Enhanced Immunogenicity of Microencapsulated Tetanus Toxoid with Stabilizing Agents[1]

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Purpose. Antigenic proteins encapsulated in biodegradable polyester microspheres (MS) can slowly denature or aggregate, which results in decreased antigenicity. In this study, we have evaluated the ability of co-encapsulated additives to protect against the loss of tetanus toxoid (TT) antigenicity.

Methods. Antibody responses were analyzed after immunization of mice with TT microencapsulated in the presence of additives (TT-MS-additive).

Results. Immunization with TT-MS-additives gave rise to higher responses than those obtained in the absence of additive. BSA, trehalose, γ-hydroxypropylcyclodextrin and calcium salts preserved the immunogenicity of the incorporated antigen with the highest efficacy. Sustained responses were obtained with mixtures of fast and slowly releasing TT-MS containing BSA plus trehalose or calcium salts.

Conclusions. The selected additives may stabilize the antigen in MS during storage and rehydration in body fluids. Regulated antigen release from MS-based vaccines permits a reduction of the antigen dose and optimization of single-dose vaccine formulations.

KEY WORDS: biodegradable microspheres; poly(D,L-lactide-co-glycolide); tetanus toxoid; vaccine; antibody response; stabilizing agents.

INTRODUCTION

Biodegradable microspheres (MS) of poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) (PLA), currently used as a parenteral drug delivery system, are also effective for vaccination (1,2). After injection, MS are degraded by nonenzymatic hydrolysis to lactic and glycolic acids, and they induce only a minimal inflammatory response (3). *In vitro*, MS have the ability to release the entrapped peptides or proteins over extended time periods at a pulsatile rate, which might be useful for mimicking conventional immunization schedules. Over the past few years, immunostimulating properties of MS have been studied using a variety of proteins (4–12), peptides (4,13–17), inactivated viruses (18,19), and proteins in association with adjuvant or cytokines (20,21), showing that specific antibody, helper and/or cytotoxic T cell responses were readily induced.

A single dose vaccine has recently been developed with tetanus toxoid (TT) encapsulated in controlled release PLA/PLGA MS. *In vitro*, TT was released in a marked pulsatile pattern coinciding with the degradation kinetics of the polymers used (4). In mice, a single injection of these TT-MS elicited strong and long lasting antibody and T cell responses, which were comparable to those induced by alum-adsorbed TT, although no significant second increase of antibody titers was observed (4,7). It was speculated that the absence of boosting effect on the primary antibody levels may be related to antigen instability within the microspheres after exposure to the physiological environment. Polymer degradation is thought to cause accumulation of acidic metabolites in the core of MS and the acidic environment could catalize TT degradation or aggregation. Further, formation of pores in the MS might facilitate penetration of proteases from the extracellular fluids and thereby promote antigen degradation. Thus, it is important to stabilize the antigen in MS to enhance prolonged antigen delivery.

In the present study, various additives were co-encapsulated together with TT into fast releasing MS, and the effect on TT immunogenicity was determined in mice by measuring antibody responses. The putative stabilizers were chosen from a previous comparative *in vitro* analysis of the antigenicity of encapsulated and released TT[1]. These included: (i) the water insoluble pH-buffering salts, calcium carbonate and calcium phosphate, expected to neutralize the acidification in the core of MS during polymer degradation; (ii) the hydrophobic poloxamers L121 and L101, expected to reduce the water uptake into the MS and thereby preventing a hypothetical protease uptake; and (iii) the commonly known protein stabilizing agents cyclodextrins, trehalose and bovine serum albumin (BSA). The MS formulations selected for *in vivo* testing all showed *in vitro* higher ELISA responsive TT encapsulation and release than the TT-MS formulation without co-encapsulated additive.

MATERIALS AND METHODS

Antigens and Polymers

Solutions of tetanus toxoid (TT), provided by WHO (Geneva, Switzerland), were from Pasteur Merieux, F-Marcy L'Etoile (conventionally prepared TT-PTC, lot n°PTC 10005:8500 Lf/ml, 2023 Lf/mg protein nitrogen) and from Massachusetts Health Biological Laboratories, Boston, MA (TT-PST, lot n° PSTxd-20: 1400 Lf/ml, 2102 Lf/mg protein nitrogen). Lf and protein N content of the two sources of toxoids were determined by the respective manufacturers. The latter TT preparation was purified by column chromatography. They were used for microencapsulation and enzyme-linked immunoabsorbent assay (ELISA). TT adsorbed on aluminum hydroxide was also used: TT-PTC-alum (prep. n° 3, 20 Lf/ml, 0.12% aluminum hydroxide, Pasteur Merieux) and TT-PST-alum. TT-PST-alum was obtained by incubating TT-PST and aluminum hydroxide (0.4 Lf/mg alum) for 30min at 4°C. After centrifugation (5 min, 3000 rpm), the TT-alum precipitate was diluted to 4 Lf/ml in PBS before use.

