymer. To improve the compatibility of the antigen with the polymer, we have devised a novel microencapsulated system consisting of a blend of PLGA 50:50 and poloxamer 188 (Pluronic® F68) and applied it to the delivery of tetanus antigen.

Methods. Tetanus toxoid was encapsulated in microspheres containing different amounts of poloxamer using an anhydrous procedure based on an *oil-in-oil solvent extraction* process. The compatibility of the polymers was studied by Fourier transform infrared (FT-IR) spectroscopy. Microspheres were assayed *in vitro* and *in vivo* for their ability to deliver active antigen for extended periods of time.

Results. Analysis by FT-IR spectroscopy evidenced the miscibility of both polymers by a hydrogen bonding mechanism. *In vitro* release studies revealed that microspheres containing poloxamer released antigenically active TT, in a pulsatile manner, for up to 50 days. In addition, it was observed that the intensity and duration of the pulses were dependent on both poloxamer content and TT loading in the microspheres. The *in vivo* evaluation of this new system showed that the neutralizing antibodies elicited by the TT encapsulated in poloxamer-PLGA microspheres were considerably higher and more prolonged than those obtained after administration of the aluminum phosphate-adsorbed toxoid.

Conclusions. These results indicate the importance of devising new microencapsulation approaches specially adapted for preserving the activity of protein antigens incorporated within PLGA microspheres.

KEY WORDS: poloxamer/PLGA microspheres; vaccine delivery; tetanus toxoid; immune response.

INTRODUCTION

In recent years, there have been several reports on the development of a single dose tetanus vaccine based on the controlled release of tetanus toxoid (TT) from biodegradable polymer microspheres (1–6). Even though most of these studies showed high levels of anti-TT IgG antibodies in animals injected with a single dose of microencapsulated TT (5,6), the values of the antitoxin neutralization titers (functional antibodies) were lower than those elicited by a single dose of aluminum phosphate-adsorbed TT (7).

Our previous work on the encapsulation of TT revealed that the antigen loaded in PLGA microspheres suffers a loss of activity, thus not allowing these devices to induce a more prolonged immune response than that observed for aluminum phosphate-adsorbed control (3). Two main stages of inactivation of the encapsulated antigen have been identified so far (8). Firstly, during the preparation of the microspheres, the use of organic solvents, strong agitation and exposure of the antigen to water/oil interfaces and to lyophilization have been found to be deleterious. Secondly, during incubation, the water uptake by the protein, the decreasing pH stemming from the formation of new carboxylic acid-end groups in the polymer chain during polyester hydrolysis and also the interaction between the protein and the polymer, have been considered as sources of protein inactivation.

Recently, we have investigated further the inactivation mechanism of tetanus toxoid encapsulated in PLGA microspheres and found that the antigen partially loses its activity during the encapsulation process. However, its inactivation is highly accelerated in the course of the polymer degradation during incubation at 37°C. More specifically, it was observed that the major cause of antigen inactivation was the interaction between the protein and the polymer and/or the polymer degradation products (9). Therefore, it is obvious that the preservation of the stability of the protein antigens during their microencapsulation and release becomes essential for the development of a successful controlled release vaccine. Despite this fact, only a few attempts have so far been aimed at the stabilization of microencapsulated antigens, most of them being focused on TT (10,11). These previous reports have not shown, however, the *in vivo* efficacy of the stabilized microencapsulated antigens.

On the basis of these considerations, the overall goal of this study was to stabilize the tetanus toxoid encapsulated in PLGA microspheres in order to achieve a high immune response after a single injection of the microspheres containing TT. For this purpose, our strategy was to design a new microencapsulated system using an *oil-in-oil solvent extraction* process. The system was composed of a blend of the PLGA with a non ionic and safe surfactant, poloxamer 188, which was supposed to prevent the interaction between the antigen and the polymer.

