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Tetanus toxoid microspheres consisting of biodegradable poly(lactide-co-glycolide)- and ABA-triblock-copolymers: immune response in mice

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Abstract

Tetanus toxoid (TT) was microencapsulated using poly(lactide-co-glycolide) (PLGA) with molar compositions of 50:50, 75:25 or an ABA-triblock-copolymer consisting of PLGA A-blocks attached to a central polyoxyethylene-B-block with a W/O/W (water/oil/water) double emulsion technique. The TT microspheres (MS) were evaluated with respect to protein integrity during antigen release in-vitro and compared with aluminum-adsorbed TT in a mouse model for in-vivo induction of tetanus-specific antibodies as well as protection against a subcutaneous tetanus toxin challenge. The more hydrophilic ABA-triblock-copolymer protected the TT against the deleterious microenvironmental conditions in the degrading MS and provided a prolonged antigen release. In spite of the distinct differences in the in-vitro release patterns MS from PLGA and ABA-triblock-copolymer did not show significant differences in the in-vivo induction of tetanus-specific antibodies. Both preparations elicited antibody titers nearly as high as conventional aluminum-adsorbed TT, which lasted for 29 weeks and were protective against a challenge with $100 \times LD_{50}$ tetanus toxin. TT-MS boosted mice which were preimmunized with aluminum-adsorbed as well as with microencapsulated TT. TT-MS are suitable candidates for single shot vaccine delivery systems which elicit a long lasting and protecting immune response.

Keywords: Tetanus toxoid; Biodegradable microspheres; Polyesters; Single-shot vaccine

1. Introduction

During the last two decades efforts were made to improve conventional vaccines based on adsorbates. Aluminum adsorbate vaccines have been reported to induce inflammation or granulomas formation (Goto et al., 1993; Kissel and Koneberg, 1996). Moreover, adsorbate vaccines are poor adjuvants for viral antigens (Allison and Byars, 1991) and often require multiple injections to achieve protective immunity (Gupta and Siber, 1995; Cox and Coulter, 1997). A typical example for an antigen adsorbed to aluminum is Tetanus toxoid (TT). Tetanus, a fatal bacterial disease

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whose clinical manifestations occur as a consequence of the release of a toxin by *Clostridium tetani*, can be prevented most efficiently by toxin neutralizing antibodies generated by an active immunization with TT. In developing countries the failure of vaccination programs is often attributed to a high dropout rate after the primary immunization. An improved TT vaccine would have great relevance for those countries where according to the World Health Organization (WHO) about 50 0000 children die annually from neonatale tetanus. A 'single-shot vaccine' for tetanus, which releases the three antigen doses, necessary for protective immunity at month 0, 1 and 12 after one single injection of a controlled release antigen delivery system would be of considerable interest.

Different antigens were microencapsulated using mainly biodegradable polymers (Powell, 1996; Kissel et al., 1997; Gupta et al., 1998). Frequently poly(lactide-co-glycolide) (PLGA), a non-immunogenic polyester with long safety record (Chandrasekaran et al., 1996; Chaudhury et al., 1996; Gupta et al., 1997; Johansen et al., 1999) was used. The degradation under in-vitro and in-vivo conditions is mainly controlled by the polymer composition and molecular weight (Thomasin et al., 1996). New polymers were synthesized which could be advantageous for protein delivery (Tobio et al., 1999), e.g. ABA-triblock-copolymers (Li and Kissel, 1993). A more hydrophilic matrix material is thought to provide more favorable conditions for antigen stability in the degrading MS avoiding an acidic microenvironment (Chang and Gupta, 1996; Xing et al., 1996b; Johansen et al., 1998).

Aims of this study were the preparation and characterization of TT loaded microspheres (MS), consisting of a new class of matrix material, the definition of suitable in vitro release conditions and the in vivo evaluation of those new TT-MS.

Therefore, a polymer with ABA-structure was used. Hydrophilic Polyoxyethylene (PEO) (B-region) domains are introduced into hydrophobic esters, like PLGA (A-region). After assessment in vitro, MS consisting of ABA-triblock-copolymer and PLGA were compared with conventional aluminum-adsorbed TT in vivo. Moreover, mixtures of fast and slow degrading TT-MS consisting of different PLGAs were studied in mice to evaluate the concept of a single-shot vaccine delivery system. The potential of microencapsulated TT to induce a booster reaction was also investigated.

2. Materials and methods

2.1. Materials

PLGA 50:50 (Mw 41 000 g/mol, type RG 503®) and PLGA 75:25 (Mw 61 000 g/mol, type RG 755®) were purchased from Boehringer (Ingelheim, Germany). The ABA-triblock-copolymer (38 mol% polyoxyethylene, lactide: glycolide ratio 4:1, Mw 23 000 g/mol) was synthesized as previously described (Li and Kissel, 1993). Poly(vinyl alcohol) (Mowiol ®1888), PVA, and bovine serum albumin (Boviserin ®) were supplied by Aventis Pharma AG (Frankfurt/Main, Germany). TT solution (21.35 mg protein per ml, specific activity: 3700 Lf/ml), TT standard (TTB 6), aluminum-adsorbed TT (Tetanol ®), tetanus toxin solution, purified anti-tetanus-rabbit-serum (No 8883, dilution 1:10 000 in PBS, pH 7.4), anti-human-IgG alkaline phosphatase conjugate and ELISA chromogen (*p*-nitrophenylphosphate) were kindly provided by Chiron–Behring, Marburg, Germany). Assay kit for total protein determination was supplied from Pierce (USA). All other chemicals purchased from Sigma were of analytical quality.

2.2. MS preparation

Microspheres were prepared by a modified W/O/W double-emulsion-technique (Bodmer et al., 1992; Kissel et al., 1996). Briefly, 400 μl of an aqueous TT solution (7.5 mg protein per ml) were emulsified in 5.6 g of an organic phase, containing the polymer (10% (w/w) in dichloromethane (DCM)). Using a rotor/stator-homogeniser (Ultraturrax 18/10 Jahnke & Kunkel, Staufen, Germany) at 20 000 rpm for 2×30 s the W/O emulsion was prepared and immediately injected with a syringe (18 G needle) in 400 ml of an aqueous PVA solution (0.4% (w/w)). After emulsification with a rotor/stator-homogeniser (Ultra-

turrax T25, Jahnke & Kunkel, Staufen, Germany) at 8000 rpm for 1 min, the system was maintained under magnetic stirring for 3 h to allow solvent evaporation. MS were collected by filtration, washed with distilled water, freeze-dried and stored at 4 °C.