Tetanus toxoid containing microspheres (TT-MS) with various additives were prepared as described by Johansen *et al.*[1] by spray-drying and coacervation methods (4,22). MS charac-

[1] **Associated paper:** Johansen et al. Improving Stability and Release Kinetics of Microencapsulated Tetanus Toxoid by Co-Encapsulation of Additives, this issue, p. 1103.

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NOTATIONS: MS, microspheres; PLGA, poly(lactide-co-glycolide); PLA, polylactide; TT, tetanus toxoid.

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teristics are shown in Table I. The biodegradable polymers used for TT microencapsulation were poly (D,L-lactide-co-glycolide) (PLGA 50:50) and poly (D,L-lactide) (PLA) from Boehringer Ingelheim, Germany. The following additives were coencapsulated: calcium carbonate (CaC) and calcium phosphate (CaP), the poloxamers Synperonic® L121 and L101 (ICI, Wilton, CT), γ-hydroxypropylcyclodextrin (γ-HPCD) and α-cyclodextrin (α-CD) (Wacker Chemie, CH-Liestal), trehalose (Tre) and BSA. Unless specified otherwise, all substances used were from Fluka, CH-Buchs.

Animals and Immunization

BALB/c female mice 8–10 weeks of age (Harlan Nederlands BV, NL-Zeist) were used in all experiments. Mice (4 per groups) received TT-PTC or TT-PST in various formulations by subcutaneous injection at the base of the tail on day 0. The formulations included various types of PLGA 50:50 or PLA MS, with or without co-encapsulated additives, and also alum adsorbed TT. TT-MS were suspended in 5% lecithin solution (Ovothin 170, Lukas Meyer, Hamburg) and TT-alum in PBS in a total volume of 100 μl before injection. Lecithin had no effect on antibody production when injected with antigen alone (7). After immunization, mouse sera were collected at an interval of 4 weeks by tail bleeding. In some cases, a boost injection of 0.2 Lf of TT in alum was performed 6 months after the first immunization. Specific anti-TT serum antibodies were measured by ELISA.

ELISA

ELISA was performed as described previously (23). Fifty microliters of TT in 0.1 M PBS pH 7.4 (10 μg/ml) were introduced into the individual wells of 96-well flat bottom immunoplates (Maxisorb, NUNC, InterMed, DK-Roskilde). After overnight incubation at 4°C, plates were washed three times with PBS-Tween 20 (0.05%) (PBS-T) and saturated with 200 μl PBS-T 5% (w/v) skimmed powdered milk (PBS-m) for 1 h at 37°C. Fifty microliters of mouse sera diluted in PBS-T were then added, and plates incubated for 2 h at 37°C. After four washes, 50 μl of a 1/4000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma, St Louis, MO) in PBS-m were added per well.

After 1 h incubation at 37°C, plates were washed four times. Then, the substrate o-phenylenediamine dihydrochloride (Sigma) was added in 50 μl of citrate buffer of pH 5.0 containing 1 μl/ml H_2O_2. The enzymatic reaction was stopped with 20 μl of 2 M H_2SO_4, and plates were read at 492 nm with Microtiter reader (MR 5000, Dynatech Produkte AG, CH-Embrach-Embraport). The antibody titer was expressed as the reciprocal of the lowest negative dilution. International Units (IU) were determined using a mouse standard anti-TT antibody from the National Institute for Biological Standards and Control (Hertfordshire, U.K.).