MATERIALS AND METHODS

Materials and Animals

The polymer D,L-PLGA 50/50 (lactic acid/glycolic acid), Resomer® RG 506, molecular weight (M_w) 98 kDa, was purchased from *Boehringer Ingelheim* (Ingelheim, Germany). Some of the carboxylic acid groups of this polymer are esterified with the catalyst used in the polymerization reaction. Purified TT, (M_w 150 kDa, 85–95% monomeric) dissolved in phosphate buffer saline, pH 7.4, was kindly donated by *Massachusetts Public Health Biologic Laboratories* (Boston, MA, USA). Cottonseed oil and soybean lecithin (type IV-S, phosphatidylcholine content: approx. 40%) were purchased from *Sigma Chemical* (Madrid, Spain). For the ELISA tests, monoclonal antibody and purified guinea-pig anti-tetanus immunoglobulin (IgG) were obtained from the *National Institute for Biological Standards and Control* (Hertfordshire, United Kingdom), rabbit anti-guinea pig IgG peroxidase conjugate and the enzyme substrate 2,2-azino-bis-3-ethylbenzthiazoline sulphonate (ABTS) were purchased from *Sigma Chemical* (Madrid, Spain), goat

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anti-mouse IgG alkaline phosphatase conjugate was purchased from *Caltag Laboratories* (San Francisco, CA, USA) and the enzyme substrate di-sodium-4-nitrophenyl phosphatase was obtained from *Fluka* (Madrid, Spain). Poloxamer 188 (Pluronic® F68) was kindly donated by *BASF Co.* (Parsippany, NJ, USA).

Female outbred mice (CD-1), 4–5 weeks old, were purchased from Charles River (Wilmington, MA, USA).

Methods

Preparation of Poloxamer 188/PLGA Microspheres

Microspheres with ratios of poloxamer/PLGA 1/10, 3/10 and 5/10 (w/w) were prepared by a modified *oil-in-oil solvent extraction* technique (12). Before microencapsulation, poloxamer 188 (10, 30 or 50 mg) was dissolved in the TT solution (1% theoretical loading of microspheres), frozen and lyophilized (primary drying: −30°C, secondary drying: 21°C; *Labconco*, Kansas City, Missouri, USA). Then, the lyophilized powder was added to a 1 ml solution of PLGA in acetonitrile (100 mg/ml). This suspension was added dropwise to 80 ml of cottonseed oil containing soybean lecithin (0.05% w/v) under mechanical agitation (700 rpm, RW 20 DZM, *IKA*, Madrid, Spain). After the system was stirred for 45 min, 50 ml of petroleum ether were added to harden the immature microspheres and stirring was continued for another 15 min. Finally, microspheres were collected by filtration, washed with petroleum ether and lyophilized.

Poloxamer/PLGA (5/10) microspheres containing TT theoretical loadings of 0.16, and 3% (mg of TT/100 mg of microspheres) were also prepared.

Morphological Characterization of Microspheres

The morphological examination of microspheres was performed using a scanning electron microscope (SEM, JSM-T220A, *Jeol*, Tokyo, Japan). Samples for SEM were mounted on metal stubs and coated with gold-palladium to a thickness of 200300 Å.

The particle size distribution of the microspheres (volume diameter, d_v) was determined by a Coulter® Multisizer II (*Coulter Electronics*, Luton, England) after suspending the microparticles in a electrolytic aqueous solution (Isoton® II, *Coulter Electronics*, Luton, England).

Determination of the Encapsulation Efficiency

For the determination of the encapsulation efficiency, microspheres were washed in PBS/Tween® 80 for 2 h immediately after preparation, and the supernatant assessed by ELISA. The amount of TT encapsulated into the microspheres was calculated by the difference between the total amount used to prepare the microspheres and the amount of TT present in the aqueous supernatant.

Physicochemical Characterization of Poloxamer 188/PLGA Blend

Fourier-transformed infrared spectra (FT-IR, *Cygnus* 100, Madison, WI, USA) of blank microspheres and the poloxamer 188 were obtained using the KBr disc technique (resolution: 4.0, scans: 32).

In Vitro Release Studies

Samples of 10 mg of microspheres were suspended in 2.5 ml of phosphate buffer saline (PBS) pH 7.4 containing 0.02% (w/v) Tween® 80 in glass tubes and incubated at 37°C (Hotcold-M, *Selecta*, Orense, Spain). At predetermined time intervals, the samples were centrifuged at 4,000 g for 15 min (*Sigma* 2–15, Madrid, Spain), 1.5 ml were removed from the supernatant and replaced by fresh release medium. The pH of the release medium was monitored and kept constant over the release study between 7.0–7.4. Antigenically active TT released was determined by the enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well flat-bottom microtitre plates (*Corning*, NY, USA) were coated with 100 µl of anti-tetanus monoclonal antibody at 1 µg/ml in carbonate buffer pH 9.6 and allowed to incubate overnight at 4°C in a humid container. The plates were washed three times between all steps with PBS containing 0.5% (w/v) Tween® 20 (PBST buffer). To minimize non-specific interactions, 100 µl of PBST containing 2.5% (w/v) of dried skimmed milk powder (PBSTM buffer) were added to all the wells, and plates were incubated for 1 h at 37°C in a humid container. After washing the plates 3 times with PBST, a reference TT preparation and test samples were diluted serially in two-fold steps in PBSTM. The plates were incubated at 37°C for 2 h in a humid container and washed. Then, 100 µl of guinea pig IgG at 25 µg/ml in PBSTM were added to the wells and allowed to react for 2 h at 37°C. After washing, 100 µl of rabbit anti-guinea pig peroxidase conjugate diluted 1:2000 in PBSTM were added to the wells, and plates were incubated for 1h at 37°C. The plates were washed and the substrate (ABTS), 0.5 mg/ml in 0.05 M citric acid, pH 4.0 was added. Following colour development (30 min) plates were read at 405 nm on a microplate reader (3550-UV, Biorad, Madrid, Spain).