2.3. MS characterization

Surface morphology and particle size of the MS were investigated by scanning electron microscopy (SEM, Hitachi S 510, Tokyo, Japan) and by laser diffractometry (MasterSizer X, Malvern Instruments, Malvern, UK). To determine TT loading and encapsulation efficiency a defined amount of MS was dissolved in 2 ml of 1 N NaOH and analyzed spectrophotometrically at a wavelength of 280 nm. Concentrations were determined from calibration curves. Each sample was run in triplicate.

2.4. TT stability during in vitro release

Solutions of TT (210 μg/ml) in water, phosphate buffers (PB pH 7.2 and PB pH 8.0) or isotonic phosphate buffer saline (PBS pH 7.2) were placed in a rotating metal block (5 ml solution in 10 ml vialsRotatherm, Liebisch, Germany) at 2 or 30 rpm and analyzed by ELISA. In order to investigate the effect of the filling level of the release vials (Pyrex Culture Tubes 10 ml) were filled with 2 or 10 ml of TT solutions and treated as described above. Solutions of 2 ml TT (105 μg/ml) In PB pH 7.2 (0.078 M) were incubated at 37 °C without movement (turned over once a day by hand) or in an horizontal shaker (1 Hz). Samples were drawn at day 0, 1, 2, 4 and 9. 50% were analyzed directly by ELISA, the other one centrifuged (12 000 rpm) and analyzed as described above. Table 1 summarizes the stability of TT during different in-vitro dissolution conditions.

2.5. In-vitro release studies of TT from microspheres

TT release studies were conducted by incubation of 30 mg MS in 1 ml PBS pH 7.2 at 37 °C without any movement only turning them over once a day by hand. Buffer was completely exchanged every 2–4 days and assayed for total protein content using a BCA-assay and ELISA-activity as described below.

2.6. Total TT amount by bicinchonic acid protein assay

Total amount of TT was determined by bicinchonic acid (BCA) assay. Therefore, 50 μl samples or standards (0–50 μg/ml TT) were added to each well in 96-well-plates (microtiter™, 2 × 8, round bottom, NUNC, Germany). Bicinchonic acid (solution A Pierce, USA) and copper ions (solution B Pierce, USA) were mixed (1:50) immediately before use and 200 μl were added to each vial and mixed again. The plates were kept at 60 °C for 1 h and absorbance was read at 570 nm wavelength with the EIA3-plate-reader (Titertek Plus MS 212, Titertek, Germany). Each unknown sample was run in duplicate together with a standard curve in duplicate. The concentration of total protein was determined from standard curves after plotting absorbance versus the concentration, by regression analysis.

2.7. TT-enzyme-linked-immunosorbent-assay

2.7.1. Evaluation of the activity of TT in in-vitro experiments

Ninety-six-well-plates with Nunc-Immuno™ modules (maxisorp™, 2 × 8, round bottom, NUNC GmbH, Wiesbaden), were coated with 60 μl (1:3000) purified anti-tetanus-rabbit-serum per well and incubated overnight at 25 °C. The plates were then washed three times with PBS (pH 7.4, 0.15 mol) containing 1% (w/w) Tween™ 20 between each step. 100 μl samples or standards were added in appropriate dilution to each well (standards from 0.08 to 2.5 μg/ml TT). The plates were kept at 37 °C for 1 h and washed three times again. Then 100 μl per well (0.11 IU/ml) human anti-tetanus IgG were added. After incubation for 1 h and washing, 50 μl anti-human-IgG alkaline phosphatase conjugate were added to each well (1:80). The plates were again incubated at 37 °C for 1 h. The plates were washed. Finally ELISA

Table 1
Stability of TT during different in-vitro dissolution conditions

Conditions	Durability of total protein [days][a]				Durability of ELISA active protein [days][b]			
Centrifugations	−		+		−		+	
Movements	None	Horizontal shaking	none	Horizontal shaking	None	Horizontal shaking	None	Horizontal shaking
Phosphate buffer pH 7.2	4	4	2	0	2	3	2	0
Phosphate buffer pH 7.2+0.44% NaCl	4	–	4	1	4	2	2	1
Phosphate buffer pH 7.2+0.1% Tween 20	2	4	2	1	2	1	0	0
Phosphate buffer pH 7.2+0.44% NaCl+0.1% Tween 20	2	4	1	0	2	–	1	0

[a] Period within total protein amounts to 95% or more of non treated sample.
[b] Period within ELISA-active protein amounts to 75% or more of non treated sample.

chromogen diluted to 1.5 mg/ml in 1 M diethanoleamine per 0.5 mM magnesium chloride buffer was added to the plates. After incubation for 30 min at 37 °C the reaction was stopped by adding 50 μl 2 N NaOH to each well. Absorbance was read at 405 nm wavelength with an ELISA-plate-reader (MS Titertek 212, ICN, Germany). Each unknown sample was run in triplicate with a standard curve in triplicate, as well. The concentration of active TT was calculated as described below.

2.7.2. Evaluation of tetanus-specific antibody titers in in-vivo experiments

Microtiterplates were coated with 100 μl per well TT (0.006 Lf/ml) and incubated for 60 min (37 °C). The plates were washed three times with PBS containing 1% (w/w) Tween 20 between each step. 100 μl sera of mice were added in appropriate dilution to well A1–G1. As positive control 100 μl anti-tetanus-human-serum (0.16 IU/ml) were pipetted in well H1. All samples were geometrically diluted from well 1 to 11. Well 12 was used as blank. The plates were kept at 37 °C for 1 h and then washed as described above. After incubation for 1 h and washing, 50 μl anti-mouse-IgG-alkaline-phosphatase-conjugate, respectively, anti-human-IgG-alkaline-phosphatase conjugate (row 12) were pipetted in each well (1:80). After incubation color development was conducted as described above. Absorbance was read at 405 nm wavelength with an ELISA-plate-reader (Dynatech 7000, Dynatech, Germany). Antibody titers were calculated and expressed as described below.