RESULTS

The immunization study was divided into three sets, in which mice received a single subcutaneous injection of various

Table I. Characteristics of TT-MS Containing Various Additives

TT-MS preparation	Additive		Mean particle size (μm)[d]	TT loading (Lf/mg MS)[a]
	Type	Amount % (w/w)		
a: TT-PTC + additives				
TT-MS-Tre	trehalose	15	n.d.	0.84
TT-MS-γ-HPCD	γ-hydroxypropylcyclodextrin	n.d.	n.d.	0.81
TT-MS-αCD	α-cyclodextrin	15	n.d.	0.69
TT-MS-L121	poloxamer L121	20	n.d.	0.62
TT-MS-L101	poloxamer L101	20	n.d.	0.45
TT-MS		—	n.d.	0.67
b: TT-PST + additive				
TT-MS-CaC	calcium carbonate	15	9.7	0.18
TT-MS-Tre	trehalose	20	6.8	0.30
TT-MS-γ-HPCD	γ-hydroxypropylcyclodextrin	20	n.d.	0.23
TT-MS-L121	poloxamer L121	22	n.d.	0.25
TT-MS-BSA/2	BSA	5	8.5	0.70[b]
TT-MS-BSA/1	BSA	1	8.2	0.70[b]
TT-MS-CaP	calcium phosphate	15	n.d.	0.11
TT-MS	none	—	7.2	0.20
c: TT-PST + additive mixture				
TT-MS-BSA/Tre	BSA + trehalose	5 + 15	n.d.	0.42[c]
$TT\text{-}MS_{hyd}$-BSA/Tre[e]	BSA + trehalose	5 + 15	n.d.	0.84[c]
$TT\text{-}MS_{sl}$-BSA/CaC[f]	BSA + calcium phosphate	5 + 15	n.d.	0.55[c]

Note: n.d.: not determined.
[a] TT loadings were measured by fluorescence spectroscopy.
[b] Theoretical loading, as the co-encapsulated BSA interfered with the fluorimetric measurement.
[c] TT loading was measured by ELISA.
[d] Particle size was determined by laser light scattering.
[e] MS_{hyd} were made of a hydrophilic form of PLGA 50:50 (Resomer RG502H).
[f] MS_{sl} are slow release MS made of PLA.

TT preparations (Table I, series a, b and c). In all groups, MS preparations were well tolerated. Neither inflammation nor granuloma were observed at the injection site.

Immunization with PLGA 50:50 MS Containing TT-PTC and an Additive

In the first set of experiments, mice (4 per group) received 20 μg of TT-PTC as previously performed corresponding to 6.5 Lf TT. TT-PTC, a tetanus toxoid used in commercial vaccine formulations, was incorporated in MS containing various additives (Table I-a) or adsorbed on alum (TT-PTC-alum). Immune responses were evaluated by measuring anti-TT antibody titers in individual sera for 5 months after immunization. Specific antibody responses were obtained in each group except for the control group that received MS-empty (data not shown). Responses induced in the TT-MS group were as high as those observed with TT-PTC-alum. In general, additives in MS failed to enhance the antibody response. Lack of any potentiating effect of additives might be related to the high dose of injected TT-PTC, which already induced nearly maximal response.

Therefore, further experiments were then designed with lower TT doses to induce suboptimal responses in control TT-alum groups. In addition, chromatography column purified TT was used which represented a more homogenous antigen preparation and was considered to be used for further human and animal studies.

Immunization with PLGA 50:50 MS Containing Low-Dose TT-PST and an Additive

In the second series of immunization, mice received 0.2 or 1.0 Lf of the purified TT-PST incorporated in MS containing various additives (Table I-b). Two groups of mice were immunized with the equivalent dose of TT-PST-alum as positive control.

The anti-TT antibody titers in individual sera were determined regularly for six months after immunization. Specific antibody responses were detected in each group. The geometric mean of individual titers of each group are shown in Fig. 1. Responses peaked at one to two months after immunization and declined thereafter. Importantly, the level of the response depended on the co-encapsulated additive and the dose of antigen injected.

Interestingly, 0.2 and 1 Lf doses of TT-MS-Tre, TT-MS-BSA/2 and TT-MS-γ-HPCD induced early antibody responses (1 and 2 months after immunization) similar to those obtained with TT-alum. Moreover, antibody titers remained almost stable over time in the groups that had received 1.0 Lf of TT-MS-CaC and TT-MS-CaP. In fact, after the initial peak, the antibody response reached a plateau level 3 months after immunization. While similar responses were obtained with 0.2 and 1.0 Lf of TT-PST in the groups MS-TT-Tre, MS-TT-BSA/2 or MS-TT-γ-HPCD, the groups of MS-TT-CaC, MS-TT-L121, MS-TT-BSA/1, MS-TT-CaP or MS-TT showed lower responses with the lower dose. On the other hand, antibody titers from TT-alum groups were stable over the entire period studied.