Immunization Protocol

A microsphere formulation prepared with poloxamer 188/PLGA (5/10) with a theoretical loading in TT of 3% was selected for the immunization study. Groups of female mice (six to ten per group) were injected subcutaneously on the left side of the abdomen with a single dose (1.65 µg, 0.55 Lf) of tetanus toxoid-containing microspheres. The same single dose was administered jointly encapsulated (1.5 µg, 0.50 Lf) and adsorbed to aluminum phosphate (0.15 µg, 0.05 Lf TT/4.4 µg $AlPO_4$). The same dose of adsorbed toxoid (1.65 µg, 0.55 Lf TT/48.7 µg $AlPO_4$) was studied as a control. Prior to injection, microspheres were suspended in 0.5 ml of an aqueous solution of 0.5% (w/v) sorbitol, 0.1% (w/v) carboxymethylcellulose and 0.02% (w/v) Tween® 80. Samples of blood were collected, by cardiac puncture, several times after administration and the sera separated by centrifugation. The sera of mice from each vaccine group were pooled and assayed for tetanus antitoxin in terms of antitoxin units (AU) by the toxin neutralization test (13,14). The values of AU/ml of serum samples were determined against US standard tetanus antitoxin (obtained from *Center for Biological Evaluation and Research*, Bethesda, MD, USA). Individual mice sera were also evaluated for IgG antibodies to tetanus toxin by the ELISA test. Briefly, 96well microtitre plates were

coated with 100 µl of purified tetanus toxin (1 µg/ml) in 50 mM sodium bicarbonate at 4°C overnight. The plates were washed three times with PBS, pH 7.4 containing 0.01% (w/v) of Tween® 20 (PBST) between each step. To minimize non-specific interactions, 200 µl of an aqueous solution of BSA (1% w/v) were added to all the wells and the plates incubated for 1 h at 37°C. After washing the plates three times with PBST, the reference solution (hyperimmune anti-tetanus toxoid mouse serum containing 0.07 µg/ml) and the test samples were diluted serially in two-fold steps in PBST containing 0.05% (w/v) of BSA (PBB). The plates were kept at 37°C for 2 h and washed. Then, 100 µl of goat anti-mouse IgG alkaline phosphatase conjugate diluted 1:1000 in PBB were added to plates and allowed to react for 2 h at 37°C. The plates were washed and the substrate p-nitrophenyl phosphate diluted to 1 mg/ml in 1 M diethanolamine, 0.5 mM magnesium chloride buffer was added to plates. The plates were kept at room temperature for 30 min and read at 405 nm wavelength on an ELISA reader. The concentration of ELISA Antitoxin Units (EAU/ml) in the serum samples was calculated against hyperimmune mouse serum by extrapolation from the standard curve.

RESULTS AND DISCUSSION

Our previous experience in the encapsulation of TT within PLGA microspheres led us to recognize the important stability problems of this antigenic protein, not only during its encapsulation, but mainly during the PLGA degradation process (15).