2.8. Immunization studies using TT microspheres

Female NMRI mice, 8 weeks old, were used in all experiments. TT-MS were suspended in a sterile vehicle consisting of 0.5% (w/w) carboxymethycellulose solution and Pluronic™ F68 (0.1% w/w)). Mice ($n = 2$) were subcutaneously immunized behind the neck with a defined amount of MS (dose: 50 μg TT = 8.8 Lf). At specified time points mice were sacrificed, blood was collected, pooled and assayed for antigen-specific antibodies by ELISA. Blood of mice without any immunization was analyzed as control at the first and the last day of each experiment.

2.8.1. Short-time kinetics of antibody formation

Groups for immunization were injected with aluminum-adsorbed TT (1), TT-MS with PLGA 50:50 (2), TT-MS with PLGA 75:25 (3), TT-MS with ABA-triblock-copolymer (4) and fluid TT (5) as reference. Blood samples were collected every 5 days until day 45. At day 45 mice of group 1–3 were injected with $100 \times LD_{50}$ tetanus toxin. Unimmunized mice were used as control (6).

2.8.2. Long-time kinetics of antibody formation

Groups for immunization were injected with aluminum-adsorbed TT (1), TT-MS with PLGA 50:50 (2), TT-MS with PLGA 75:25 (3) and TT-MS with ABA-triblock-copolymer (4). Blood samples were collected every 20 days until day 200. A challenge test was conducted with all groups at day 200 as described above.

2.8.3. Effect of booster injections on preimmunized animals

Groups for immunization were injected with aluminum-adsorbed TT (1), TT-MS with PLGA 50:50 (2) and TT-MS with PLGA 75:25 (3). In the first study (a) animals were immunized with Tetanol® and boosted with Tetanol® or microencapsulated TT. In the second study (b) mice were immunized with microencapsulated TT and all groups were boosted with Tetanol®. In the third study (c) mice were immunized with microencapsulated TT and boosted with TT-MS. Blood was collected at day 15 and 30 (after first immunization) and at day 45 (after booster injection).

2.8.4. Induction of antibodies after administration of MS mixtures

Groups for immunization were injected with TT-MS with PLGA 50:50 (2) or TT-MS with PLGA 75:25 (3) or a mixture of both (1:1). They were immunized with a dose of 50 μg (= 8.8 Lf) or 100 μg (17.6 Lf) TT. Blood samples were collected every 5 days until day 60.

2.9. Computation and statistics

The concentration of immune-reactive TT was determined from standard curves after plotting absorbance versus the concentration, by regres-

sion analysis (r, 0.990, range, 0.027–0.6 Lf/ml). The limit of detection for TT was 0.027 Lf/ml.

The concentrations of anti-tetanus titers were calculated by fitting each curve to a polynomial after plotting absorbance against log sample dilution. The dilution for OD = 0.2 was interpolated using the MIKROTECH software package (Dynatech, Germany). Antibody titers were expressed as the reciprocal of the estimated serum dilution. The significance of the difference between the mean response of groups of mice was calculated by a two sample Student's t-test.

3. Results

3.1. MS preparation and characterization

TT was microencapsulated using PLGA 50:50 or 75:25 or ABA-triblock-copolymer by W/O/W double-emulsion-microencapsulation-technique. Typical characteristics of these MS are summarized in Table 2. The mean size of the MS ranged from 11.3 (PLGA 50:50) to 25.0 μm (ABA-triblock-copolymer). The yield which refers the quantity of MS recovered from the microencapsulation process was for all three batches above 79%. Encapsulation efficiency ranged from 75.4% (PLGA 50:50) to 87.8% (ABA-triblock-copolymer). Loading was slightly higher for ABA-triblock-copolymer-MS (0.8%) than for PLGA-MS (0.5%) due to an increased interaction of the protein with the more hydrophilic ABA-triblock-copolymer. Scanning electron micrographs showed round spheres with a smooth surface for PLGA-MS (Fig. 1a), whereas ABA-triblock-copolymer-MS (Fig. 1c) have a rough surface with pores.

Scanning electron micrographs show that PLGA 50:50 MS lost their round shape during release studies and became porous (Fig. 1b), whereas there was nearly no change for PLGA 75:25 MS. In contrast, MS with ABA-triblock-copolymer show deep fissures and a highly porous structure (Fig. 1d) at day 45 of in vitro release.

3.2. TT stability under release conditions

The effects of different methods of agitation, pH and incubation times on ELISA activity of TT are presented in Fig. 2. After only 1 day of incubation (rotating speed of 10 rpm) the ELISA-activity for all buffers was decreased to 10% (Fig. 3a). Therefore, the rotation speed in all further experiments was reduced from 10 to 2 rpm, where after 2 days between 20 and 40% of the initial ELISA-activity was retained. Different volumes of various buffers, thus different buffer-air interfaces, showed no significant effects on protein activity (Fig. 2 b). However, ELISA activity of TT is highly affected by the intensity of the motion, carried out by the rotating release apparatus (Fig. 2a). Similar results were obtained using horizontal or orbital mixers (data not shown).

3.3. In vitro release of TT from MS

Graphs in Fig. 3 compare the release profiles of TT from MS with PLGA- and ABA-triblock-copolymer. The release of ELISA-active TT from

Table 2
Characteristics of TT loaded microspheres

Polymer	Loading [μg TT per mg MP][a]	Encapsulation efficiency [%]	Yield [%]	Mean diameter [μm][b]	Morphology[c]
PLGA 50:50	5.3	75.4 ± 9.2	86.8	11.3	smooth
PLGA 75:25	5.0	67.8 ± 6.7	91.6	17.3	smooth
ABA-triblock-copolymer	8.1	87.9 ± 2.2	79.3	25.0	rough and porous

[a] Calculated from UV-spectroscopy.
[b] Laser light diffraction.
[c] Scanning electron microscopy.

