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Release of tetanus toxoid from adjuvants and PLGA microspheres: How experimental set-up and surface adsorption fool the pattern

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Abstract

The classical adjuvants alum and Freund's Incomplete Adjuvant (IFA) are frequently used as references for the design of new adjuvants and antigen delivery systems, e.g., microspheres (MS). Poly(DL-lactic-co-glycolic acid) (PLGA) MS have been proposed for delivering antigen booster doses in vivo after a single injection. However, as antigen release kinetics from conventional adjuvants are generally unknown, it appears presumptuous to propose a desired antigen release pattern from PLGA MS. Therefore, we have studied the tetanus toxoid (Ttxd) in vitro release from alum, IFA formulations and MS in four different test systems. The results showed a stronger Ttxd association to alum than to IFA, and the release from both formulations lasted between 3–9 days. The total of ELISA-responsive antigen released was 60–85% of the actual dose. Both the total amount and the prolongation of release depended on the Ttxd dose. Furthermore, the incomplete in vitro release of Ttxd from the adjuvants and also from PLGA 50:50 MS was shown to be partly due to experimental conditions. Typically, Ttxd adsorbed on the glass vials used for the release test and also on the surface of the PLGA 50:50 MS, wherefrom it was released. In conclusion, the test system depending rate and quantity of release observed evidence the limitations of in vitro release data. Finally, for mimicking conventional vaccination schedules, i.e. injections typically at time points 0, 1, 3, and 12–24 months, PLGA MS should release antigen doses at the corresponding time points, and the release pulse should only last for a few days. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Biodegradable poly(lactic acid) (PLA) or poly-(lactic-co-glycolic acid) (PLGA) microspheres are considered attractive for controlled antigen delivery [1]. It is currently assumed that the frequently observed pulsatile release pattern extending over several months may mimic booster doses of conventional vaccines and obviate the need for repeated injections in young children. Surprisingly, the antigen release kinetics of PLA/PLGA microspheres is probably better established than that of conventional adjuvants, such as alum and Freund's Incomplete Adjuvant (IFA). Thus, it appears rather presumptuous to propose an optimised antigen release pattern for microspheres potentially suitable for boosting, considering that the release kinetics of standard vaccine formulations is not even known. A further

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difficulty for the rational design of pulsatile release microspheres is the frequently observed incomplete protein release from microspheres, as typical for albumin [2], carboxyanhydrase [3], interferon [4], and toxoids [5–7]. In case of tetanus toxoid (Ttxd), the incomplete release was found to be partly due to stability problems [7].

Release experiments with PLA/PLA microspheres are commonly performed in glass or plastic tubes by dispersing microspheres in a buffer and subsequent incubation at 37°C under agitation [8]. However, it has been demonstrated that the experimental set-up exerts a great effect on the release of indomethacin from PLA microspheres [9]. In case of protein drugs, a major problem may also arise from the acidic polymer degradation products, which can compromise the stability of entrapped and released protein [5,10]. Typically, aggregation and loss of activity may be important sources of incomplete release of intact protein [11–13]. Thus, to circumvent this effects, it was suggested to eliminate continuously the mono- and oligomeric acids formed from the release medium by dialysis [14]. Finally, protein adsorption on surfaces (container for release test and polymeric microspheres) may influence the release observed in a given release test. Mechanisms of and conformational changes upon protein adsorption on hydrophobic surfaces were described by Norde and Favier [15]. More specifically, protein adsorption on PLGA microspheres was examined by Calis et al. [16] and Crotts et al. [17]. The latter study concluded that non-specific protein adsorption is a critical factor in controlling protein release kinetics from PLGA microspheres and that it can be suppressed by adding sodium dodecyl sulphate to the release medium.

The goal of this study was to characterise first the in vitro release kinetics of tetanus toxoid adsorbed on alum or dispersed in IFA. We believe that this information may be useful for designing pulsatile delivery systems mimicking priming and booster doses. Secondly, we examined whether the incomplete in vitro release of tetanus toxoid is caused by adsorption to polymeric particles or borosilicate glass surfaces. For this, we studied the release from fast degrading PLGA microspheres under different experimental conditions as well as the adsorption of the toxoid on placebo PLGA particles.

