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Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres

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Abstract

The development of a single-dose tetanus vaccine based on Poly(Lactic acid) (PLA) or Poly(Lactide-co-Glycolide) (PLGA) microspheres has been complicated due to the instability of tetanus toxoid (TT) inside these systems. Herein we report an attempt to re-design PLGA microspheres by co-encapsulating TT in the dry solid state together with potential protein stabilizers, such as trehalose, bovine serum albumin, alginate, heparin, dextran or poloxamer 188 and by using an appropriate microencapsulation technique. These newly developed PLGA microspheres were able to release in vitro antigenically active TT for at least 5 weeks, the amount released being highly dependent on the stabilizing excipient used. More specifically, results showed that dextran and heparin provided a particularly stabilizing environment for TT inside the microspheres during the polymer degradation process. The efficacy of this strategy was demonstrated by the high, long lasting titers of neutralizing antibodies achieved after in vivo administration of dextran-containing microspheres with a small amount of alum-adsorbed TT, as compared to the commercial adsorbable tetanus vaccine. These findings suggest that future developments in the area of vaccinology depend on ability to combine a detailed knowledge of the microencapsulation technology with a rational choice of stabilizing excipient or combination of excipients. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Controlled-release; Single-dose vaccines; Microspheres; Tetanus toxoid; Poly(Lactide-co-Glycolide); Protein stabilization

1. Introduction

Although microencapsulation has been used extensively in the pharmaceutical and chemical industries, the technology remains far from being fully developed. As an example, results obtained to date on the idea of controlling the release of therapeutic or antigenic proteins from biodegradable Poly(Lactic acid) (PLA) or Poly(Lactide-co-Glycolide) (PLGA) microspheres are not yet satisfactory. A crucial issue in the development of such formulations is the difficulty of controlling the manner and timing of protein delivery while

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preserving its bioactivity. Future developments in this area greatly depend on the ability to overcome the instability of microencapsulated proteins. In this respect, the main hurdle is related to the complex structure inherent to protein molecules, which make them highly susceptible to physical and chemical instabilities (Squire and Himmel, 1979; Volkin and Klibanov, 1989). Compared to small molecule drugs, whose biological activity can only be lost as a consequence of a chemical modification, in the case of proteins it is also essential to maintain the native conformation of the macromolecule and to avoid aggregation processes (Constantino et al., 1994).

Most of the effort under way to stabilize proteins inside PLGA microspheres is being devoted to antigenic proteins, because of their potential in the development of single-dose vaccines (Aguado, 1993; Khan et al., 1994). In this respect, tetanus toxoid (TT) is one of the most challenging candidates for microencapsulation. However, it has been reported that microencapsulation techniques may create mechanical, thermal and chemical stresses which affect TT stability (Alonso et al., 1994; Gopferich et al., 1994; Chang and Gupta, 1996). In addition, it is known that the moisture and acidic pH of the microenvironment created inside PLGA microspheres during polymer degradation, the PLGA hydrophobicity and reactivity of the corresponding degradation products and, finally, the prolonged exposure at body temperature, provide a harmful microenvironment for the encapsulated proteins in general (Constantino et al., 1994; Lu and Park, 1995). In the particular case of TT it has been observed that the pH and the interaction of the toxoid with the polymer play a key role in the TT inactivation when in contact with PLGA degrading microspheres (Tobío and Alonso, 1998). On the basis of these considerations, it is likely that the development of a single-dose tetanus vaccine will depend largely on the introduction of further advances in formulation procedures. Here, we report the design of new TT-loaded PLGA microspheres containing various stabilizing excipients coencapsulated with TT in the dry solid state.

2. Materials and methods

2.1. Materials and animals

The Poly(D,L-Lactide-co-Glycolide) 50/50 (PLGA), molecular weight 34 KDa (Resomer® RG 503) and 98 KDa (Resomer® RG 506), was purchased from Boehringer Ingelheim (Germany). Purified tetanus Toxoid (TT, M_w 150 KDa, 85–95% monomeric) dissolved in phosphate buffer saline, pH 7.4, was kindly donated by Massachusetts Biologic Laboratories (Boston, MA). Cottonseed oil, soybean lecithin (type IV-S), polyviniylalcohol (M_w 30 000–70 000), trehalose, dextran (M_w 72 200), bovine serum albumin, alginate (M_w 80 000–120 000) and heparin were purchased from Sigma (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, UK), 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 anti-mouse IgG alkaline phosphatase conjugate was purchased from Caltag Laboratories (San Francisco, CA) and the enzyme substrate di-sodium-4-nitrophenyl phosphatase was obtained from Fluka (USA). Poloxamer 188 (Pluronic® F68) was kindly donated by BASF Co. (Parsippany, NJ).