To evaluate the effect of stabilizing agents on the relative TT-PST-MS antigenicity, antibody titers from TT-PST-MS-additive groups were compared to those from the control groups injected with TT-PST-MS without additive. Calculated titer

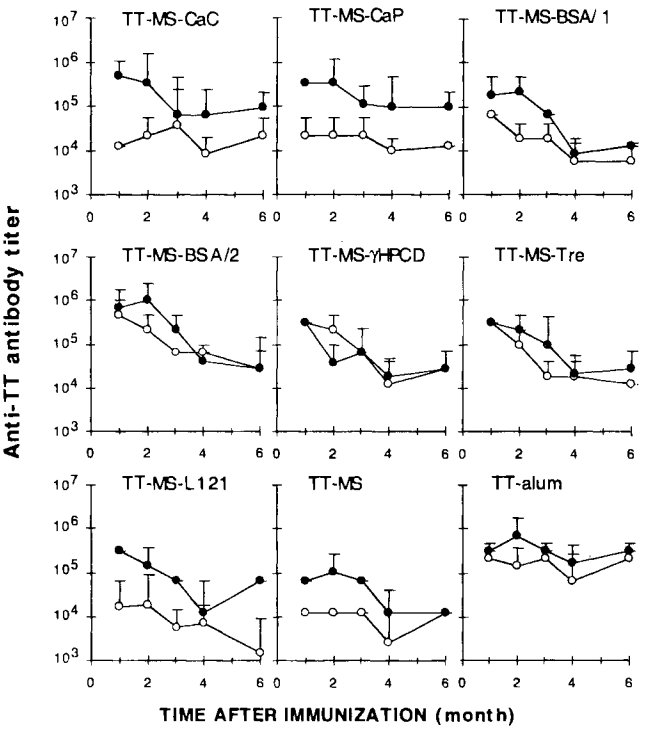

Fig. 1. Time course of antibody responses in BALB/c mice immunized with TT-PST-MS containing various additives. For each formulation, TT-MS or TT-alum, groups of four mice received one dose of 0.2 (○) or 1.0 (●) Lf of TT-PST. Immunizing doses were calculated from the values reported in Table I. Specific antibody responses at 1, 2, 3, 4 and 6 months are expressed as the geometric mean of titers obtained in each group.

indices are shown in Table II. In general, for the same dose of TT-PST, immunization with TT-PST-MS-additive induced higher antibody responses than the MS without stabilizer (indices varied between 0.9 for TT-MS-L121 to 15.8 for TT-MS-BSA/2). All groups considered, an averaged 4.8 fold increase in antibody titer was observed, but the effect was more marked with the low dose of TT as compared to the higher dose (average index of 3.4).

The effect of co-encapsulated additives was further analyzed by comparing the titers of the low dose TT-PST-MS-additive groups to that of the high dose TT-PST-MS (with or without additive) groups (Table II, index[b]). The determined titer index was clearly greater than unity in the groups having been injected with TT-PST-MS containing Tre, γ-HPCD or BSA/2. Thus, because of the presence of these additives, the 0.2 Lf MS induced a higher response than 1.0 Lf formulations without stabilizers.

To examine the boost effect, all groups of mice received 0.2 Lf of TT-PST-alum 6 months after the first injection. Figure 2 shows the antibody responses obtained before and 2 and 7 weeks after the boost injection. In all groups, the booster effect was most pronounced with antibody titers increasing from a range of 0.3–3.1 IU/ml before to a range of 22–224 IU/ml after the boost. The average increase was 103 ± 92 fold in TT-PST-MS and 12 ± 8 fold in TT-alum groups. Moreover, the response persisted for at least 7 weeks after the boost injection. Finally, for the same first immunization dose, boosting responses reached similar high level in TT-PST-MS and TT-PST-alum

Table II. Evaluation of the Efficacy of Additives in TT-PST-MS on the Antibody Response in BALB/c Mice