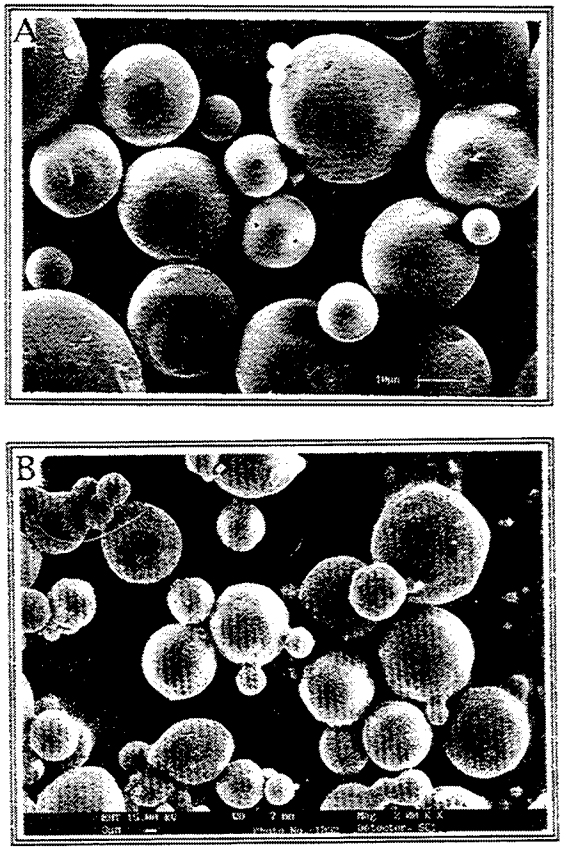

Fig. 1. Microphotographs obtained by SEM of poloxamer/PLGA (A) 1/10, and (B) 5/10 microspheres.

Table I. Particle Size and Loading Efficiency of PLGA and Poloxamer/PLGA TT-Loaded Microspheres

Microspheres' composition	TT theoretical loading (%)	Mean particle size*, µm (σ)	TT loading efficiency (%)
PLGA	1	24 ± 2(8)	35.3 ± 1.1
polox./PLGA 1/10	1	26 ± 2(10)	43.2 ± 1.9
polox./PLGA 3/10	1	35 ± 3(13)	65.1 ± 3.6
polox./PLGA 5/10	0.16	44 ± 1(17)	93.7 ± 2.1
polox./PLGA 5/10	1	43 ± 2(14)	54.8 ± 1.8
polox./PLGA 5/10	3	44 ± 2(10)	34.9 ± 1.1

* Average (dv) ± SD (n = 4) (population SD).

Looking for the major causes of inactivation we found that TT interacted irreversibly with the polymer and, especially, with its acidic degradation products; therefore, we concluded that the use of a blocking agent which interacts favourably with PLGA would prevent the interaction of the antigen thereby improving its stability. Among the agents evaluated, poloxamer 188 exhibited excellent properties to block the interaction between tetanus toxoid and PLGA polymers (8). Thus, the incorporation of this surfactant was thought to be a good approach to stabilize TT encapsulated in PLGA microspheres.

Poloxamers have been previously used in the preparation of PLGA microspheres by the w/o/w solvent evaporation technique (16–18). In these previous studies the surfactant was incorporated in the oily phase with the only goal of stabilizing the emulsion, whereas the protein was incorporated in the aqueous phase.

In the present work we propose, as a new approach for improving the stability of microencapsulated proteins, the development of poloxamer/PLGA blend microspheres according to an anhydrous procedure. The efficacy of this system in preserving the immunogenicity of TT was assessed *in vitro* and *in vivo*.

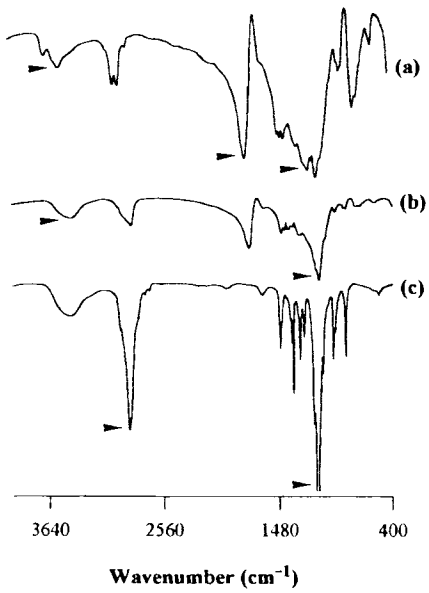

Fig. 2. IR spectra of (a) PLGA (Resomer® RG 506) microspheres, (b) poloxamer/PLGA (5/10) microspheres, and (c) poloxamer 188.

