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In vivo distribution of radioactivity in mice after injection of biodegradable polymer microspheres containing ^{14}C-labeled tetanus toxoid

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Radiolabeled tetanus toxoid (TT) was prepared by detoxifying chromatographically purified tetanus toxin with ^{14}C-labeled formaldehyde. ^{14}C-TT was encapsulated inside poly(D,L-lactide-co-glycolide, 50/50) microspheres (MS) of varying average size (~10 μm and ~50 μm). Balblc mice were injected subcutaneously with 5 Lf (~15 μg) of ^{14}C-TT, encapsulated in MS, mixed with blank MS without encapsulated antigen, as soluble antigen or adsorbed onto aluminum phosphate ($AlPO_4$) and radioactivity was monitored at the site of injection, draining lymph nodes, blood, liver, spleen, and kidneys at various intervals. At one day, ~95% and 90% radioactivity disappeared from site of injection for soluble TT or blank MS mixed TT and $AlPO_4$ adsorbed TT, respectively, whereas ~55% and 70% radioactivity disappeared from site of injection for MS of average size ~50 μm and ~10 μm, respectively. By 7 days, 99% of radioactivity disappeared from site of injection for soluble TT or blank MS mixed TT, whereas 2–3% radioactivity persisted at the site of injection for $AlPO_4$ adsorbed TT for 4 weeks. In contrast, ~20% radioactivity stayed at the site of injection for MS injected mice up to 4 weeks. At all time points, large MS (~50 μm) showed more radioactivity at the site of injection than small MS (~10 μm). Other organs showing radioactivity were draining lymph nodes and kidneys. Small MS with encapsulated TT showed highest level of radioactivity in lymph nodes at 4 h. In kidneys, soluble and $AlPO_4$ adsorbed TT showed a peak of radioactivity at 4 h whereas TT encapsulated in MS showed a peak of radioactivity at 7 days. These results indicate that $AlPO_4$ did not act as a depot for TT at the site of injection, but TT encapsulated in MS did form a depot for approximately 1 month. Copyright © 1996 Elsevier Science Ltd.

Keywords: Microspheres; tetanus toxoid; aluminum phosphate; radioactivity; distribution

In recent years, development of controlled release vaccines using biodegradable polymer microspheres (MS) received much attention due to their promise of continuous release of vaccine antigens from the site of injection for extended periods, thus avoiding repeated injections to achieve protection[1–3]. Depot formation by the adjuvanted vaccines at the site of injection is considered one of the mechanisms of enhanced immune response by several adjuvants including MS (10 μm) made up of poly(D,L-lactide-co-glycolide) (PLGA)[3,4]. *In vitro* release studies of proteins from MS preparations at 37°C have shown sustained release for extended periods depending upon the characteristics of MS[5–7]. The release of small drugs, peptides, hormones from MS has been controlled from a few hours to weeks and even for months both *in vitro* and *in vivo*[8–10]. As far as we know, there is no *in vivo* study about distribution of large proteins such as tetanus toxoid (TT) when injected to mice as encapsulated within PLGA MS. In the present study, we describe distribution of radiolabeled (^{14}C) TT after its injection to mice as soluble protein, adsorbed onto aluminum phosphate ($AlPO_4$) or encapsulated in PLGA MS.

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MATERIALS AND METHODS

Reagents

Chromatographically purified tetanus toxin (purity 2000 Lf mg^{-1} protein nitrogen) was provided by Bradford Rost of our Laboratories. ^{14}C-labeled formaldehyde (radioactivity 43.6 mCi mmol^{-1}) and gelatin (type A, porcine, 300 bloom) were purchased from

Sigma Chemical Company, St. Louis, MO. PLGA (lactide-glycolide ratio of 50:50; inherent viscosity 0.65 dl g^{-1}) was from Birmingham Polymers, Birmingham, AL. Methylene chloride was from J.T. Baker, Phillipsburg, NJ, polyvinyl alcohol (88% hydrolyzed, M_w ~78000) from Polysciences, Inc., Warrington, PA, tissue solublizer (Solvable) from Du Pont, Garden City, NY and liquid scintillation cocktail (Ultima Gold) from Packard Instruments, Meriden, CT.

Mice

Female Balb/c mice, 4 weeks old, were purchased from Charles River, Wilmington, MA.