2. Materials and methods

2.1. Materials

Tetanus toxoid (Ttxd) in aqueous solution (lot No. PTC 10005: 8500 Lf/ml or 26.3 mg/ml, Pasteur Mérieux, F-Lyon) and adsorbed on aluminium oxide (lot No. 10005: 19.7 Lf/ml) were provided by WHO. Poly(DL-lactic-co-glycolic acid) (PLGA 50:50) with a M_W of approx. 12 kDa was purchased from Boehringer Ingelheim, D-Ingelheim (Resomer® RG502H). Incomplete Freund's Adjuvant (IFA) was from Sigma Chemical, St. Louis, MO. Monoclonal anti-tetanus antibodies (TT010) and guinea pig anti-tetanus IgG were from Wellcome Biotechnology, UK-Beckenham. Rabbit anti-guinea pig IgG horse radish peroxidase conjugate and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) were from Sigma Chemical, St. Louis, MO. Unless specified otherwise, all other substances used were of pharmaceutical or analytical grade and purchased from Fluka, CH-Buchs.

2.2. In vitro release of tetanus toxoid from adjuvants

The release of Ttxd from alum and IFA was studied according to model D in Table 1. Briefly, 50 μl of Ttxd (20 or 200 Lf/ml) formulated in alum or IFA were embedded in 250 μl of 2% agarose at 60°C. A second layer of 250 μl agarose was added on the top of the first one. Finally, 3.4 ml of isoosmolar 67 mM PBS of pH 7.4, containing 0.2% BSA to prevent toxoid adsorption on the glass vials, were added as receiver medium. Samples were taken at regular intervals and replaced with fresh buffer. As a control, the same experiment was carried out with an aqueous solution of Ttxd.

2.3. Preparation of microspheres

PLGA microspheres (MS) were prepared by spray-drying (Büchi 190, CH-Flawil) a dispersion of aqueous toxoid solution in a 5% (w/w) solution of PLGA in ethyl formate as described elsewhere [8]. For improving the antigenicity of Ttxd, BSA (5%) and trehalose (15%) were co-encapsulated [7].

Table 1
Experimental conditions for the release of tetanus toxoid from PLGA 50:50 microspheres

Release system and medium[a]	Sampling volume
A: MS suspended in 67 mM PBS[b]	1 ml
B: MS suspended in 67 mM PBS +0.2% BSA[b]	1 ml
C: MS suspended in 67 mM PBS +0.2% BSA[b]	2 ml
D: MS immobilised in agarose and 67 mM PBS+0.2% BSA[c]	1 ml

[a] The volume of the release medium was 3.4 ml, the pH 7.4 and the osmolarity 300 mosmol/kg.
[b] MS were suspended directly in 3.4 ml buffer and the vials rotated vertically.
[c] MS were suspended in 40 μl 5% soya lecithin and immobilised in 250 μl of 2% agarose in the vials. Another 250 μl of 2% agarose was added to separate the gel-embedded MS from the recipient release medium. The vials were horizontally shaken.

2.4. In vitro release of tetanus toxoid from microspheres

Toxoid release experiments from 10 mg MS were conducted at 37°C in 5 ml borosilicate vials (Chromacol, GB-London) sealed with a Teflon cap, according to the experimental conditions given in Table 1. A medium with pH-buffering capacity was chosen to neutralise the acidic degradation products from the polymeric microspheres. At regular intervals (see Fig. 1), samples were assayed by ELISA.