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

2.2. Preparation of PLGA microspheres containing TT

Before microencapsulation, a stabilizing agent (trehalose, bovine serum albumin, alginate, heparin, dextran, poloxamer 188 or sodium chloride) was dissolved in the TT solution (weight ratio stabilizing agent: TT of 10:1) and lyophilized. Afterwards, the lyophilized powder was used to prepare the microspheres. PLGA microspheres with theoretical loadings in TT of 1% (mg of TT/100 mg of microspheres) were prepared using

two different microencapsulation techniques, conveniently modified.

Oil-in-oil (O/O) solvent extraction/evaporation technique: 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 agitation (700 rpm, RW 20 DZM, IKA, Spain). After the system was stirred for 30 min, microspheres were collected by filtration, washed with petroleum ether and lyophilized.

Oil-in-water (O/W) solvent extraction/evaporation technique: The lyophilized powder was suspended in 1 ml of ethyl acetate containing 100 mg of PLGA. This suspension was added dropwise to 50 ml of an aqueous solution of polyvinylalcohol (PVA, 1% w/v) containing 3 ml of ethyl acetate. The system was maintained under magnetic agitation for 30 min to allow the organic solvent to evaporate. After, microspheres were collected by centrifugation, washed three times with distilled water and lyophilized.

2.3. Physicochemical characterization of microspheres

The morphological examination of microspheres was performed using a scanning electron microscope (SEM, JSM-T220A, Jeol, Japan). Samples for SEM were mounted onto metal stubs and coated with gold palladium to a thickness of 200–300 Å.

The particle size distribution of the microspheres 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).

2.4. Determination of the encapsulation efficiency

Three different methods were used:

2.4.1. Extraction method

This method involves the dissolution of the PLGA in an organic solvent, followed by extraction of the entrapped TT into water. Briefly, 20 mg of microspheres were dissolved in 0.6 ml of ethyl acetate with the aid of a vortex. Then, the TT was extracted by three 0.6 ml vol. of PBS containing 0.05% Tween® 20, pH 7.2 (PBST buffer) with the aid of a vortex and subsequent centrifugation. The extracted antigenically active TT was determined by using the ELISA technique described in the In vitro release studies section, and the total protein was determined using the Lowry method (Lowry et al., 1951).

2.4.2. Filtration method

This method involves the dissolution of the PLGA in an organic solvent in which the TT is not soluble, followed by the filtration and recovery of the entrapped TT in a filter. Briefly, 20 mg of microspheres were dissolved in 0.6 ml of ethyl acetate with the aid of a vortex. Then, this organic phase was filtered using a 0.22 μm filter with low protein affinity and previously incubated with a protein (BSA) for 12 h. This filter was subsequently washed three times with PBST buffer and incubated with this buffer under magnetic stirring for 30 min to dissolve the TT. The antigenically active TT and the total protein were determined as previously described.

2.4.3. Digestion method

This method involves alkaline hydrolysis of the microspheres and determination of the TT recovered. Briefly, 10 mg of microspheres were shaken overnight with 10 ml of 5% (w/v) SDS in 0.1 M sodium hydroxide solution (NaOH/SDS). Following centrifugation, the protein content was determined by the Lowry method.

2.5. Microspheres water uptake

Microspheres (30 mg) were suspended in 5 ml of PBS (pH 7.4) containing 0.02% (w/v) Tween® 80 at 37°C for 24 h to measure their water uptake. After they were collected by filtration, weighed immediately (W_1) and dried to a constant weight (W_2). Water uptake of microspheres was calculated using Eq. 1:

$$\text{Water uptake (\%)} = [W_1 - W_2)/W_2] \times 100$$

2.6. 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, Spain). At predetermined time intervals, the samples were centrifuged at $4000 \times g$ for 15 min (Sigma 2-15, Spain), 1.5 ml were removed from the supernatant and replaced by fresh release medium. Total TT release (being antigenically active or not) was determined by the Lowry method. Antigenically active TT release was determined by the enzyme-linked immunosorbent assay (ELISA). Briefly, 100 μl of equine tetanus antitoxin in PBS, pH 7.2 was added to flat-bottom micro-titration plates (Corning, NY) and allowed to incubate overnight. The plates were washed three times between all steps with PBS containing 0.05% Tween® 20, pH 7.2 (PBST buffer). A reference TT preparation and test samples were diluted serially in 2-fold steps in phosphate blocking buffer (PBB, PBS/0.5% bovine serum albumin/0.1% Brij® 35, pH 7.4). The plates were incubated at room temperature for 2 h and washed. Then, 100 μl of human anti-TT IgG (1:10 000 dilution) in PBB were added to the wells and allowed to react for 1.5 h, followed by 100 μl of goat anti-human IgG alkaline phosphatase diluted 1:1000 in PBB for another 1.5 h. The plates were washed and 100 μl of the substrate (di-sodium-4-nitrophenyl phosphate, 1 mg/ml in a 1 M diethanolamine, 0.5 mM $MgCl_2$ buffer, pH 9.8) were added. Following color development (15 min.), plates were read at 405 nm on a microplate reader (3550-UV, Biorad, Spain).

2.7. Immunization protocol

Groups of female mice (6–10 per group) were injected subcutaneously on the left side of the abdomen with a single dose (1.65 μg, 0.55 Lf) of TT-loaded microspheres containing dextran as an stabilizer. The same single dose was administered jointly encapsulated (1.5 μg, 0.50 Lf) and adsorbed onto aluminum phosphate (0.15 μg, 0.05 Lf TT/4.4 μg $AlPO_4$). The same dose of alum adsorbed toxoid (1.65 μg, 0.55 Lf TT/48.7 μg $AlPO_4$) was studied as a control formulation. 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. Blood samples were collected, by cardiac puncture, several times after administration and the sera separated by centrifugation. The mice sera from each vaccine group were pooled and assayed for tetanus antitoxin in terms of antitoxin units (AU) by the toxin neutralization test (Relyveld, 1977; Gupta et al., 1985). 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). Individual mice sera were also evaluated for IgG antibodies to TT by the ELISA test. Briefly, 96-well microtitre plates were coated with 100 μl of purified TT (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-TT mouse serum containing 0.07 μg/ml) and the test samples were diluted serially in 2-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 phosphatase diluted to 1 mg/ml in 1 M diethanolamine, 0.5 mM magnesium chloride buffer was added to plates. The plates were kept to 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.

3. Results and discussion

A large amount of literature on protein formulation has focused upon protein stabilization under typical pharmaceutical conditions including lyophilization, freezing and drying, storage, and rehydration (Constantino et al., 1994). However, in recent years the challenge has been to devise means of preserving the integrity (i.e. stability) of proteins formulated in controlled release systems such as Poly(Lactide-co-Glycolide) (PLGA) microspheres. In fact, nowadays it is becoming clear that the efficacy of these microspheres as protein delivery systems is highly dependent on the stability of the protein during microencapsulation and also in the course of the polymer degradation process (Crotts and Park, 1997). This fenomenon has been particularly investigated in the case of TT becasue of the potential of PLGA microspheres for the development of a single-dose tetanus vaccine (Alonso et al., 1993, 1994). Among the various approaches attempted until now to stabilize TT, we observed that the PLGA coated oily microcapsules provided not only a stabilizing environment for the toxoid but also a pulsatile release behavior (Sanchez et al., 1996). More recently, we showed that the antigenicity of this toxoid could be highly preserved using anhydrous microencapsulation procedures and by including gelatin or poloxamer 188 as stabilizers (Tobío et al., 1999a,b). At the same time, other authors proposed formulation approaches based on the co-encapsulation of additives such as albumin, trehalose and cyclodextrin by the spray-drying technique (Johansen et al., 1998; Audran et al., 1998).

In the present work our objective was to evaluate comparatively the potential of several protein stabilizing agents and two different encapsulation procedures in order to preserve the activity of TT and, hence, elicit an enhanced and prolonged immunogenic response. For this purpose, we selected different kinds of protein stabilizing excipients such as bovine serum albumin (BSA), several saccharides and polysaccharides (trehalose, alginate, heparin and dextran), a nonionic surfactant (poloxamer 188) and a salt (sodium chloride).