Development and Characterization of Poloxamer/PLGA Microspheres

With the aim of investigating the effect of poloxamer in the PLGA microspheres, we prepared poloxamer/PLGA blend microspheres by the *oil-in-oil solvent extraction* technique. This anhydrous procedure was supposed to be very convenient for the encapsulation of proteins since it is known that the presence of water during exposure of the protein to the organic solvent can cause its denaturation followed by its aggregation (19,20). Both polymers, poloxamer 188 and PLGA, were blended in different ratios (poloxamer/PLGA 1/10, 3/10, and 5/10 w/w) by dissolving them in the organic solvent, acetonitrile, prior to the formation of the microspheres. For the preparation of TT containing poloxamer/PLGA microspheres, TT was first lyophilized in the presence of poloxamer 188 to facilitate its dispersion in the PLGA organic solution. The initial antigen loading of the poloxamer/PLGA (1/10, 3/10, 5/10) microspheres was 1% (mg TT/100 mg microspheres). Poloxamer/PLGA (5/10) microspheres were also prepared at loadings of 0.16 and 3%.

Figures 1.a and 1.b show the SEM microphotographs of microspheres made of 1/10 and 5/10 poloxamer/PLGA ratios. Microspheres were spherical and formed a very homogeneous population; however, as the poloxamer content in the microspheres increased, their surface became more irregular. Results in *Table I* indicate that the mean size of the microspheres increased from 25 to 40 μm as the poloxamer content in the microspheres did. However, the particle size distribution was narrow, irrespective of the microspheres composition (σ values between 8–17). In addition, it was found that the loading efficiency of the process was affected by both the poloxamer content and the loading in TT. More specifically, the loading efficiency was enhanced by increasing the amount of poloxamer in the formulation and reducing the theoretical loading in TT. The latter effect was expected since high initial loadings lead to the accumulation of protein molecules on the surface of the microspheres which are then eliminated in the washing steps. It should be noted that these loading efficiency values were calculated by an indirect method (see methods section) and therefore, there is a possibility that some of the encapsulated antigen molecules are not ELISA-reactive.

Physicochemical Characterization of the Poloxamer 188/PLGA Blend: FT-IR Spectroscopy

The modification of the physicochemical properties of PLGA copolymers by poloxamers was previously reported. More specifically, a plasticizing effect of these surfactants on the PLA and PLGA polymers was described (18,21). In some preliminary experiments, we confirmed the plasticizing effect of poloxamer on PLGA in films made of both polymers. In this respect, we found that the glass transition temperature (Tg) of a PLGA film was shifted to a lower Tg in a poloxamer/PLGA film, thus suggesting that poloxamer acts as a plasticizer (data not shown).

In order to characterize further the poloxamer 188/PLGA blend, blank microspheres made of poloxamer/PLGA (5/10) were analyzed by FT-IR spectroscopy. As shown in *Fig. 2*, poloxamer 188 showed characteristics bands at 2890 and 1120 cm^{-1}, which correspond to alkyl and ether groups, respectively. PLGA microspheres showed the peak of the OH stretching of carboxylic and alcohol groups at 3510 cm^{-1}, and those corresponding to carbonyl and ester groups at 1760 cm^{-1} and 1220 cm^{-1}, respectively. In the case of the poloxamer/PLGA microspheres, the peaks representative of the OH stretching of the PLGA carboxylic and alcohol groups and of the ether group of poloxamer showed a displacement to a lower wavelength (from 3510 to 3400 cm^{-1} and from 1120 to 1100 cm^{-1}, respectively). These results suggest the formation of hydrogen bonds between the OH groups of PLGA and the ether groups of poloxamer. This mechanism was previously suggested by *Park et al.* (21) for PLA/poloxamer blends, since it is known that the polymers containing carboxylic acid groups are readily miscible with polyethers via hydrogen bonding.

In Vitro Release Studies

Since the release profile has been indicated to be a relevant factor in determining the immune response elicited by the encapsulated TT (5,7), it is crucial to evaluate the release of antigenically active toxoid under adequate *in vitro* conditions. In this respect, our previous studies showed that the inactivation of TT in the presence of PLGA degrading microspheres was clearly dependent on the microspheres' concentration in the

Table II. Percentages of Antigenically Active TT Released *In Vitro* in Different Time Intervals from PLGA Microspheres with Different Poloxamer Content