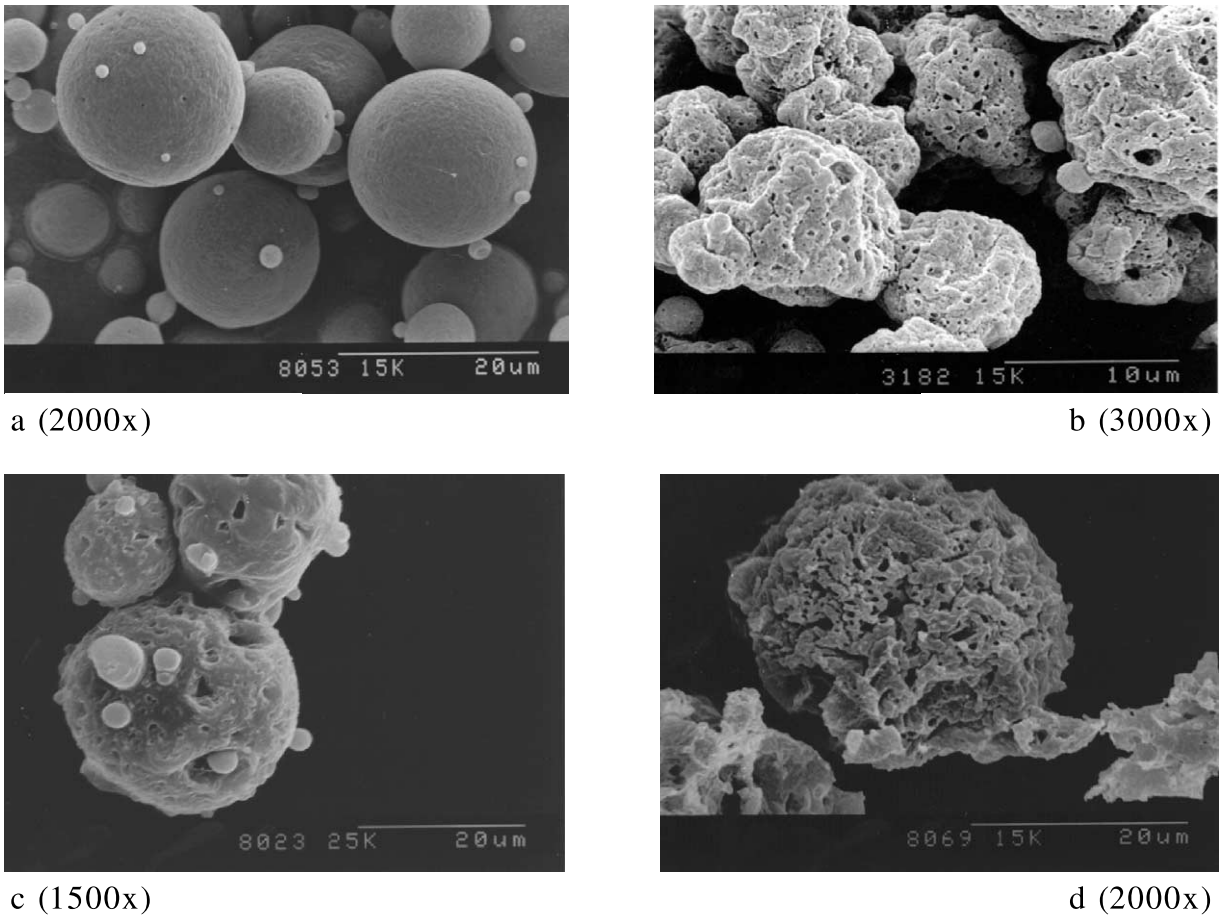

Fig. 1. TT-MP with PLGA 50:50 before (a) after; (b) and with ABA-triblock-Copolymer before; (c) after; (d) antigen release.

both, PLGA 50:50 and PLGA 75:25 MS was characterized by a biphasic profile. After a burst release of TT with $20.5 \pm 0.5\%$ (50:50), respectively, $6.1 \pm 0.8\%$ (75:25) no further release could be detected up to 84 days, neither with a protein assay nor with an ELISA (Fig. 3a). There was no difference between both PLGA types tested except of the extent of the bursts. In contrast, MS consisting of ABA-triblock-copolymer showed a continuous release of TT for a period of about 20 days (Fig. 3b). About 60% of the protein was released in an ELISA-active form. The in-vitro release patterns are compatible with the known degradation behavior of both polymer types. Due to the hydrophobic nature of PLGA, TT is mainly adsorbed onto the MS surface leading to an increased initial burst in release. In case of the more hydrophilic ABA-triblock-copolymer, phase separation took place and TT is distributed in more favorable hydrophilic microdomains leading to a stable matrix like state of dispersion thus reducing the initial burst.

3.4. In vivo studies using TT-MS

3.4.1. Experiment 1: short-time kinetics of antibody formation

The antibody levels induced in mice after subcutaneous immunization with a TT solution, TT aluminum and microencapsulated TT are shown in Fig. 4a. After a lag-time the antibody titers of all preparations with exception of the TT solution

began to increase sharply until day 20. Between day 20 and 45 the antibody titer leveled off slowly. The induction of antibodies by TT solution showed a lag-time of 10 days and was significantly lower ($P < 0.05$) than those of the other preparations. Comparing all microencapsulated preparations with each other, the difference between the preparations did not reach statistical significance ($P < 0.05$). A challenge test with $100 \times LD_{50}$ tetanus toxin at day 45 with mice preimmunized with aluminum-adsorbed and microencapsulated TT (PLGA 50:50 and ABA-