With respect to the microencapsulation technique, the water-in-oil-in-water (W/O/W) double emulsion solvent technique has been commonly employed to encapsulate proteins within PLGA microspheres (Alonso et al., 1993). However, it is being increasingly accepted that this technique does not provide a good environment for preserving protein stability. In fact, this technique requires the protein to be dissolved in an aqueous phase and in contact with organic solvents, a situation that is known to cause denaturation followed by aggregation (Volkin and Klibanov, 1989). Thus, considering that proteins are far less reactive in the dry solid state, it was our thought that a solution to this problem could be found in a method of encapsulation which does not expose the flexible protein to an organic solvent. In general, whenever pre-formulation studies indicate that sufficient macromolecule stability cannot be achieved in aqueous liquid formulations, lyophilization provides the most attractive alternative (Constantino et al., 1994). Taking this account, we selected an anhydrous oil-in-oil (O/O) solvent extraction/evaporation technique, in which the toxoid is dispersed in a dry solid state conveniently stabilized. Thus, we first lyophilized TT together with the selected stabilizers in a ratio stabilizer: TT of 10:1. The mass ratio between the stabilizer and TT was decided by taking into account that concentrations exceeding 5% (w/w) are needed to maximize stabilization with some stabilizing agents (Schein, 1990). We judged the success of the stabilization during the lyophilization step by examining the protein antigenicity by

Table 1
Percentage of antigenically active TT remaining after lyophilization with the different stabilizing agents

Stabilizing agent	Antigenically active TT remaining (%)
Dextran	80.5 ± 1.3
Albumin	76.4 ± 2.2
Alginate	57.3 ± 1.5
Trehalose	89.1 ± 1.7
ClNa	68.4 ± 1.9
Poloxamer	77.4 ± 1.6
Heparin	90.9 ± 1.5

ELISA. Results presented in Table 1 indicate that dextran, trehalose and heparin are efficient stabilizing excipients, since 80–90% antigenically active TT remained active after the lyophilization process. On the other hand, these excipients were also effective in stabilizing TT upon exposure to acetonitrile, the most widely used organic solvent in O/O microencapsulation techniques. More specifically, it was found that direct exposure of TT to acetonitrile for 30 min caused insoluble precipitates and an important loss of antigenicity (90% of antigenicity loss, as determined by ELISA). However, following exposure of TT, in dry solid state and stabilized with the above mentioned agents, to acetonitrile only a 25% of antigenicity loss was detected. Consequently, these conditions were selected for further studies.

The general appearance of the microspheres developed by the above mentioned non-aqueous oil-in-oil (O/O) solvent extraction technique is shown in Fig. 1A. Microspheres had the same appearance irrespective of the stabilizer incorporated into them. In addition, the mean size and the behavior of these microspheres in terms of water uptake and release properties was also similar (Table 2) independent of the stabilizer co-encapsulated with TT. The high values of the antigenically active TT released after a one-day incubation period agree well with the water uptake values. In fact, the high burst effect observed for these formulations could be explained by the osmotic effect caused by the stabilizing excipients. It was therefore understood that water entered the microspheres and saturated the TT-stabilizing excipient depots, generating an osmotic pressure which caused them to release the TT content in a burst manner. A similar behavior was previously observed for the gelatin core-coated microspheres prepared by the same anhydrous procedure (Tobío et al., 1999a). From these data it was concluded that these new formulations did not display adequate in vitro release properties, even when the stabilization of the encapsulated antigenic protein was successful.

From the above discussion, it becomes clear that a rational modification of the PLGA microspheres was required to achieve a controlled release of the stabilized antigen. With this purpose in mind, we used a modified oil-in-water (O/W) solvent extraction/evaporation technique, which is simple and, as described in the Section 2 section, allows the processing of TT in the dried solid state and its co-encapsulation with the selected stabilizing excipients.