Time interval (days)	Antigenically Active TT Released,[a] %							
	PLGA		polox./PLGA 1/10		polox./PLGA 3/10		polox./PLGA 5/10	
	%	Amount	%	Amount	%	Amount	%	Amount
0–1	56.72 ± 2.81	20.02 ± 0.99	21.49 ± 1.65	9.26 ± 0.71	10.25 ± 0.89	6.67 ± 0.58	9.87 ± 2.08	5.90 ± 1.24
2–7	9.95 ± 0.54	3.51 ± 0.19	17.81 ± 2.10	7.68 ± 0.91	3.42 ± 0.50	2.23 ± 0.33	2.22 ± 0.45	1.32 ± 0.25
8–14	n.d.	n.d.	n.d.	n.d.	0.87 ± 0.21	0.57 ± 0.14	n.d.	n.d.
15–21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22–28	n.d.	n.d.	1.32 ± 0.14	0.57 ± 0.06	1.56 ± 0.23	1.02 ± 0.15	2.36 ± 0.47	1.41 ± 0.28
29–35	n.d.	n.d.	n.d.	n.d.	1.39 ± 0.10	0.90 ± 0.06	3.35 ± 0.13	2.00 ± 0.07
36–42	n.d.	n.d.	n.d.	n.d.	4.19 ± 0.30	2.73 ± 0.19	0.55 ± 0.05	0.33 ± 0.03
43–50	n.d.	n.d.	n.d.	n.d.	0.85 ± 0.13	0.55 ± 0.08	2.20 ± 0.51	1.32 ± 0.31

[a] Average ± SD (n = 3); n.d.: not detectable.

incubation medium (9). Consequently, in this study a very low concentration of microspheres (4 mg/ml) was selected in order to minimize the degradation of the toxoid during the *in vitro* release process.

Taking into consideration that the PLGA and poloxamer 188 form a miscible blend, it could be expected that the presence of poloxamer would affect the release of the macromolecule, as previously reported by *Park et al.* (21) for BSA. *Table II* shows the percentages of antigenically active TT released from several formulations containing different amounts of poloxamer and the same theoretical loading, at various times after incubation at 37°C. Results show that the presence of poloxamer in the microspheres helps reducing the burst effect and providing significant amounts of active antigen after long-term incubation periods. The formulation without poloxamer released a significant amount of the encapsulated antigen during the first week of incubation and no active antigen was further detected for up to 50 days; contrarily, the formulations with poloxamer exhibited a pulsatile release behaviour, the intensity and duration of the pulses being dependent on the poloxamer content. The burst effect is normally related to the dissolution of the protein molecules located near the microspheres' surface (22,23). Consequently, results of this work suggest that the blend of poloxamer and PLGA led to a better entrapment of the toxoid in the polymer matrix. On the other hand, in the case of the formulations containing poloxamer a second pulse of release started one month later and lasted for 1 to 4 weeks depending on the poloxamer content in microspheres. This prolonged release of active TT from poloxamer/PLGA microspheres could be attributed to an stabilizing effect of the poloxamer.

With respect to the mechanism that governs the release, "a priori" it could be expected that the hydrophilic poloxamer would quickly leach out from the microspheres in the initial stage of incubation. Nevertheless, the prolonged release observed, which could be controlled by the amount of poloxamer incorporated into the microspheres, suggests that poloxamer forms a miscible blend with PLGA thus prolonging the release of the antigen for up to 50 days. SEM photographs obtained from poloxamer/PLGA (3/10) after incubation (*Fig. 3.a–3.c*) revealed that, despite the high amount of poloxamer in the polymer matrix, the erosion process advances relatively slowly, thus confirming the good miscibility between PLGA and poloxamer.

Results presented in *Table III* compare the *in vitro* release behaviour of poloxamer/PLGA (5/10) microspheres containing different antigen loadings (0.16, 1 and 3%). The three preparations exhibit a pulsatile release behaviour, the intensity and duration of the pulses being dependent on the TT loading of the microspheres. The amount of TT released over the first day increases as the TT loading does, due to the more important amount of poorly entrapped antigen. After the burst effect, the release of active TT extends for a period of 7–14 days for the formulations with higher TT loading (1 and 3%). In all cases, a second pulse of release was observed, the intensity and duration of which varied according to the antigen loading: whereas microspheres with the loading of 0.16% did not exhibit release after 28 days, formulations with 1% and 3% loadings provided a second pulse of release which lasted from day 28 to the end of the experiment (day 50). This could be attributed to a higher amount of antigen that is available for release, as the TT content in the microspheres increases.