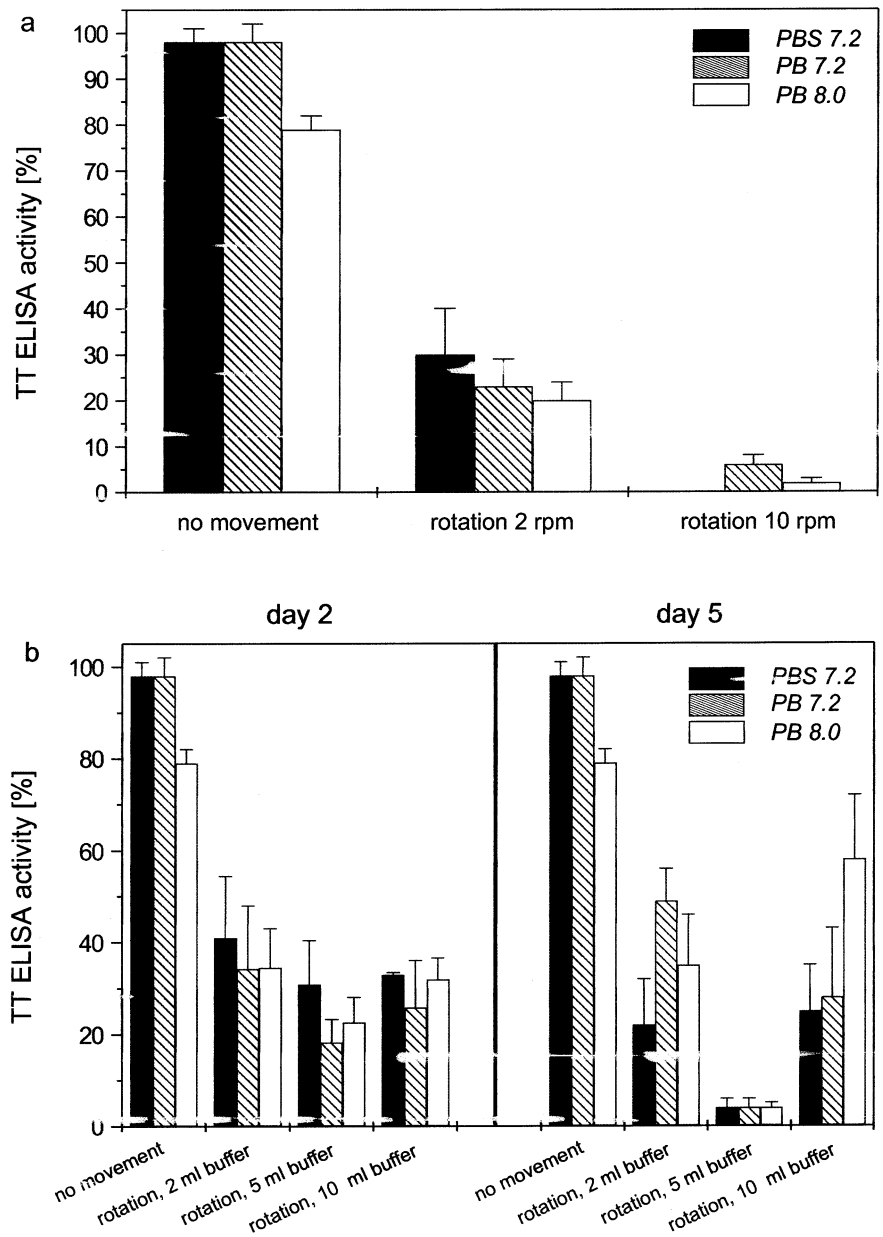

Fig. 2. ELISA-activity of TT (a) after 24 h incubation (rotating metal block/37 °C/2 rpm/48 h) in different buffer volumes and (b) after 24 h incubation under different movements and different filling volumes.

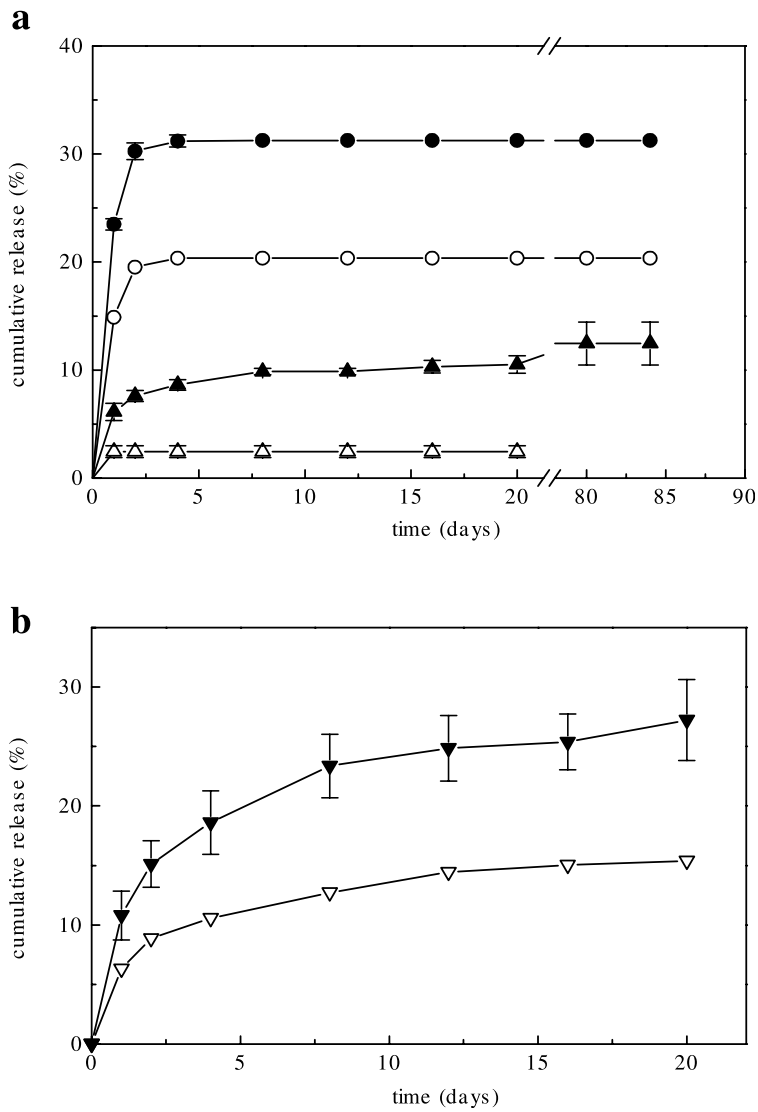

Fig. 3. In-vitro release of total (solid symbols) and ELISA-active (open symbols) TT from (a) PLGA 50:50- (●/○), PLGA 75:25- (▲/△) and (b) ABA-triblock-copolymer-MP (▼/▽).

triblock-copolymer) confirmed these findings and demonstrated the protective properties of the antibody response. All immunized mice (groups 1–5) survived, whereas all control animals (group 6) died.

3.4.2. Experiment 2: long-time kinetics of antibody formation

Fig. 4b shows the development of antibodies against tetanus antigen after one subcutaneous immunization with aluminum-adsorbed or microencapsulated TT. All titers reached a plateau after 50 days, which was maintained until day 200 (= 29 weeks). The challenge test (100 LD_{50}) with mice of every group (1–4) showed protective properties of the induced immune response. Just as in experiment 1 (45 days after immunization) 100% of the immunized mice (groups 1–4) sur-

vived, whereas all control animals (group 6) died.

An important prerequisite for successful protection is a sufficient antibody production within a few days after a second antigen dose. Boosting is possible if circulating memory cells are formed after first antigen contact. To characterize the boosting potential we investigated several protocols using TT-MS.

3.4.3. Experiment 3: effect of booster injections on preimmunized animals

Fig. 5a–c shows the results of a booster immunization on the immune response induced by microencapsulated TT. Aluminum-adsorbed TT was used as positive control.