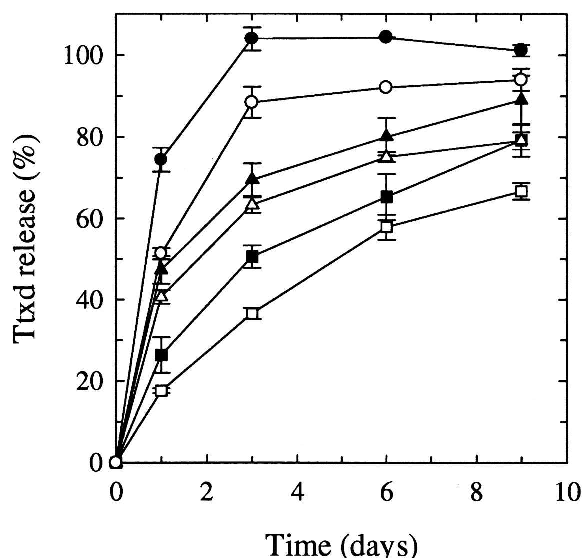

Fig. 1. Release of 1 Lf (filled symbols) or 10 Lf (open symbols) tetanus toxoid from the adjuvants IFA (triangles) and alum (squares). Vaccine formulations were immobilised in agarose, and toxoid determined in a recipient compartment of BSA-containing PBS buffer. As controls toxoid solutions (circles) were embedded in the agarose without any adjuvant ($n=3$).

For this, the release medium with the MS was centrifuged at 3500 rpm (2000 G) for 10 min, and the particle free supernatant withdrawn for analysis and replaced with fresh buffer. The pH of the medium was monitored and found to remain constant. All experiments were repeated five times.

2.5. Adsorption of toxoid

Adsorption of Ttxd on the borosilicate vials used for the release study and on placebo MS (15 mg), loaded or not loaded with BSA, was carried out either in PBS or in PBS with 0.2% BSA at pH 7.4. Varying concentrations of Ttxd in 3.4 ml solution were added to the vials and incubated at 37°C for 24 h under horizontal rotation. The equilibrium concentration was assayed by ELISA after separating the particles from the supernatant by centrifugation at 3500 rpm for 10 min at room temperature.

2.6. ELISA of tetanus toxoid

Tetanus toxoid antigenicity was measured by ELISA. Briefly, flat-bottom 96 well microtiter plates (Nunc-Immuno Plate Maxisorb, Nunc, DK-Roskilde) were filled with 100 μl of 1 μg/ml monoclonal anti-tetanus IgG (TT010) in 0.05 M carbonate buffer of pH 9.6, and incubated overnight at 4°C. The plates were washed three times with 300 μl of 0.05% Tween 20 in PBS of pH 7.4 (PBST) after each incubation step. After 1 h incubation at 37°C with 150 μl 2.5% milk powder in PBST (PBSTM), the plates were incubated at 37°C for 2 h with serial dilution of standard and test solutions of tetanus toxoid. Guinea pig IgG (25 μg/ml) was added to

each well in 100 μl of PBSTM, and plates incubated for another 2 h. Then, rabbit anti-guinea pig peroxidase conjugate (1/8000 dilution) in 100 μl PBSTM was added to each well, and plates incubated for further 1 h. Finally, 100 μl of 0.5 mg/ml peroxidase substrate 2,2′-azino- bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.05 M citric acid of pH 4.0 was added to the wells, and the endpoint optical density measured at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA) after 30 min incubation at room temperature.

3. Results and discussion

3.1. Release of tetanus toxoid from adjuvants

Classical adjuvants have not been considered so far as delivery systems with controlled release properties. Hence, in vitro release or dissociation of antigens from adjuvants (alum or IFA) has attracted only minor experimental interest. As the combination of antigen and adjuvant is crucial to elicit an efficient immune response, the release kinetics might also play a role in this process. One might speculate, that if the antigen dissociates too rapidly, it might be degraded enzymatically and escape before its recognition and uptake by antigen presenting cells.