TT-PST formulations	Titer index		
	0.2 Lf-additive[a]	1.0 Lf additive[a]	0.2 Lf additive[b]
	0.2 Lf	1.0 Lf	1.0 Lf
TT-MS-CaC	1.9	3.9	0.4
TT- MS-Tre	8.7	2.6	1.8
TT-MS-γ-HPCD	11.9	1.8	2.4
TT-MS-L121	0.9	2.3	0.2
TT-MS-BSA/2	15.8	7.9	3.2
TT-MS-BSA/1	2.1	1.8	0.4
TT-MS-CaP	1.6	3.6	0.3
TT-alum	15.8	7.0	15.8
	6.1[c]	3.4[c]	1.3[c]

The efficacy of the presence of additives in MS was expressed as:
[a] ratio between the mean of the antibody titers (months 1 to 6) of each TT-PST-MS-additive group and the mean of the antibody titers (months 1 to 6) of the TT-MS (without additive) groups, for both doses of TT-PST injected (0.2 or 1.0 Lf).
[b] ratio of the titers of the 0.2 Lf TT-PST-MS-additive groups over that of the 1.0 Lf TT-MS (without additive) group.
[c] mean index for TT-PST-MS-additive groups.

groups, suggesting that almost maximal responses were obtained in these experimental conditions.

Immunization with Different MS Types Containing TT-PST and an Additive Mixture

Having determined that BSA and trehalose increased the encapsulation efficiency of antigenic TT and presented higher responses at later time points than TT in MS without additives, the next step was to prepare fast and slow release MS to obtain high and sustained antibody responses throughout the time course of investigation. For this purpose, TT was encapsulated together into three different polymer types, i.e., the conventional end-group esterified (capped) PLGA 50:50 used previously, a more hydrophilic (uncapped) PLGA 50:50 carrying free hydroxyl and carboxyl end groups, which hydrolyse very fast, and a high molecular weight less hydrophilic PLA, which degrades slowly (4). BSA and trehalose were co-encapsulated into the capped and uncapped PLGA 50:50 MS. For the slow release PLA MS, we speculated that co-encapsulation of a pH stabilizer was more appropriate. Therefore, in the last series of immunization, mice received TT-PST incorporated in three types of MS: fast releasing capped PLGA 50:50 MS containing BSA and trehalose as additives (TT-MS-BSA/Tre), very fast releasing hydrophilic uncapped PLGA 50:50 MS (TT-MS_{hyd}-BSA/Tre) and slowly releasing PLA MS containing BSA plus calcium carbonate (TT-MS_{sl}-BSA/CaC) (Table I-c). Four groups received 0.2 or 1.0 Lf TT-PST in mixtures of very fast and slowly releasing MS, or in fast and slowly releasing MS. Two other groups received 0.2 or 1.0 Lf TT in the single PLGA 50:50 and two control groups were injected with 0.2 or 1.0 Lf of TT-alum. Immune responses were analyzed over a period of 10 months (Table III). Elevated early antibody responses were detected in all groups except that injected with 0.2 Lf TT-MS-BSA/Tre. In the groups immunized with 0.2 Lf of TT-MS, the mixtures gave better results than TT-MS-BSA/Tre alone. In the groups immunized with 1.0 Lf TT-MS, only minor differences in the antibody response were detectable in the first 6 months. However, 10 months after immunization, the group immunized with TT-MS_{hyd}-BSA/Tre + TT-MS_{sl}-BSA/CaC mixture showed a more sustained antibody response than in the group immunized with TT-MS-BSA/Tre + TT-MS_{sl}-BSA/CaC ($p < 0.05$). Nonetheless, the slow release MS in the two mixtures did not induce a boost antibody response by a delayed antigen release. Finally, the mixture of very fast and slowly releasing MS provided best long term immune responses, similar to those obtained with TT-alum.