First, the effect of a booster using aluminum-adsorbed TT on mice which were primed with microencapsulated TT was studied. After con-

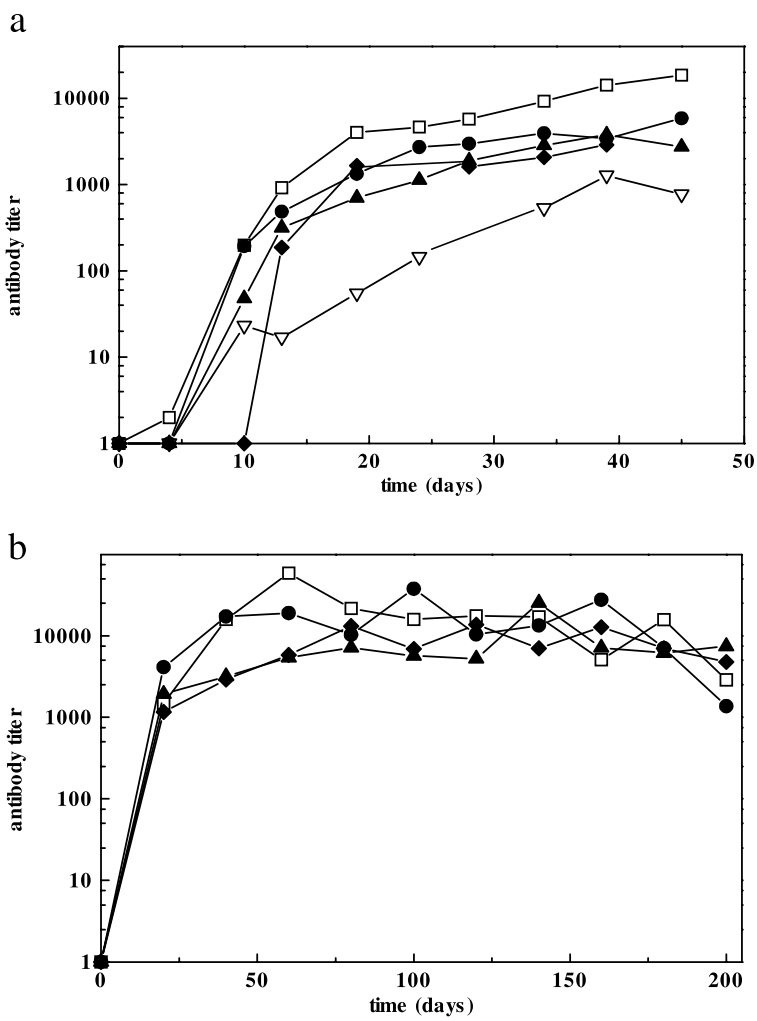

Fig. 4. Short-term- (a) and long-term-study (b) for induction of antibodies in mice after a single injection (s.c.) of alum-adsorbed TT (□), TT-ABA-triblock-copolymer-MP (▲), TT-PLGA 50:50-MP (●), TT-PLGA 75:25-MP (◆) and TT in phosphate buffered saline (▽).

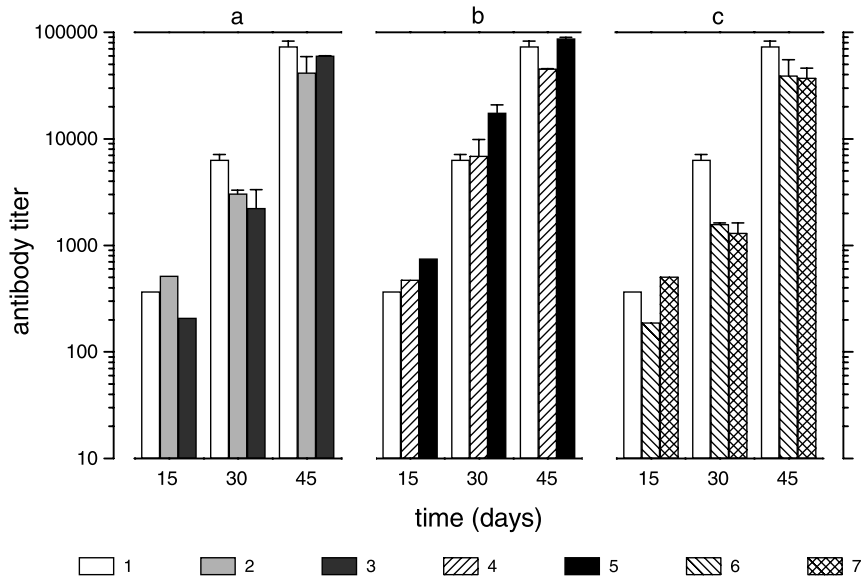

Fig. 5. In-vivo immune response after two s.c. injections (0 and day 30) of TT-vaccines in mice (dose per mouse: 5 μg TT).(a) First injection alum-adsorbed TT (1), TT-MP with PLGA 50:50 (2) and ABA-triblock-copolymer (3), second injection alum-adsorbed TT.; (b) First injection alum-adsorbed TT, second injection alum-adsorbed TT (1), TT-MP with PLGA 50:50 (4) and ABA-triblock-copolymer (5).; (c) First injection alum-adsorbed TT (1) TT-Mp with PLGA 50:50 (6) and ABA-triblock-copolymer (7) second injection same one as first.

tact with microencapsulated antigen antibody titers increased up to day 45 (Fig. 5a).

As represented in Fig. 5b microencapsulated TT also allows boosting of mice which were primed with aluminum-adsorbed TT, demonstrating that after first contact with microencapsulated antigen circulating memory cells are formed.

Lastly, data of Fig. 5c prove that homologous boosting also results in an increase in tetanus-specific antibodies. All antibody levels achieved were in a similar range as the positive control with aluminum-adsorbed TT.

Since boosting the immune response was possible with the dose of TT release from MS, we investigated the possibility to use a mixture of fast and slow degrading MS releasing TT as a consequence of the polymer erosion. If TT remained intact one would expect to reach the same antibody titers as after repeated injections.

3.4.4. Induction of antibodies after administration of MS mixtures

Fig. 6 shows the kinetics of antibody development after one injection of microencapsulated TT (PLGA 50:50 or PLGA 75:25) or a mixture (1:1) in comparison. In order to evaluate the influence of the applied dose in Fig. 6b twice the dose of MS as before was used. After a lag-time of 5 days antibody titers increased and reached a plateau at day 30. There was no significant difference between the three preparations. The mixture did not result in a boosting effect when the MS eroded. Moreover, these antibodies did not reach the level of antibodies induced after a booster at day 30 (Fig. 5a–c).