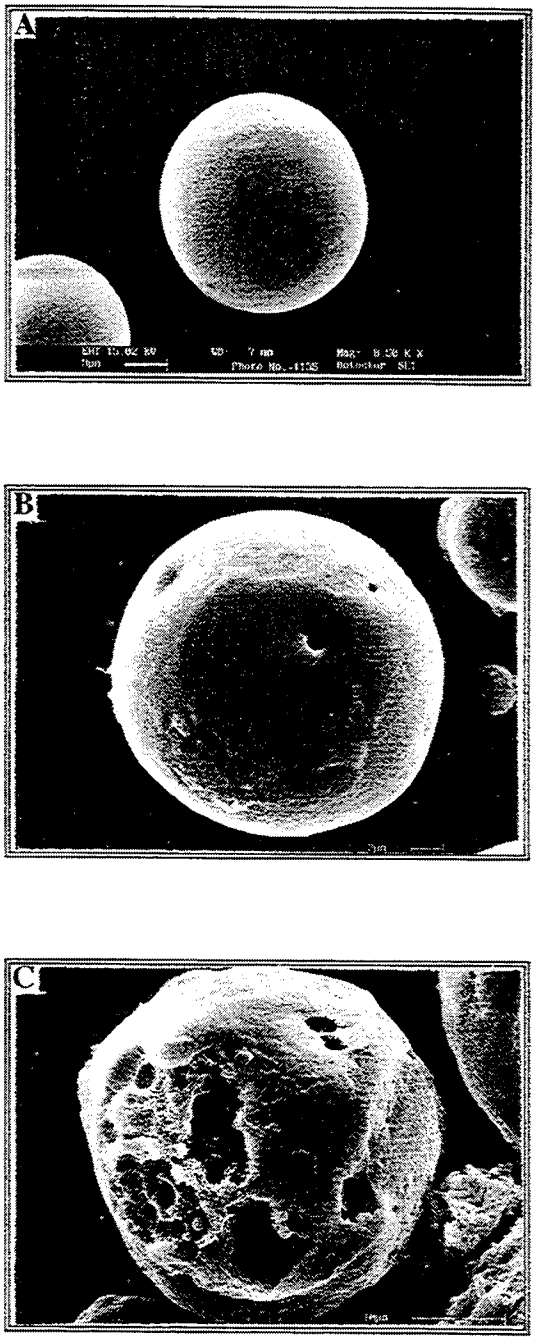

Fig. 3.A–C. SEM microphotographs of poloxamer/PLGA (3/10) microspheres at different times during the *in vitro* erosion process: (A) 4 hours, (B) 7 days and (C) 15 days.

Immunization Study

The stabilizing effect of poloxamer in the microspheres led us to select the formulation poloxamer/PLGA 5/10 (theoretical loading in TT: 3%) for *in vivo* evaluation.

The specific tetanus toxin IgG antibody levels induced in mice after subcutaneous immunization with a single dose of

Table III. Percentages of Antigenically Active TT Released *In Vitro* in Different Time Intervals from Poloxamer/PLGA 5/10 Microspheres, Prepared with Different Theoretical Antigen Loadings

Time interval (days)	Antigenically Active TT Released,[a] %					
	0.16% loading		1% loading		3% loading	
	%	Amount	%	Amount	%	Amount
0–1	7.79 ± 2.21	1.16 ± 0.32	9.87 ± 2.08	5.90 ± 1.24	11.95 ± 1.29	12.53 ± 1.36
2–7	n.d.	n.d.	2.22 ± 0.45	1.32 ± 0.26	0.49 ± 0.08	0.51 ± 0.08
8–14	n.d.	n.d.	n.d.	n.d.	0.98 ± 0.21	1.03 ± 0.22
15–21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22–28	0.42 ± 0.18	0.06 ± 0.02	2.36 ± 0.47	1.41 ± 0.28	1.66 ± 0.37	1.74 ± 0.39
29–35	n.d.	n.d.	3.35 ± 0.13	2.00 ± 0.07	0.59 ± 0.03	0.61 ± 0.03
36–42	n.d.	n.d.	0.55 ± 0.05	0.33 ± 0.03	0.45 ± 0.12	0.47 ± 0.13
43–50	n.d.	n.d.	2.20 ± 0.51	1.32 ± 0.31	0.82 ± 0.23	0.86 ± 0.24

[a] Average ± SD (n = 3); n.d.: not detectable.