Preparation of ^{14}C-labeled tetanus toxoid (^{14}C-TT)

^{14}C-TT was prepared by detoxifying chromatographically purified toxin with formaldehyde according to slightly modified procedure used in our laboratories for preparing stable TT. Briefly, ^{14}C-labeled formaldehyde was added to tetanus toxin (500 Lf ml^{-1} in 1% sodium bicarbonate solution, pH 8.55) to get a 0.008% final concentration of formaldehyde. The mixture was incubated at 37°C for 1 week with an aim of putting maximum molecules of ^{14}C-formaldehyde onto tetanus toxin. Then cold formaldehyde was added to achieve a final concentration of 0.07% to assure complete detoxification of the toxin and the mixture was incubated at 37°C for two more weeks. The solution was exhaustively dialyzed against normal physiological saline (0.85% NaCl) containing 0.01% thimerosal to remove free formaldehyde and sterile filtered with 0.22 μm filter. The radioactivity in ^{14}C-TT was 11100 counts per minute (c.p.m.) per μg of protein. We could put approximately 16 molecules of ^{14}C-formaldehyde to each molecule of tetanus toxin. In a completely detoxified toxin a total of 60 molecules of formaldehyde bound to each molecule of toxin[11] indicating that we put approximately one fourth molecules of ^{14}C-formaldehyde and remaining molecules of cold formaldehyde on tetanus toxin. More ^{14}C-formaldehyde molecules could be bound to tetanus toxin by using only radiolabeled formaldehyde for complete detoxification. This would be very expensive and since the levels of radioactivity on ^{14}C-TT prepared by our method were suitable for our studies, we did not attempt to put more radiolabeled formaldehyde on TT.

^{14}C-TT at 10 Lf ml^{-1} was adsorbed onto freshly prepared $AlPO_4$ gel (4 mg ml^{-1}) as described[12]. The adsorption of TT was 95% when assayed by monitoring radioactivity and antigenically active TT (by ELISA[12,13]) in the supernatant of adsorbed preparation.

The stability of the ^{14}C label on the toxoid was evaluated after storing ^{14}C-TT at 4°C for 6 months. ^{14}C-TT was precipitated with 50% saturated ammonium sulfate solution and radioactivity was monitored in the precipitates and supernatant. More than 90% of radioactivity was found in the precipitate whereas ^{14}C-formaldehyde treated similarly with saturated ammonium sulphate showed 100% radioactivity in the supernatant. Secondly, $AlPO_4$ adsorbed ^{14}C-TT after storage at 4°C for 6 months was evaluated for antigenically active TT in supernatant by ELISA[12,13] and monitored for radioactivity in the gel and the supernatant. The supernatant showed 3% antigenic TT and 7% radioactivity indicating 95% of radiolabeled formaldehyde associated with TT on the gel. As a control, ^{14}C-formaldehyde adsorbed onto $AlPO_4$ under similar conditions as ^{14}C-TT showed 98% radioactivity in the supernatant indicating nonadsorption of free formaldehyde onto $AlPO_4$ gel. Storage of $AlPO_4$ adsorbed ^{14}C-TT at 37°C for 5 days did not show any increase in radioactivity in supernatant further confirming that ^{14}C-label on TT was stable. Additional evidence on the stability of ^{14}C-label on TT was from nonreversion of TT to toxin when mice injected with ~15 μg of ^{14}C-TT did not show any symptoms of tetanus for 3 months. We have observed that partially detoxified purified tetanus toxin shows reversion when injected to animals in doses even lower than 15 μg per mouse[14] (unpublished data).

Preparation of ^{14}C-TT microspheres

^{14}C-TT MS were prepared by the double emulsion water-in-oil-in-water method using solvent evaporation technique[5,6,13]. Briefly, 200 μl of ^{14}C-TT or saline (for blank MS without encapsulated antigen) and 0.35 mg gelatin were emulsified in 2 ml of methylene chloride containing 400 mg PLGA by homogenization at 15000 revs min^{-1} for 30 sec at 4°C. The water-in-oil emulsion was added to 15 ml of 1% polyvinyl alcohol and homogenized at 6000 revs min^{-1} for 40 sec for large MS or at 15000 revs min^{-1} for 45 s for small MS. This double emulsion was poured into 150 ml water and stirred for 2 h to evaporate the solvent. The MS were collected by centrifugation, washed with distilled water, and freeze-dried.

The size of the MS preparations was determined by a light microscope with micrometer against calibrated polystyrene MS (Polysciences, Inc., Warrington, PA). The surface morphology was evaluated by scanning electron microscopy and the protein loading was determined by complete digestion of MS with 6 N HCl at 60°C overnight[13]. The *in vitro* release of total protein from MS was studied by incubating the MS preparations in phosphate buffered saline, pH 7.2 (PBS) at 37°C on a shaker[13]. At various intervals, the suspension was centrifuged, the supernatant assayed for total protein by micro-BCA (Pierce, Rockford, IL) and MS suspended in fresh PBS.