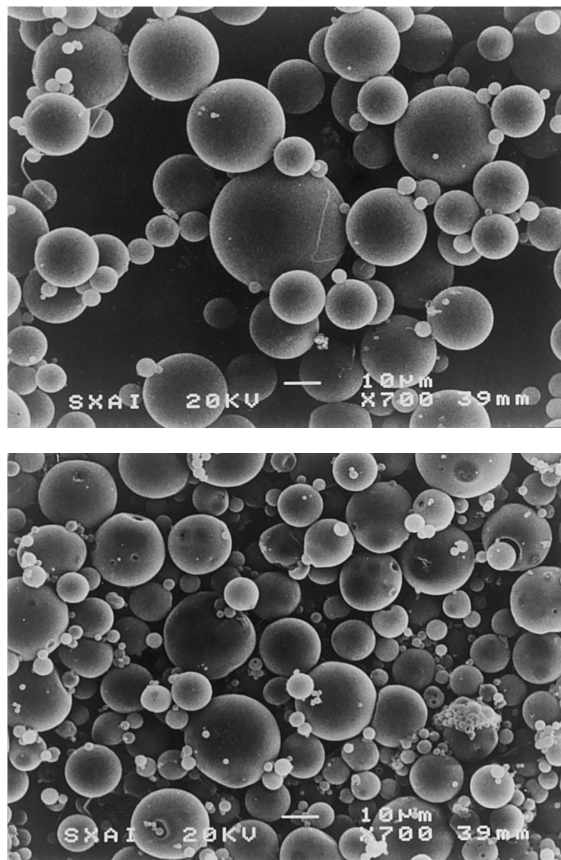

Fig. 1. Scanning electron micrographs of tetanus toxoid-loaded PLGA RG 506 microspheres containing dextran as a stabilizing agent and prepared by an oil-in-oil (A) or an oil-in-water (B) solvent extraction/evaporation technique.

The preliminary step was to select an organic solvent in order to ensure mild microencapsulation conditions. TT in the presence of the stabilizers was readily soluble and showed full antigenic activity following exposure for 5 min to either methylene chloride or ethyl acetate. However, a loss of antigenicity was observed after 30 min, this loss being higher for methylene chloride than for ethyl acetate. Therefore, the exposure of TT to ethyl acetate in the presence of any of the stabiliz-

ers and in the dry solid state was found to be aceptable for the preparation of the microspheres. An additional advantage of ethyl acetate is that it is much less toxic than methylene chloride, and therefore more suitable for the clinical use of a PLGA vaccine.

Fig. 1B and Table 3 show, respectively, the microspheres containing TT stabilized with dextran developed by the O/W technique and the characteristics of these formulations. With respect to their size, it can be observed that microspheres made of high molecular weight PLGA (RG 506) were larger than those made of low molecular weight PLGA (RG 503). On the other hand, the encapsulation efficiency (EE) was lower when using the O/W solvent extraction/evaporation method compared to the O/O method (Table 2). This was probably a consequence of the TT diffusion towards the external aqueous phase during the emulsification step. Similar results were obtained when using trehalose or heparin as stabilizing agents (results not shown). Table 3 also shows the EE values determined either by quantification of total protein or antigenically active protein. Total protein could be determined by a digestion method whereas antigenically active protein could only be determined following the extraction and filtration methods. These data revealed that the most accurate method of determining antigenically active protein is the filtration method. In contrast, the extraction method resulted in an underestimation of the entrapped TT, which was attributed to the concentration of the protein at the water/oil interphase, as previously reported (Sharif and O'Hagan, 1995). Finally, the digestion method is an accurate technique for the determination of the levels of total protein entrapped in PLGA microspheres, however, it leads to the inactivation of the toxoid. Similar results were obtained with trehalose and heparin (results not shown).

The release patterns observed for PLGA RG 506 microspheres (Figs. 2–4) can be interpreted in terms of a biphasic process, as usually described for PLGA microspheres. In the initial phase (first day) a slow penetrating water front dissolves the TT-stabilizing excipient at the surface or in the vicinity of the microspheres surface. After this, the increasing porosity of the microsphere due to the PLGA degradation leads to a slow release of TT and stabilizing excipients into the release medium. An important observation is that the encapsulated antigen is released in the antigenically active form for at least 5 weeks. It should, however, be noted that the percentages of total protein and antigenically active protein released are quite different. This suggests that TT was not fully protected by any of the stabilizers assayed during the in vitro release. Nevertheless, results in Fig. 5 show the important differences in stabilization achieved with the various stabilizers, suggesting that the selection of the stabilizing excipient is a key issue in the preservation of the activity of TT inside PLGA microspheres. As shown in Fig. 5, the stabilizing effect of the saccharides varied depending upon their molecular weight and struc-

Table 2
Microspheres size, percentage of antigenically active TT released after one day of in vitro release, and water uptake during this period of time for PLGA microspheres prepared by the oil-in-oil (O/O) solvent extraction/evaporation method containing different stabilizers ($n = 4$)