encapsulated TT, aluminum phosphate-adsorbed TT and a mixture of encapsulated and adsorbed TT are displayed in *Table IV*. The geometric mean antibody levels achieved for the encapsulated toxoid (MS) were initially (4–8 weeks) higher than those obtained after injection of adsorbed toxoid ($AlPO_4$-TT). At more prolonged times, the encapsulated TT elicited lower IgG levels than the adsorbed toxoid. A remarkable fact in this study is that the administration of a mixture of encapsulated and adsorbed TT (MS+$AlPO_4$) led to significantly higher and prolonged IgG levels than those elicited by the adsorbed antigen. Despite the known complexity of the mechanisms of the immune response by TT-containing microspheres, it is presently accepted that the adjuvant capacity of the microspheres is related to a combination of physicochemical factors such as the polymer properties, the microspheres' size and the antigen-release profile (5,7). With respect to the aluminum compounds, their adjuvancity has been attributed also to a complex mechanism that includes the formation of a depot at the site of the injection from which the antigen is released slowly (24). Based on this information, it is our hypothesis that the aluminum phosphate acts as a depot of the vaccine released from the microspheres, thus potentiating its immune response. In addition, it should be also considered that the aluminum adjuvants cause inflammation at the site of the injection, attracting immunocompetent cells thus favouring the capture of the microspheres by them (24). This is an interesting achievement since the amount of aluminum phosphate administered with the microspheres is ten times lower than that used as a control. Consequently, this approach leads to a high and long lasting immune response while minimizing one of the major limitations of the aluminum compounds, which are the chronic local tissue reactions (7).

Figure 4 shows the neutralization titres of tetanus antitoxin following administration of the encapsulated TT (MS) and aluminum-phosphate adsorbed toxoid. It is shown that the titres of tetanus antitoxin elicited by the microspheres were up to four times higher than those measured in the control (adsorbed toxoid). Furthermore, while the neutralizing antibodies decreased after six weeks for the aluminum adsorbed-TT, in the case of the encapsulated antigen, and contrarily to what was observed for the IgG levels, they remained constant over the entire period studied. This observation may be explained by progressive affinity maturation of tetanus antibodies induced by prolonged exposure to low concentrations of tetanus toxoid. These results clearly demonstrate the efficacy of this novel microencapsulation approach for the delivery of TT.

In conclusion, the encapsulation of TT in a system composed of poloxamer/PLGA led to a successful improvement in the stability of the encapsulated antigen which elicited levels of neutralizing antibodies higher and more prolonged than those obtained for the aluminum phosphate adsorbed tetanus toxoid.

Table IV. Tetanus Toxin IgG Antibodies (ELISA Antitoxin units/ml) in Sera After Subcutaneous Administration of a Single Dose (1.65 μg) of Encapsulated TT (MS), Aluminum Phosphate-Adsorbed TT (TT – $AlPO_4$), and Jointly Encapsulated (1.50 μg) and Adsorbed (0.15 μg) TT (MS + $AlPO_4$)

Formulation	IgG Antibodies (EAU/ml)[a]					
	4 weeks	8 weeks	10 weeks	12 weeks	20 weeks	26 weeks
MS	2.52 (0.99–6.43)	2.29 (0.38–13.98)	N.D.[b]	0.54 (0.04–7.02)	0.71 (0.05–9.96)	0.55 (0.04–7.34)
MS + $AlPO_4$	3.65 (1.59–8.37)	2.51 (1.04–6.06)	N.D.	1.73 (0.64–4.66)	2.17 (0.73–6.45)	2.06 (0.7–9.04)
TT – $AlPO_4$	1.00 (0.50–2.10)	N.D.	2.00 (0.60–6.51)	N.D.	1.50 (1.50–2.20)	1.80 (0.69–2.32)

[a] Geometric mean (c.i. 95%).
[b] Not determined.

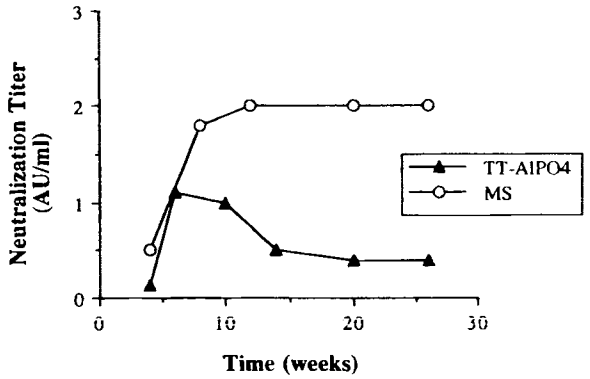

Fig. 4. *In vivo* neutralizing antibody response (antitoxin units/ml) obtained in mice after administration of a single dose (1.65 μg) of encapsulated (MS), and aluminum phosphate-adsorbed tetanus toxoid ($TT\text{-}AlPO_4$). The neutralizing titres were determined in the pooled sera of six to ten mice.

Therefore, this novel approach has a great potential in achieving a single dose tetanus vaccine formulation.

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