Release of Ttxd from alum and from IFA was studied by immobilising the formulations in agarose to mimic a subcutaneous or intramuscular injection site [18]. At a dose of 1 Lf Ttxd, the amounts released from alum and IFA after one day were 26 and 47%, respectively, and 79 and 89% after nine days (Fig. 1). At a dose of 10 Lf, the release profiles were similar to 1 Lf, except for the Ttxd fraction released during the first day, which was lower with the 10 Lf dose. In a control experiment, 1 and 10 Lf Ttxd solution were directly embedded in the agarose gel. Approx. 75% (1 Lf) or 51% (10 Lf) of the dose were released within one day and 90 to 100% after three days.

The initially faster release of Ttxd from IFA as compared to alum might be due to a poor physical entrapment of the W/O-emulsion in the agarose gel. Indeed, during the experiment, mineral oil appeared in the receiver compartment. Thus, through the escape of some of the W/O-emulsion from the gel, a certain amount of Ttxd became immediately available. This suggests a certain limitation of the agarose release model for such emulsions. On the other hand, the release of Ttxd from alum was more linear in shape as compared with Ttxd released from IFA. Although adsorption of proteins at interfaces generally occurs through weak interaction forces of the van der Waals type, hydrophobic interactions and hydrogen bonds may in some cases result in irreversible adsorption. The slow and incomplete release of Ttxd from alum suggests the involvement of such strong interactions between Ttxd and alum. In contrast to the Ttxd release profiles observed here, the release of ovalbumin (10%, w/w) from alum in PBS was reported to be of nearly zero order over 30 days with a total of 45% albumin released [19], although this release experiment was performed by dispersing the formulation directly in a buffer (e.g., system A, Table 1). Apparently, ovalbumin adsorbs stronger on alum than Ttxd does. Further, it was reported that polyvalent cations such as Al^{3+} were able to neutralise negatively charged proteins [20] and thereby, making them more hydrophobic at neutral pH. A subsequent change in the tertiary structure of a protein can influence the kinetics and the reversibility of the adsorption and desorption processes [21]. By the same token, the release of Ttxd from alum may be partly controlled by such changes in interaction.

The analysis of the release kinetics revealed an approximate linear square root-of-time dependency with both alum ($r^2=0.990$) and the IFA ($r^2=0.934$ for 10 Lf, and $r^2=0.968$ for 1 Lf). However, as the release occurs by a two step process, i.e., dissociation of Ttxd from the adjuvant and diffusion through the 3–6 mm 2% agarose gel, it would be rather speculative to interpret a diffusion controlled release of Ttxd. By extrapolating the square root-of-time plot to time zero, a pronounced burst release appeared with IFA, but not with alum, which clearly demonstrates the higher affinity of Ttxd for alum than for IFA.

3.2. Release of tetanus toxoid from microspheres using different test systems

The release kinetics of Ttxd from PLGA micro-

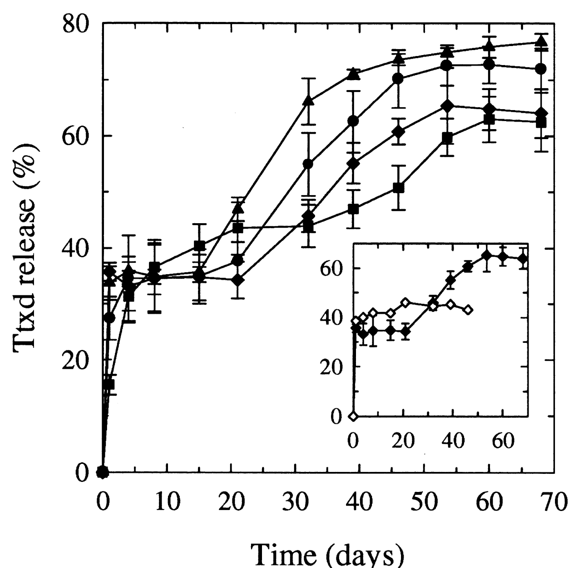

Fig. 2. Release profiles of antigenic Ttxd from PLGA MS obtained in the four release test systems described in Table 1. System A: PBS (♦); System B: PBS–BSA – 1 ml (●); System C: PBS–BSA – 2 ml (▲); System D: agarose gel (■). The inset compares the Ttxd release in system A obtained from MS containing 5% BSA and 15% trehalose (♦) with MS containing only 15% trehalose (◇) as additives ($n=3$).