PLGA	Coencapsulated stabilizer	Size (μm)	% Active TT released (day 1)	Water uptake (%) (day 1)
RG 503	None	23.14 ± 2.34	51.24 ± 3.27	43.2 ± 2.7
RG 503	Trehalose	23.80 ± 3.73	85.64 ± 5.31	77.7 ± 5.4
RG 503	Dextran	25.17 ± 2.21	84.47 ± 8.86	75.1 ± 5.9
RG 503	Heparin	24.15 ± 2.45	84.40 ± 8.84	74.9 ± 6.0
RG 506	None	29.16 ± 2.89	56.72 ± 2.81	39.2 ± 4.9
RG 506	Trehalose	30.18 ± 3.45	87.32 ± 4.67	79.7 ± 6.3
RG 506	Dextran	32.12 ± 2.31	83.48 ± 6.95	76.9 ± 5.1
RG 506	Heparin	31.45 ± 3.21	78.16 ± 8.48	75.8 ± 6.2

Table 3
Encapsulation efficiency (E.E.) of TT in PLGA microspheres and mean size of these microspheres prepared by the oil-in-water (O/W) solvent extraction/evaporation method containing dextran as a stabilizing agent, as determined by an extraction, filtration or digestion method ($n = 4$)

PLGA	Stabilizer	Mean size (μm)	E.E. (%) Extraction[a,b]	E.E. (%) Filtration[a,b]	E.E. (%) Digestion[a,c]
RG 503	Dextran	16.25 ± 1.42	25.43 ± 2.52	36.43 ± 4.68	39.57 ± 6.36
RG 506	Dextran	24.23 ± 1.50	34.50 ± 6.36	54.59 ± 6.63	67.24 ± 4.51

[a] Methods used to determine the TT E.E., as described in Section 2.
[b] Determined by ELISA.
[c] Determined by Lowry method.

ture. These results could be explained by the release of the stabilizing excipients from the microspheres, which was probably slower for those with a higher molecular weight (dextran and heparin). The molecular weight could also have an influence on the viscosity of the protein solution created inside the microspheres upon contact with an aqueous medium (Schein, 1990). Finally, it should taken into account that non-reductor disaccharides (trehalose) could easily be hydrolyzed in the acidic microenvironment created inside the microspheres thereby producing reductor sugars, which may be harmful for stabilization purposes (Crowe et al., 1987).

Based on the above mentioned results, we selected the PLGA RG 506 microspheres prepared by the O/W solvent extraction/evaporation technique containing TT stabilized with dextran as good candidates to perform an in vivo study. We injected to a group of mice a single dose of PLGA microspheres containing stabilized TT (0.55 Lf), to a second group a mixture of these microspheres (0.50 Lf) and a small amount of alum-adsorbed TT ($AlPO_4$) (0.05 Lf) and to a third group the same total dose of TT as alum-adsorbed TT (0.55 Lf).

Table 4 shows the specific TT IgG antibody levels induced in mice after subcutaneous immunization with the above formulations. Antibody levels achieved for the encapsulated TT were lower than those achieved for the alum-adsorbed vaccine. However, a substantially higher response was attained following the administration of a mixture of microspheres and a very low amount of alum-adsorbed vaccine. In fact, IgG antibody levels elicited by this mixture during the period of time studied (26 weeks) were substantially higher than those obtained for the commercial tetanus vaccine.

In order to evaluate if the developed PLGA microspheres are clinically useful and provide additional advantages over the commercial tetanus vaccine, we decided to use the neutralization titres of tetanus antitoxin as a marker of clinical efficacy and the commercial adsorbable vaccine as a control. Table 5 shows that the neutralization titres of tetanus antitoxin elicited by the mixture of TT containing microspheres plus alum adsorbed TT were up to four times higher than

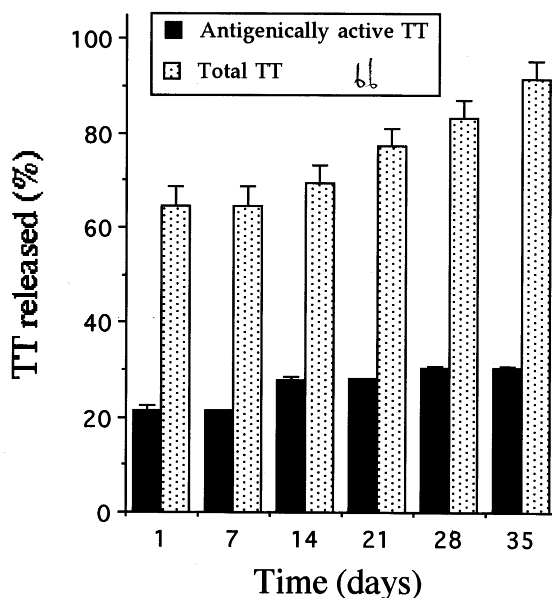

Fig. 2. In vitro release profiles of TT from PLGA microspheres containing dextran as a stabilizing agent.