4. Discussion

4.1. In vitro characterization

The characterization of the three MS batches, prepared by the double emulsion technique, showed that MS size of ABA-triblock-copolymer-MS was higher than for PLGA. This may be caused by a swelling of the polymer during microencapsulation using a ABA-triblock-copolymer, due to their high swelling rate these MS

showed increased particle sizes (Kissel et al., 1996). This observation was confirmed by scanning electron micrographs which showed that ABA-triblock-copolymer-MS had a rough surface which may be attributed to the swelling process described above. In contrast, PLGAs are more hydrophobic and did not take up water readily. The microencapsulation efficiency and loading of the MS with TT was in the expected range for all batches.

Mechanical movement during incubation and particle separation before analysis, pH and incubation times showed a distinct effect on ELISA activity of TT, which may be explained by the continuous formation of air-buffer-vial-interfaces caused by the over-the-top rotating movement of the vials. It is known that due to their amphiphilic structure proteins tend to interact with hydrophobic interfaces. Processes in shaken solutions are special cases of interactions at interfaces. The hydrophobic segments of the protein are able to contact the interface directly. As a result of hydrophobic adhesion, adsorption of parts of the protein can take place. The hydrophilic segments in contrast are left protruding into the surrounding solution. This often results in an unfolding of the protein and thereby in a loss of its conformation and activity (Maste et al., 1997). The adsorption can be reversible or irreversible. In a reversible process there is a continuous exchange of adsorbed and free protein (Norde and Lyklema, 1978; Norde, 1986). These studies indicate that the intensity/type of movement and the ratio between buffer volume and vial volume seem to play an important role for stability of TT during in-vitro release experiments. Due to the instability of TT to rotating movement we decided to incubate the vials without any movement only turning them over once a day manually.

An important difference was noted studying the release of TT from MS under in-vitro conditions. The release of PLGA-MS generally is characterized by a tri-phasic process (Thomasin et al., 1996), a rapid initial release phase (burst effect) caused by drug molecules in the vicinity of the surface of MS followed by a lag-time with slow

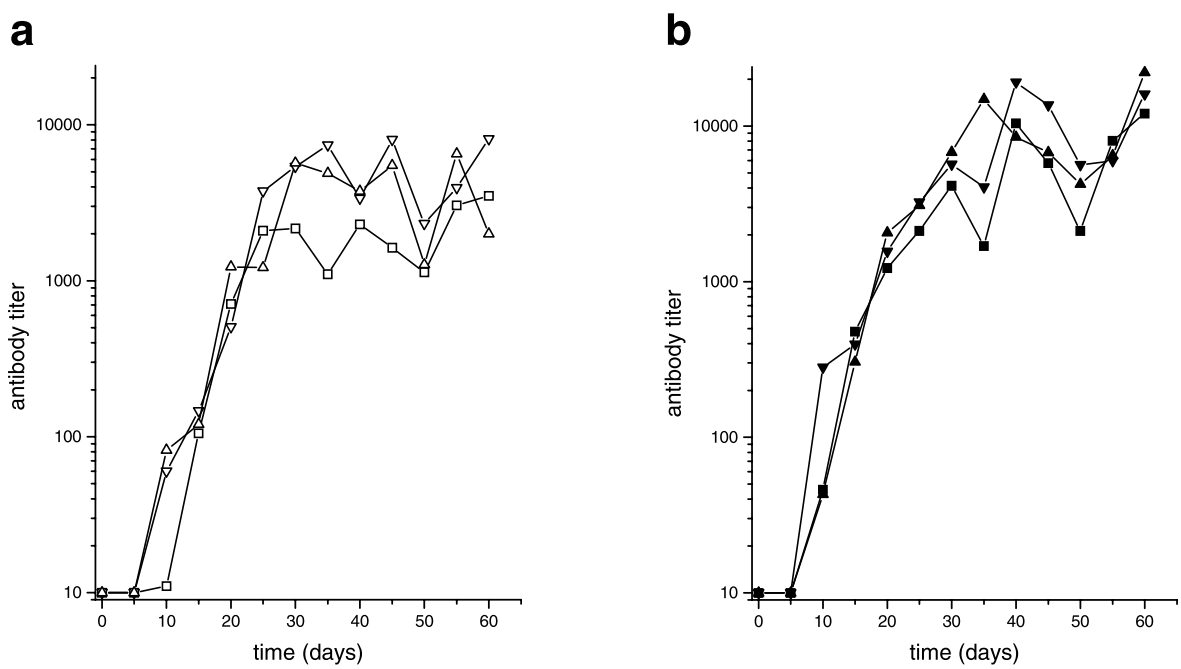

Fig. 6. Kinetics of antibody formation after single injection of TT-MP with PLGA 50:50 (▲/△) or PLGA 75:25 (▼/▽) separately or as mixture (■/□). Dose per mouse: 5 μg (open symbols) or 10 μg (solid symbols).

TT release (second phase), where the MS are slowly hydrated and, thirdly, again a phase of rapid release due to polymer degradation and erosion. The second phase is already affected by polymer degradation so that small molecules can diffuse through pores which are formed within the MS.

In the case of TT-MS, the release of the antigen from PLGA MS only showed phase one and two. After a burst no further protein was released for a period of 84 days. A similar behavior was reported by Yan et al. (Yan et al., 1994) for the release of FITC-BSA from PLGA 50:50 (Mw 50–75 kg/mol) MS. This release profile might be caused by two effects. For one, PLGAs (Shah et al., 1992) are known to show bulk hydrolysis. In phase two the polymer is subjected to hydrolytic chain cleavage (Wang et al., 1990), but the formed pores are too small for large proteins, therefore, diffusion is less likely. Moreover, it is known that proteins tend to aggregate when exposed to small amounts of water (Sluzky et al., 1991; Costantino et al., 1994) or acidic conditions. The molecular weight of such aggregates will be higher than that of TT reducing the diffusion rate dramatically.

In contrast, MS composed of the ABA-triblock-copolymer continuously released TT which retained nearly 60% of is ELISA-activity up to 20 days. The ABA-triblock-copolymer is hydrated quickly because of its polyoxyethylene B-block so that a hydrophilic environment for the microencapsulated protein is formed within minutes (Bodmer et al., 1992; Li et al., 1994). Due to the high content of polyoxyethylene-B-blocks (38% w/w) in the ABA-triblock-copolymer large pores are formed allowing diffusion of large proteins, such as TT (150 000 g/mol) (Kissel et al., 1996).