spheres (MS), containing the additives BSA and trehalose, depended on the test system used (Fig. 2). In general, when the MS were directly suspended in the buffer solution (test systems A, B and C), a triphasic release pattern was seen, consisting of a initial burst release phase, a dormant period, and a second release pulse. This contrasts the more regular release pattern of MS immobilised in 2% agarose where 27–35% of the actual Ttxd dose were released within the first 24 h. This burst phase was followed by a dormant period of 14–20 days, before a second pulse of Ttxd was released over a period of 15–30 days. The total fraction released from these samples lay in the range of 64–77% of the total dose. The effect of the release test system was most marked during the second and third release phases. Typically, the second phase lasted slightly shorter when the MS were dispersed in PBS containing 0.2% BSA (PBS–BSA) as compared to PBS without BSA. Further, the rate of release in the third phase and the maximum fraction released were higher in PBS–BSA samples (72–77%) than in PBS alone (64%). The effect of aliquot volumes withdrawn and replaced with fresh buffer at each sampling time (test system B, 1 ml, and C, 2 ml) was most important in the third phase. The second release pulse began slightly earlier, and the total amount of Ttxd released was slightly higher when 2 ml of medium were replaced (system C) instead of 1 ml.

In previous experiments, we observed that the total fraction of Ttxd released from PLGA 50:50 MS was lower than the percentage measured here (previously, 5–40% depending on the co-encapsulated additives used; here, 64–77% depending on the experimental conditions). Since previous investigations indicated a major effect of co-encapsulated BSA on the total Ttxd amount released, one MS batch with, and a second without the additive BSA as stabiliser, were studied here (Fig. 2, Inset). The two batches showed a comparable burst release, but there was no further Ttxd release from MS without BSA. At present, it remains unknown whether this is due to Ttxd adsorption on the release vials and the polymeric material itself (see below), or to the deterioration of Ttxd inside the MS prior to release. Such a deterioration of antigenic Ttxd may indeed occur in PLGA MS [5].

Various studies have demonstrated the importance of the in vitro test systems and conditions for prolonged drug release from biodegradable MS. Conti et al. [9] evaluated quite systematically different dissolution methods and test conditions for the release of indomethacin from PLA MS. It appeared that the drug release kinetics depended greatly on the test parameters. For peptides and proteins, release from PLA/PLGA MS is generally not solely diffusion controlled, but follows a pulsatile pattern. It is assumed that this is due to strong interactive forces between peptide or protein compounds and polyesters. Thus, this very complex release behaviour is particularly sensitive to the actual in vitro test conditions.

3.3. Adsorption characteristics of tetanus toxoid

A better understanding of in vitro protein release from PLA/PLGA microspheres may require knowledge about protein adsorption on the glassware used for the release test and on the surface of the MS themselves. The adsorption isotherms of Ttxd indi-