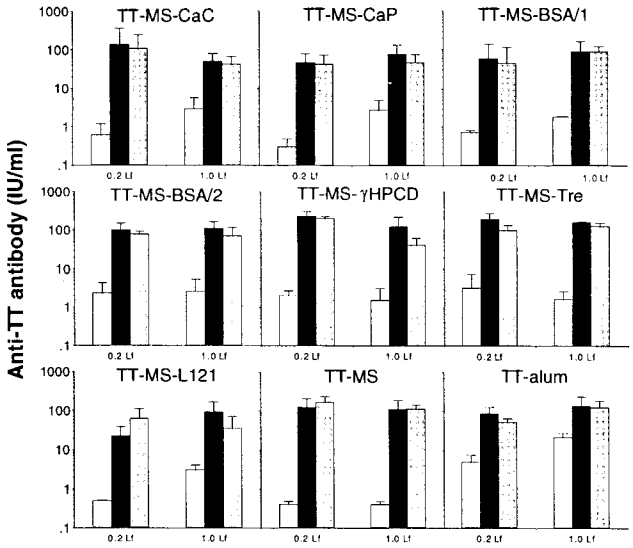

Fig. 2. Booster effects in BALB/c mice immunized with TT-PST-MS containing various additives. Mice were injected first with 0.2 or 1.0 Lf of TT-PST in various formulations and then, all were boosted with 0.2 Lf TT-PST- alum 6 months later. Immunizing doses were calculated from the values reported in Table I. Mouse sera were collected before (□) and 2 (▦) and 7 (■) weeks after the boost injection. Results are expressed as arithmetic mean IU/ml ± s.d.

DISCUSSION

When encapsulated proteins are exposed to extracellular fluids at 37°C for a long time period, denaturation or aggregation may occur, possibly by a result of an acidic environment due to polymer hydrolysis. These changes could decrease antigenicity (24). In this study, we have evaluated the effect of co-encapsulating various additives in TT containing MS on the specific antibody response. The additives used are known for their protein or pH stabilizing properties. We found that immunization with TT-PST-MS-additives gave rise to antibody responses higher than those obtained in the absence of stabilizer and that the presence of additives elicited at least equivalent antibody responses with a 5-fold lower dose of TT-PST. Further experiments showed that sustained responses were obtained with mixtures of fast and slowly releasing TT-PST-MS-additives.

The additives calcium carbonate (CaC) and calcium phosphate (CaP) showed similar effects, i.e. increasing the response shortly after immunization and sustaining the antibody response for the following three months. It has been reported (25) that the intensity of initial antigen burst depends on the antigen loading, because of the large amount of protein located near the MS surface and, consequently, available for initial release. The low antigen loading in these TT-MS-CaP and TT-MS-CaC

Table III. Antibody Response in BALB/c Mice Immunized with Mixtures of Fast and/or Slowly Releasing TT-PST-MS Containing Additive Mixture

TT-PST-MS mixtures		Anti-TT antibody					
		0.2 Lf			1.0 Lf		
Components	% (w/w)	month 1	month 6	month 10	month 1	month 6	month 10
TT-MS_{hyd}-BSA/Tre	*50%*						
TT-MS_{sl}-BSA/CaC	*50%* (0.66)[a]	7.42 ± 1.54	1.24 ± 0.36	0.99 ± 0.17	9.18 ± 8. 18	2.59 ± 1.85[b]	2.17 ± 0.39[b]
TT-MS-BSA/Tre	*60%*						
TT-MS_{sl}-BSA/CaC	*40%* (0.47)[a]	7.79 ± 9.41	1.13 ± 0.13	0.47 ± 0.16	6.13 ± 0.61	0.69 ± 0.41	0.45 ± 0.27
TT-MS-BSA/Tre	*100%* (0.42)[a]	1.43 ± 0.77	0.12 ± 0.09	0.04 ± 0.01	12.5 ± 5.14	1.58 ± 0.97	0.61 ± 0.21
TT-alum	—	12.0 ± 4.65	3.50 ± 1.07	1.81 ± 1.00	11.5 ± 1.57	3.66 ± 1.91	4.69 ± 2.79

Note: Mice (4 mice per group) received one injection of 0.2 or 1.0 Lf of TT-PST-MS in 3 different formulations (Table I) or the same dose of TT-PST-alum as positive control. Specific antibody responses at months 1, 6 and 10 after immunization are expressed as the arithmetic mean of individual antibody international units ± s.d.

[a] TT content in the mixture (Lf TT/mg Ms).

[b] significant difference with the corresponding values of the (TT- MS_{hyd}-BSA/Tre + TT-MS_{sl}-BSA/CaC) group ($p < 0.05$.)