Table 4
Evolution of geometric mean tetanus toxin IgG antibody levels in mice injected with a single dose of TT encapsulated in PLGA microspheres (TT dose = 0.55 Lf) (TT-PLGA), a mixture of these microspheres (TT dose = 0.50 Lf) and a small amount of TT adjuvanted by aluminum phosphate (TT dose = 0.05 Lf) (TT-PLGA + TT-$AlPO_4$), or the same total dose of TT (0.55 Lf) as the commercial adsorbable vaccine (TT-$AlPO_4$)

Formulation	Dose (Lf)	4 Weeks	6 Weeks	10 Weeks	14 Weeks	20 Weeks	26 Weeks
TT-PLGA (stabilizer:dextran)	0.55	0.005 (0–0.63)	0.08 (0.01–0.66)	0.059 (0.006–0.60)	0.049 (0.006–0.40)	0.1508 (0.08–0.36)	0.03 (0.003–0.306)
TT-PLGA + TT-$AlPO_4$ (stabilizer:dextran)	0.50 + 0.05	1.71 (1.15–2.54)	3.20 (2.62–3.92)	3.16 (2.27–4.39)	2.29 (1.46–3.59)	3.59 (1.05–12.3)	2.12 (0.76–5.96)
TT-$AlPO_4$	0.55	1.00 (0.50–2.10)	1.70 (0.90–2.90)	2.00 (0.60–6.51)	1.10 (0.71–1.60)	1.50 (1.50–2.20)	1.80(0.69-2.32)

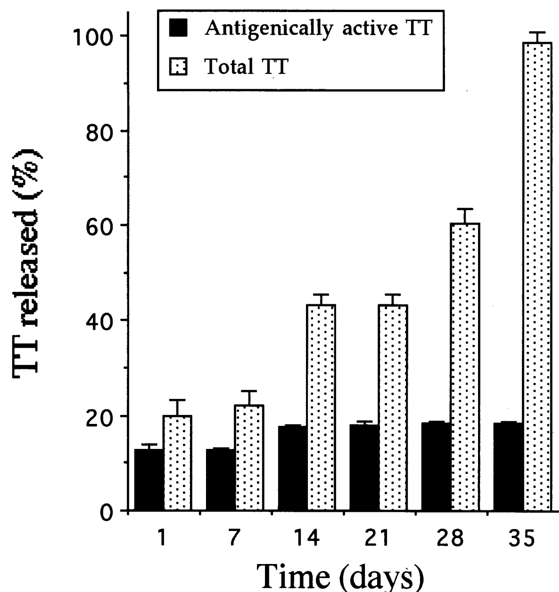

Fig. 3. In vitro release profiles of TT from PLGA microspheres containing heparin as a stabilizing agent.

those elicited by the commercial vaccine. Furthermore, while the titters corresponding to the absorbed vaccine started to decrease at 10 weeks, those achieved for the mixture microspheres/ $AlPO_4$-TT continuously increased for up to 20 or 26 weeks. The same administration protocol consisting of a mixture of encapsulated antigen (0.5 Lf) (no stabilizers were introduced in the formulation) and alum-adsorbed antigen (0.05 Lf) was recently reported by Gupta et al. to elicit a immune response which was comparable to that achieved for the commercial vaccine (Gupta et al., 1998). Singh et al. also showed that the combination of alum adsorbed TT and microencapsulated antigen (no stabilizers were introduced in the formulation) provided the best immune response (Singh et al., 1997). Nevertheless, these previous data cannot be directly compared with those attained in this work because of the different administration protocol (they administered to rats a TT dose of 15 Lf half of which was adsorbed and half of which entrapped in microspheres). On the other hand, Audran et al., reported that the immune response could be enhanced by the co-encapsulation of stabilizers with TT (Audran et al., 1998), however, the response elicited by their for-