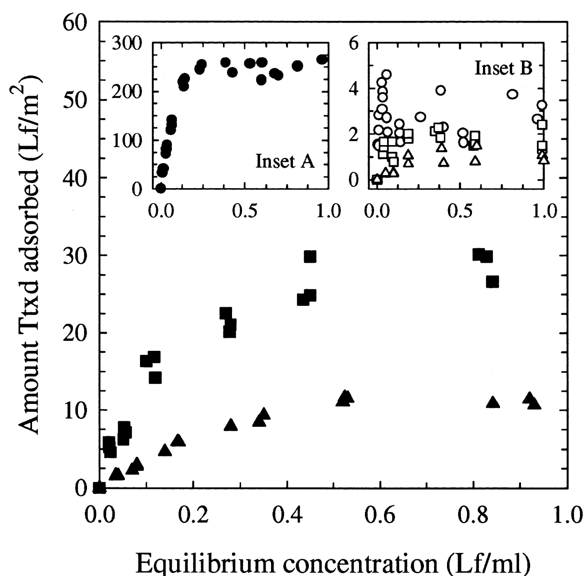

Fig. 3. Adsorption isotherms of Ttxd on PLGA placebo MS containing trehalose (■) and on PLGA MS containing BSA and trehalose (▲) in PBS at 37°C. Inset A: adsorption of Ttxd in PBS on borosilicate glass vials (●). Inset B: adsorption of Ttxd in PBS containing 0.2% BSA on borosilicate glass vials (○), PLGA placebo MS containing trehalose (□), and on PLGA MS containing BSA and trehalose (△) ($n=3$).

cate a Langmuir type monomolecular adsorption on both the glass vials used here and on placebo MS (Fig. 3). Adsorption on both materials was rather important when PBS was used as incubation medium, but became negligible when the medium contained BSA.

The adsorption data were also plotted along the Langmuir equation

$$\frac{x}{m} = \frac{abc}{1 + bc}$$

where c is the Ttxd concentration (Lf/ml) at equilibrium and x the amount (Lf) of toxoid adsorbed to the surface area m (m^2). The value of the Langmuir constant a (Lf/m^2) is a measure of the adsorptive capacity of a particular surface (glass or MS) for the toxoid, and b (ml/Lf) is related to the enthalpy of adsorption. The adsorption parameters for the experiments in PBS are summarised in Table 2. The adsorption in PBS containing 0.2% BSA did not follow the non-linear Langmuir model ($r^2<0.68$) and, therefore, the Langmuir parameters were not

Table 2

Langmuir parameters a and b and correlation coefficient r^2 of the non-linear Langmuir transformation of the adsorption of tetanus toxoid on borosilicate glass and PLGA (50:50) microspheres (MS) in PBS[a]

Surface Type	a (Lf/m^2)	b (ml/Lf)	r^2
Borosilicate glass	280.1	16.1	0.961
PLGA MS	33.4	6.9	0.974
PLGA MS with co-encapsulated BSA	15.6	3.7	0.964

[a] All adsorption data were plotted along the Langmuir equation. Adsorption in PBS containing 0.2% BSA did not follow the Langmuir model. ($r^2<0.68$).
The determined surface area of glass and of MS samples (15 mg) were approx. 0.0027 and 0.0250 m^2, respectively.

calculated. It should also be noticed that the values do not exclusively reflect protein adsorption, but adsorption of ELISA-responsive toxoid. Considering that conventional Ttxd solutions contain, besides the antigen itself, substantial amounts of accompanying proteins, i.e., up to 60%, the values have more immunochemical rather than pure physicochemical significance. The calculated Langmuir parameters clearly demonstrate the influence of BSA on the adsorption of Ttxd onto the materials studied. Firstly, the addition of 0.2% BSA to the buffer solution produced a significant reduction of the adsorption capacity, a, of both borosilicate glass and PLGA MS. Secondly, the surface area related adsorption capacity of glass for Ttxd in PBS was approx. eight-fold higher than that of PLGA. However, this difference diminished upon adding 0.2% BSA to PBS. Importantly, the Ttxd adsorption capacity of MS containing 5% encapsulated BSA was reduced to less than half of that of the PLGA MS without co-encapsulated BSA.

Borosilicate vials showed an adsorption capacity for Ttxd in PBS of 280 Lf/m^2 at an equilibrium toxoid concentration of approx. 0.2 Lf/ml (Fig. 3, Inset A). Assuming monomolecular adsorption as well as a quantitative correlation between ELISA-responsive toxoid and protein content (323.2 Lf/mg protein), this should correspond to a molecular dimension of approx. 268 nm^2 (surface occupied by one adsorbed Ttxd molecule). This is larger than the expected dimension of dissolved Ttxd in its native state (for comparison, albumin has a dimension of 31