Bio-distribution of ^{14}C-TT in mice

Groups of mice were injected subcutaneously, anterior the right hind limb, with 5 Lf (~15 μg of protein) of ^{14}C-TT as soluble protein, adsorbed onto $AlPO_4$, encapsulated in MS of average size ~10 μm and ~50 μm and mixed with blank MS of average size ~10 and ~50 μm. The site of injection was marked with 0.5% picric acid. A group of normal mice served as a control for background radioactivity of various organs. Two animals from each group were killed at day 1, 3, 7, 14, 28, and 70 and tissue from the site of injection, local draining lymph nodes (popliteal, inguinal, iliac and renal), blood, liver, and kidneys were collected from each animal. A portion of blood was used for serum collection. All the tissue samples were chopped and digested with tissue solubilizer and radioactivity was counted on a liquid scintillation counter (Packard Instruments) and mean of radioactivity counts for two animals was calculated. The serum samples were assayed for tetanus toxin IgG antibodies by ELISA[12]. As

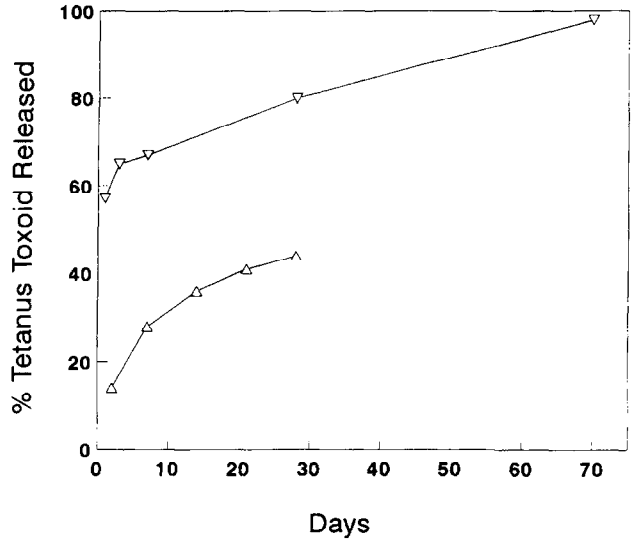

Figure 1 *In vitro* release of total protein in PBS pH 7.2 at 37°C (△) and clearance of radioactivity from the site of injection in mice (▽) injected with MSs (average size ~50 μm) containing ^{14}C-TT

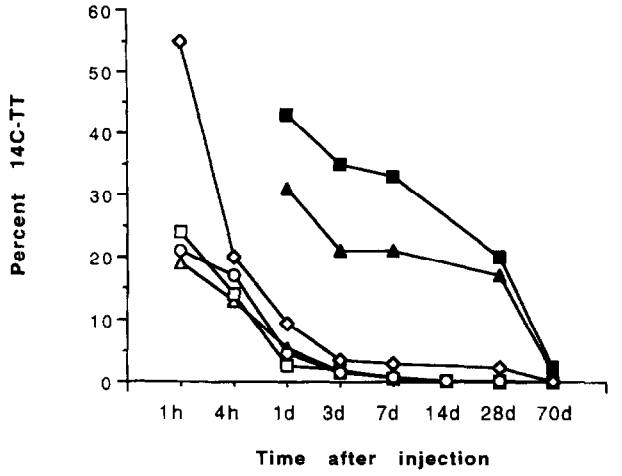

Figure 2 Radioactivity at the site of injection in mice inoculated subcutaneously with 5 Lf (~15 μg) of soluble TT (○), $AlPO_4$ adsorbed TT (◇), large MS-TT (■), small MS-TT (▲), mixture of large blank MS and TT (□) and mixture of small blank MS and TT (△)

majority of radioactivity had disappeared from the site of injection within 24 h for mice injected with soluble TT, $AlPO_4$ TT, or TT mixed with blank MS, another experiment was set up with these groups where the tissues samples were collected at 1 and 4 h after injection.

RESULTS

Characteristics of microspheres

The large MS preparations, both blank and with ^{14}C-TT, had an average size of ~50 μm and the small MS preparations had an average size of ~10 μm. All the MS preparations were spherical with smooth surface as observed by scanning electron microscope. The *in vitro* release studies on large MS containing TT (average size ~50 μm) showed continuous release of protein for 4 weeks with an initial burst of 14% (*Figure 1*).