4.2. In vivo studies

In the present study we compared the in-vivo performance of TT-MS prepared from biodegradable PLGA- and ABA-triblock-copolymer, since a retrospective analysis of literature data proved to be very difficult. Injection sites and schedules as well as vehicles for MS administration vary considerably (Esparza and Kissel, 1992; Men et al., 1995; Gupta et al., 1996; Kersten et al., 1996; Tobio et al., 1999), therefore, it is not surprising, that antibody titers, duration of immune response and type of immune reaction are not consistent. Secondly, there is no consensus on a generally accepted 'standard dose' for animal models and a dose response relationship for microencapsulated antigens has not been established to our knowledge. Thirdly, and most importantly, there seems to be little agreement in the literature on a 'standardized immunization protocol' for in-vivo studies. Positive and negative controls, time schedule for boosting and time intervals for blood sampling differ considerably from group to group.

In these studies, the antibody titer development against TT was investigated both in a short- and a long-term experiment in mice (Fig. 4a). TT-MS elicited specific anti-tetanus-antibodies after a single injection. The antibody titers were comparable to the positive control, aluminum-adsorbed TT, and clearly higher than TT solution without adjuvant, suggesting that microencapsulated TT shows significant adjuvant activity. The TT solution induced a clear antibody response, underscoring the fact that TT itself is a potent antigen (Cox and Coulter, 1997). Interestingly TT-MS prepared from slowly degrading PLGA 75:25 showed a lag-time of 10 days, whereas other batches had only a lag-time of 5 days, suggesting that the release has an influence on the kinetic of antibody formation. The antibody titers induced after application of TT-MS with PLGA is consistent with data of other groups (Esparza and Kissel, 1992; Alonso et al., 1994; Men et al., 1995; Gupta et al., 1996; Kersten et al., 1996; Audran et al., 1997; Kissel et al., 1997). TT-MS prepared from ABA-triblock-copolymer which exhibited a faster in-vitro-release rate induced antibody titers in the same range as PLGA. In this study, we found no clear correlation between the in-vitro release kinetics of TT from different MS preparations and antibody formation. Contrary to the single shot vaccine concept, no booster effect was observed when TT is released from MS in the erosion phase, usually reached after 20–30 days in PLGA with a 50:50 composition. These findings suggest that the amount of TT which is released in the initial burst phase is sufficient for priming

the mice, but the quantity of antigen released during erosion phase does not seem to be sufficient for boosting. Different formulations seem to affect only the time point when plateau titers were reached. Furthermore, slightly different MS sizes did not influence the immune response in mice which confirmed studies of others (Alonso et al., 1994) using PLGA-MS.

Moreover, we demonstrated using a tetanus toxin challenge test in mice that the induced antibodies by TT-MS, were protective even against $100 \times LD_{50}$ of tetanus toxin which is known as one of the most potent toxins (Schiavo and Montecucco, 1997). Long-time kinetics (Fig. 4b) demonstrate that the antibody titers persisted for a period of at least 29 weeks. They protected the immunized animals against tetanus toxin challenge after one single administration even after this long period of time. These findings suggest that TT-MS induce a potent and long lasting immune response, which is protective in nature.

The ultimate goal of single shot vaccines for TT based on biodegradable MS is a pulsatile release profile of an antigen. Using mixtures of fast and slow degrading TT-MS antigen release can occur after 1 and 6 months when the biodegradable polymers are eroded. A pulsatile in-vitro antigen release pattern was reported for TT-MS (Sanchez et al., 1996; Schwendeman et al., 1998; Johansen et al., 2000b,a).

For an effective boosting of the primed mice the formation of memory cells after the first injection is a prerequisite, which can not be predicted from the antibody titers after priming. Therefore, we studied different protocols for boosting the immune response using two injections of TT-MS and/or aluminum-adsorbed TT (Fig. 4a–c). TT-MS were able to booster the anti-TT response both after preimmunization using aluminum-adsorbed TT or TT-MS. These results, which are in agreement with Singh et al. (Singh et al., 1997) demonstrate the induction of memory cells after one injection of microencapsulated TT. For successful boosting sufficient priming followed by induction of memory cells, rapidly proliferation and production of neutralizing antibodies on one hand and a second contact with an antigen pulse after a defined period of time on the other hand seem to be essential. As shown above, TT-MS meet the first requirement, namely sufficient boosting capacity due to their adjuvanticity. Consequently it should be possible to design single-shot vaccine with TT-MS if the controlled, pulsatile release of TT at a second contact with the antigen after approximately 4 weeks, is feasible. Therefore, we injected a mixture of fast (PLGA 50:50) and slow (PLGA 75:25) degrading TT-MS in mice (Fig. 6).

We found no difference between mixtures of the two batches and the application of single batches, which suggests that the mixture did not induce a sufficient booster effect. These results are in conflict with studies of Gander et al. (Schiavo and Montecucco, 1997), but confirm these of Men (Men et al., 1995) and Xing (Xing et al., 1996a).

For successful booster reactions two aspects are important: (a) induction of memory cells, (b) the second immunization should take place as a pulsatile release of a defined dose of antigen. Fig. 4 demonstrates that the induction of memory cells was achieved. Therefore, the missing booster reaction seems to be connected with insufficient release of intact TT at later time points. In-vitro release profiles suggested that no pulsatile release of TT from PLGA-MS was achieved, although this profile could be shown for FITC-Dextrans with 150 000 and 40 000 g/mol (data not shown). The erosion of MS after 4 weeks seems to be too slow to release a real pulse of antigen. This may be caused by the matrix structure of the MS caused by the double-emulsion-method. A second aspect is that during polymer degradation the pH within the MS decreases. Stability studies showed clearly that TT experiences a loss in ELISA activity when exposed at pHs below 7 (data not shown).

In conclusion, the single injection of TT in biodegradable MS has the capacity to elicit a long lasting and protecting immune response. Conventional PLGAs did not seem to be suitable for the design of a single-shot vaccine, due to their bulk erosion behavior on one hand and their hydrophobic nature on the other hand. ABA-triblock-copolymers were studied as a new class of polymers for vaccination and proved to be promising candidates.

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