nm^2 in water [15]). The difference might be caused by a partial toxoid unfolding upon adsorption, or by a preferential adsorption of accompanying proteins. Upon adsorption, hydrophobic interactions between the protein and the adsorbent and also between neighbouring protein molecules can cause conformational rearrangements which may lead to dimensional changes. Therefore, the number of adsorbed molecules in a monolayer will be inversely proportional to the protein's ability to unfold, i.e., its flexibility. Such unfolding properties of Ttxd have been observed previously (unpublished results). When 0.2% BSA was added to the incubation solution (Fig. 3, Inset B), an approx. 100-fold decrease in adsorption was observed and the plateau was reached at a Ttxd concentration of less than 0.1 Lf/ml. However, at this very low adsorption level, scattered values rather than distinctive isotherm were observed. The reduced toxoid adsorption most likely resulted from competitive adsorption of albumin to the glass and MS surfaces.

Similar to borosilicate glass, placebo PLGA MS adsorbed well Ttxd (Fig. 3). In PBS, a plateau adsorption capacity of 33 Lf/m^2 (Table 2) was reached at an equilibrium concentration of 0.6 Lf/ml Ttxd. Further, the adsorption capacity was much higher (approx. 20-fold) in PBS than in PBS containing BSA (Fig. 3, Inset B), which probably reflects again competitive adsorption between the two proteins. Competitive adsorption was also observed for both calcitonin and BSA adsorption on PLGA microparticles in the presence of ionic and nonionic surfactants [17,22]. It has indeed been shown that surfactants displace protein molecules from polymer surfaces by competitive hydrophobic interaction [23]. In the present study, BSA (also surface active) appeared to interact more strongly than the toxoid with glass or MS surfaces.

Finally, when Ttxd adsorption was measured on PLGA MS containing co-encapsulated BSA, it was clearly lower than the adsorption on placebo MS (free of protein) (Fig. 3 and Table 2). An apparent plateau of approx. 16 Lf/m^2 was reached at an equilibrium concentration of 0.6 Lf/ml in PBS, but the low gradient slope of the isotherm may indicate a non-Langmuir type process. The low adsorption of Ttxd from PBS may be explained by the concomitant release of BSA from the MS and subsequent competition for adsorption within the period of time of the adsorption experiment. Hence, the more complete release of Ttxd from the MS containing co-encapsulated BSA, as compared to those without BSA, can be explained by the concomitant release of BSA which competes with the toxoid for adsorption. In this competition, BSA might adsorb preferentially, because it is expected to be released faster from the MS than Ttxd, thereby getting prior access to the particle surface.

For in vitro release experiments, protein adsorption on glassware and polymers is of high relevance as the measured amounts of released protein may be greatly falsified by concomitant surface adsorption. In light of this, significant adsorption on PLA/PLGA microspheres has previously been reported for proteins such as BSA, IgG, calcitonin and Ttxd [16,20,24,25]. Particular attention should be given to this process when release experiments are performed over a prolonged period of time during which only minute amounts will be released. We can speculate that the incomplete Ttxd release from PLGA MS shown in Fig. 2 is a result of such competitive processes. Fig. 2 also reveals that the differences in the measured Ttxd release was minor after one day (burst release), but became increasingly important during the later stages. As the release medium was replaced by fresh medium at regular intervals, low toxoid concentrations were maintained. Therefore, we expect adsorption to become more important than release. When freely available Ttxd in solution (equilibrium concentration) was plotted as a function of initial Ttxd concentration, a hyperbolic relationship was observed (Fig. 4). This illustration emphasises the practical importance of the in vitro release medium. In PBS, the maximum fraction of available Ttxd varied between 40 and 100% depending on the initial toxoid concentration. It appears from Fig. 3 and Fig. 4 that adsorption of Ttxd on glass and on MS in PBS is very important at low toxoid concentrations and that the presence of MS exerts only a minor additional effect. When MS with co-encapsulated BSA were co-incubated, 80 to 90% of the toxoid remained available, i.e., nonadsorbed, almost independently of the initial toxoid concentration.