Distribution of radioactivity in mice

Figure 2 shows mean radioactivity at the site of injection in mice inoculated with various formulations. The majority of the soluble TT or TT mixed with blank MS (~80%) disappeared from the site of injection within 1 h of injection whereas 80% of TT adsorbed onto $AlPO_4$ was cleared in 4 h. At day 1, ~95, 90, ~55, and 70% of radioactivity disappeared from the site of injection for soluble TT or blank MS mixed TT, $AlPO_4$ adsorbed TT, large MS with encapsulated TT (~50 μm) and small MS encapsulating TT (~10 μm), respectively. By 7 days, most of radioactivity disappeared from site of injection for soluble TT or blank MS mixed TT and 2–3% radioactivity persisted at the injection site for $AlPO_4$ adsorbed TT for 4 weeks. In contrast ~20% radioactivity stayed at the site of injection up to 4 weeks for mice injected with MS encapsulating TT. For the first 4 weeks, large MS with encapsulated TT showed more radioactivity at the injection site than small MS. Gross evidence of $AlPO_4$ crystals were observed at the injection site 1 year after injection whereas no MS were found at the site of injection after 6 months.

Other organs showing significant levels of radioactivity were draining lymph nodes and kidneys (*Figure 3*). Soluble and $AlPO_4$ adsorbed TT appeared in lymph nodes at 1–4 h after injection with declining levels of radioactivity thereafter. Small MS with encapsulated TT showed highest level of radioactivity in lymph nodes. Radioactive levels in kidneys peaked at 4 h for soluble and $AlPO_4$ adsorbed TT and at 7 days for TT encapsulated in MS. Spleen and blood samples did not show significant levels of radioactivity above background levels in normal mice for all groups of mice. Liver samples from mice injected with soluble TT, $AlPO_4$ adsorbed TT or blank MS mixed TT showed significant levels of radioactivity at 4 h after injection, the levels declined at day 1 and were close to background levels at day 3 (data not shown).

Tetanus toxin IgG antibody response

Mice injected with soluble TT did not show detectable levels of tetanus toxin IgG antibodies and those injected with mixture of TT and blank MS showed low levels of tetanus toxin IgG antibodies at 10 weeks (*Table 1*). In contrast, mice injected with TT encapsulated in MS and adsorbed onto $AlPO_4$ showed high levels of tetanus toxin IgG antibodies at 4 and 10 weeks.

DISCUSSION

We have found that mice injected with TT encapsulated in MS showed sustained release of labeled protein from the site of injection as compared to soluble TT or $AlPO_4$ adsorbed TT. The aluminum adjuvants have been considered to act by depot formation at the site of injection by Glenny[15], but the depot theory was challenged by Holt[16] when it was shown that antibody formation continued even after removal of adjuvant–antigen depot from the site of injection. In the present study also, though $AlPO_4$ adsorbed TT stayed at the site of injection slightly longer than soluble TT, its clearance from the site of injection was much faster than TT encapsulated in MS. Aluminum adjuvants appear to act by a number of mechanisms, not only by depot formation at the site of injection[17]. Late peak (7 days) of radioactivity

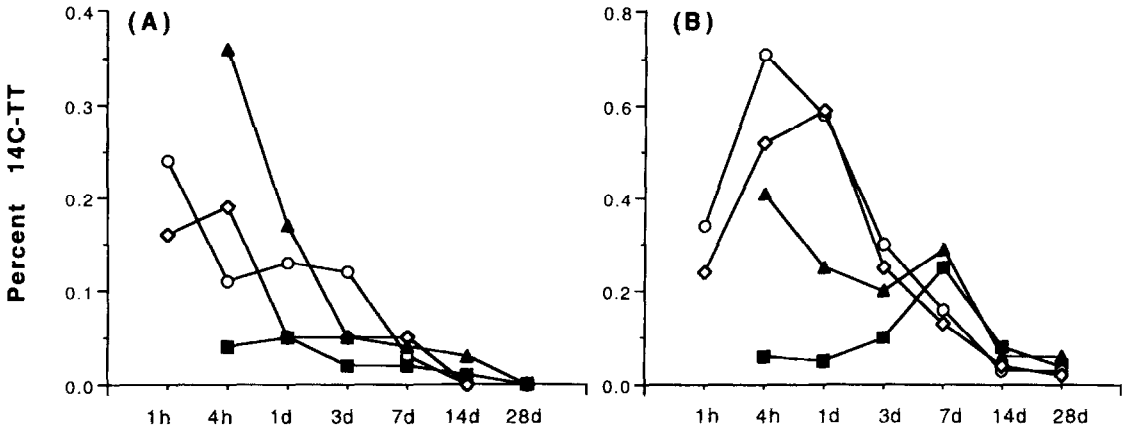

Figure 3 Radioactivity in draining lymph nodes (A) and kidneys (B) of mice injected subcutaneously with 5 Lf (~15 μg) of soluble TT (○), $AlPO_4$ adsorbed TT (◠), large MS-TT (■), small MS-TT (▲). Results for mixture of TT and blank MSs being very similar to those for soluble TT are not shown