(0.11 and 0.18 Lf/mg, respectively, Table I), as determined by fluorimetry, may explain the modest immunogenicity observed when the low dose of 0.2 Lf was injected. The sustained action of MS containing the calcium salts may be due to the availability of the intact antigen during MS degradation. We can assume that the stabilization of the antigen structure results from the buffering action of the calcium salts and the prevention of acid catalized polymer degradation.[1]

Interestingly, addition of BSA, trehalose and cyclodextrin was associated with higher antibody responses, especially in the early phase, since the resultant antibody titers were at least equal or even superior to those obtained with TT-alum (Fig. 1). Moreover, these additives were particularly efficient since even the low antigen dose (0.2 Lf) induced high antibody responses (Table II, titer index). TT-MS- BSA released *in vitro* more antigenic TT than MS without BSA.[1] Chang and Gupta previously showed that serum albumin reduced the water content in MS, which might stabilize the antigen in MS during storage (26). On the other hand, γ-HPCD and trehalose did not preserve encapsulated TT antigenicity, as determined by ELISA after TT extraction from MS.[1] However, these two oligosaccharides yielded an increase in TT loading efficiency and mediated the highest *in vitro* burst release, which could therefore explain their *in vivo* efficacy to induce high antibody responses. Thus, BSA and trehalose were subsequently co-encapsulated together in PLGA MS. The combination of BSA and trehalose did not lead to a synergistic effect on TT-PST encapsulation efficiency in PLGA-MS, but the immune response appeared to remain more stable with TT-MS-BSA/Tre (Table III) than with TT-MS-BSA/2 or TT-MS-Tre throughout the time of investigation (Fig. 1). In addition, these two additives exerted a synergistic effect on the TT-PST loading efficiency in hydrophilic PLGA MS.[1] This efficient TT loading was accompanied by an increase in antibody response as compared to the response induced with TT in standard PLGA MS-BSA/Tre.

Among the additives used in this study, L121 is an anionic blockcopolymer surfactant which stimulates the humoral response through complement activation (27) and by enhancing the ability of macrophages to present antigens to T cells (28), and preferentially induces a Th_1 response (29). Despite these properties, no enhancement in antibody response could be detected by the co-encapsulation of L121 in TT-MS, at least under the experimental conditions used.

Importantly, an efficient and sustained boost effect was achieved after injection of a low dose of TT-alum 6 months after the first immunization. Despite the differences in antibody levels in the various groups prior to injecting the booster dose, comparable boost responses were obtained in all TT-MS and TT-alum groups. These results suggested the importance of the TT preparation on the quality of the immune responses. Previous TT-MS immunization schedules (7) did not induce such high concentrations of anti-TT antibodies after boosting injection with TT-alum (10–20 IU/ml with a standard TT versus 22–224 IU/ml with the TT-PST used here). In our previous study, a standard TT of commercial source was used, which contained 85 Lf/mg protein, whereas the TT-PST used here contained 333 Lf/mg protein. So, a lower amount of contaminants in TT preparations may improve the specificity of the response, by avoiding the formation of antibodies against contaminant proteins. In addition, for TT-PST preparation, immune responses were enhanced by the presence of additives (Table II).

These data suggest that a chromatographically purified TT and the mixture of very fast and slowly releasing MS represent key elements for a single injection vaccine. Immunization with such a preparation (TT-MS_{hyd}-BSA/Tre + TT-MS_{sl}-BSA/CaC, Table III) induced indeed a high and long lasting antibody response even with a low TT-PST dose. Our results also indicate that the additives BSA, trehalose, γ-HPCD and calcium salts, in combination with the use of hydrophilic PLGA 50:50, improved the *in vitro* release of antigenic TT. Combination of hydrophilic PLGA, BSA and trehalose achieved the highest loading of TT-PST and, in combination with slow release MS, the highest level of antibody titer and IU 6 and 10 months after immunization. As the selected additives may stabilize the antigen in MS during storage and rehydration in body fluids, their use raises the possibility of decreasing the antigen dose

in MS-based vaccines. However, the presence of additives in MS was not essential for eliciting a strong secondary response by a booster injection. In conclusion, our data suggest that it is important to concentrate on the design of delayed delivery MS preparations containing additives and capable to mimic a booster immunization 3 to 6 months after the first inoculation. Since a low dose of TT was sufficient to induce a high and sustained boost response, additives may be essential in the manufacturing of delayed delivery particles because of the need to protect the antigen against moisture during a long-lasting latent period prior to antigen release. The combination of some of these stabilizing agents and the use of PLGA/PLA MS mixtures with various antigen delivery profiles may allow the optimization of single-dose vaccine formulations.

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