Considering the continuous release pattern observed in the in vitro release test system D (MS immobilised in agarose) (Fig. 2), one might specu-

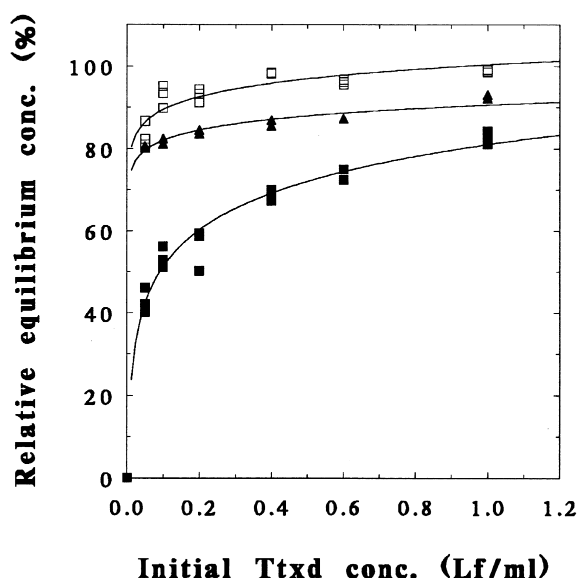

Fig. 4. Free Ttxd in solution after 24 h incubation at 37°C with PLGA placebo MS containing trehalose in PBS (■), PLGA placebo MS containing BSA and trehalose in PBS (▲), or with PLGA placebo MS in PBS containing 0.2% BSA (□) ($n=3$). The lines represent a logarithmic fit to visualise the trend and do not reflect a physical evaluation. The equilibrium Ttxd concentration is expressed relative to the initial Ttxd concentration (%).

late that the pulsatile release obtained in the test systems A, B, and C (MS freely suspended in the buffer) can be ascribed, at least partly, to adsorption of the released Ttxd. Furthermore, the earlier onset of the second release pulse and the higher total amount of Ttxd released, when twice the sampling volume of the release medium was replaced at each time point, also suggest that adsorption and desorption phenomena may interfere. On the other hand, adsorption can only be partly responsible for the pulsatile release pattern because the pulsatile behaviour appears in both PBS and PBS containing 0.2% BSA. Hence, the presence of BSA in the medium did not fundamentally alter the Ttxd release pattern, although it prevents adsorption to a great extent.

A parameter not considered here that should be of great importance for protein adsorption on glass and microspheres surfaces is the pH of the solution [15,26]. Affinity normally increases with decreasing repulsion between the protein and the sorbent, and maximum adsorption is generally observed at the isoelectric point of the protein. When studying release from PLA and PLGA matrices, the pH in the release medium will drop, depending on the buffer capacity, as the polymer degrades. If the release medium is not replaced regularly, this pH-change may enhance protein adsorption as pH will approach the p*I* of the protein, which is in the range of 4–6 for most toxoids.

4. Conclusions

This study showed that the observed in vitro release of tetanus toxoid from PLGA microspheres (MS) depends on the model chosen for this purpose. As the toxoid strongly adsorbed to hydrophobic surfaces such as glass and MS, attention should be paid to the experimental conditions for the purpose of investigating such release. The adsorption capacity of borosilcate glass for Ttxd was significantly higher than that of PLGA MS and should particularly be taken into consideration when designing a release experiment. However, the use of BSA in the release medium or co-encapsulated in MS competed with the adsorption of Ttxd on borosilicate glass and MS and improved the release characteristics of the toxoid. Further, MS immobilised in an agarose gel (model for subcutaneous injection site) produced a quite continuous release pattern, whereas particles freely dispersed in the buffer gave a pulsatile release. This evidences the limitations of release data, which depend not only on the formulation, but also on the test system. Finally, the release of tetanus toxoid from the adjuvants alum and IFA showed a stronger association to alum than to IFA. Interestingly, the release was relatively slow and lasted between three and nine days. Therefore, a single dose vaccine formulation mimicking the priming and one or two booster injections, might require similar kinetics to alum vaccine at the conventional vaccination time points.

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