Table 1 Tetanus toxin IgG antibodies in sera of mice injected with 5 Lf (~15 μg) ^{14}C-TT as soluble protein, adsorbed onto $AlPO_4$, mixed with blank MSs (MS+TT) or encapsulated in MSs (MS-TT)

Preparation	Mean tetanus toxin IgG antibodies (EAU ml^{-1})[a] at: 2 weeks	4 weeks	10 weeks
Soluble TT	N.D.	<0.001	<0.001
$AlPO_4$ TT	<0.001	0.65	2.70
MS-TT (~50 μm)	0.13	2.86	2.55
MS-TT (~10 μm)	0.36	1.26	3.53
MS+TT (~50 μm)	<0.001	N.D.	0.03
MS+TT (~10 μm)	<0.001	N.D.	0.06

N.D., Not done. [a]Mean of tetanus toxin IgG antibodies from two mice. The antibodies were determined by ELISA in ELISA Antitoxin Units (EAU) per ml as described[12]

in kidneys for TT encapsulated in MS in contrast to early peak (4 h) for soluble and $AlPO_4$ adsorbed TT also supports the slow release of antigen from MS preparations. From the present study, it appears that MS preparations are more efficient in forming a depot at the site of injection, thus slowly releasing the antigen over time which may be useful for developing vaccines requiring fewer injections to achieve protection. Another significant finding of this study is that $AlPO_4$ without antigen stayed at the site of injection for 1 year whereas MS disappeared from the site of injection in 6 months. Visscher *et al.*[18] reported disappearance of PLGA MS from the site of injection in mice at 8 weeks. The disappearance of PLGA MS from the site of injection depends upon the degradation rate of polymer. It is not known whether or how aluminum is metabolized in the body[19] and thus aluminum adjuvants are not considered biodegradable[17].

The amount of protein released from TT-containing MS in the *in vitro* release study was lower than the disappearance of radioactivity from the site of injection suggesting that protein is released faster from MS inside the body. Similar results were found by Alleman *et al.*[20] with a drug savoxepine. TT encapsulated in MS elicited tetanus toxin IgG antibodies in mice similar to that adsorbed onto $AlPO_4$ adjuvant. Though this aspect was not focus of this study, the antibody response was studied to show that the MS formulations used in this study show enhanced immunogenicity as found in our earlier reports[5,6]. Though MS preparations of varying size (large and small) showed differences in the release of antigen from the site of injection, there were no significant differences in the antibody levels elicited by MS of varying size[5,6]. Large MS (10 μm) are considered to form a depot at the site of injection whereas small MS ($\leq$10 μm) have additional adjuvant properties, such as targeting antigens to antigen presenting cells[2,21]. The antibody levels elicited by MS preparations, though significantly higher than those elicited by the soluble toxoid, were very similar to those elicited by the routine $AlPO_4$ adsorbed TT[6]. Therefore, it may be argued that MS preparations with encapsulated TT do not offer any advantage over the conventional $AlPO_4$ adsorbed vaccine. However, due to sustained release properties of MS formulations it may still be possible to reduce the number of injections required for primary immunization for routine vaccines by mixing aluminum adjuvants with MS formulations. Antigen adsorbed onto aluminum adjuvants would elicit a strong primary response and sustained release of antigen from MS preparations would act as a boost for the immune response. We are currently evaluating MS formulations mixed with aluminum adjuvants.

Finally, we conclude that the mechanism of adjuvant action of MS is different from that of aluminum adjuvants in that MS form a depot at the injection site from which antigen is released over prolonged periods of time whereas aluminum adjuvants do not. This finding does not preclude the possibility that MS have additional adjuvant mechanisms such as targeting antigens for antigen presenting cells[2,21] or activating such cells for more effective presentation and lymphokine production. Nevertheless, the qualitatively different mechanism of action of MS suggests that they might offer advantages over aluminum adjuvants in humans. Since the immunologic effects of adjuvants in humans cannot be predicted precisely from animal experiments[22], we propose that MS preparations be considered for human evaluation.

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