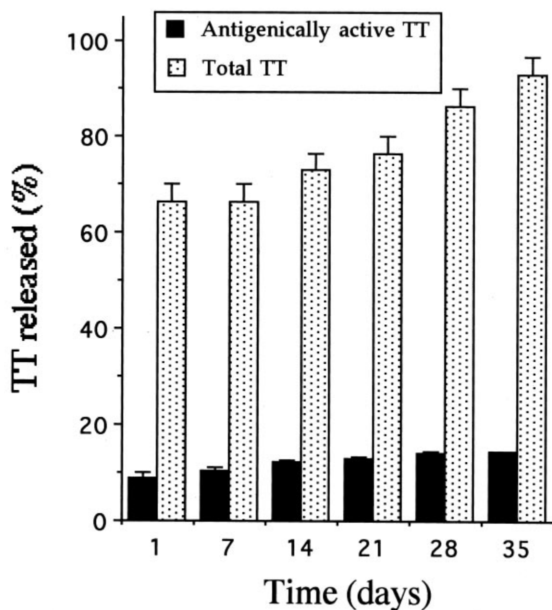

Fig. 4. In vitro release profiles of TT from PLGA microspheres containing trehalose as a stabilizing agent.

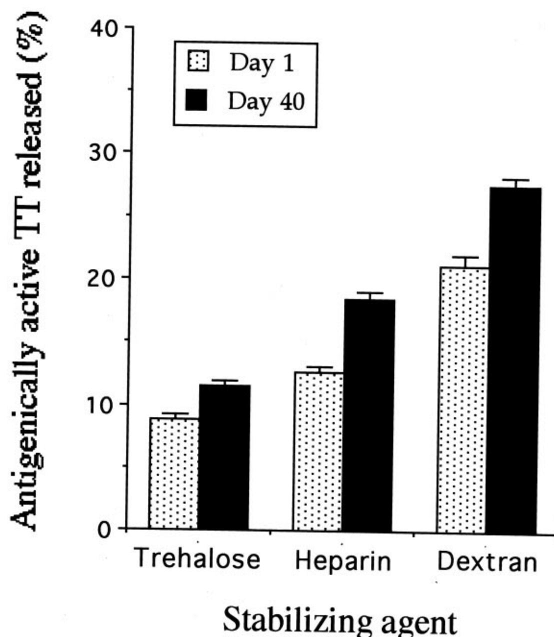

Fig. 5. Antigenically active TT released from PLGA microspheres containing the different stabilizing agents (dextran, heparin and trehalose).

Table 5
Evolution of TT neutralizing titers in mice injected with a single dose of TT encapsulated in PLGA microspheres (TT dose = 0.55 Lf) (TT-PLGA), a mixture of these microspheres (TT dose = 0.50 Lf) and a small amount of TT adjuvanted by aluminum phosphate (TT dose = 0.05 Lf) (TT-PLGA + TT-$AlPO_4$), or the same total dose of TT (0.55 Lf) as the commercial adsorbable vaccine (TT-$AlPO_4$)

Formulation	Dose (Lf)	4 Weeks	6 Weeks	10 Weeks	14 Weeks	20 Weeks	26 Weeks
TT-PLGA (stabilizer:dextran)	0.55	<0.010	0.020	0.012	0.013	0.013	0.012
TT-PLGA + TT-$AlPO_4$ (stabilizer:dextran)	0.50 + 0.05	>0.24	1.00	1.50	1.60	1.60	1.60
TT-$AlPO_4$	0.55	0.12	1.10	1.00	0.50	0.40	0.40

mulations was lower than that observed for the alum-adsorbed TT. Even though it would not be appropriate to compare the immune responses reported in these previous studies, there are two main conclusions derived from them which are both corroborated by the data presented here: first, that the administration of a mixture of microspheres with a minimum amount of alum enhanced immune response and second, that this response can also be improved by stabilizing the encapsulated antigen. The positive effect of the alum-adsorbed TT when administered together with the microspheres could be explained by the ability of aluminium salts to attract macrophages to the site of injection (Gupta et al., 1995, 1998). Therefore, it could be assumed that macrophages phagocyte the microspheres and target them to the immune system, thus leading to an enhanced antibody production.

From the discussion above it is possible to conclude that polysaccharides and, in particular dextran, can stabilize TT inside PLGA microspheres and provide an enhanced and prolonged immune response. In addition, this antigen stabilization approach together with the co-administration of a very small amount of alum-adsorbed toxoid was found to be a good way towards the optimization of the vaccination protocols against tetanus. Furthermore, the formulation approach presented here is based on products which are commercially available and biocompatible and is easily amenable to industrial-scale. Perhaps most importantly, our findings show that a detailed knowledge of the microencapsulation technology combined with a rational choice of stabilizing excipients may lead to the optimization of protein/vaccine delivery systems using PLGA microspheres.

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