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Immunogenicity and Protection in Small-Animal Models with Controlled-Release Tetanus Toxoid Microparticles as a Single-Dose Vaccine

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Tetanus toxoid (TT) was encapsulated in microparticles prepared from polylactide-co-glycolide polymers by a solvent-evaporation technique. Combinations of small- and large-sized microparticles with controlled-release characteristics were used to immunize Sprague-Dawley rats, and the antibody responses were monitored for 1 year. For comparison, control groups of rats were immunized at 0, 1, and 2 months with TT adsorbed to alum. The antibody responses generated by the TT entrapped in microparticles were comparable to those generated by TT adsorbed to alum in control groups from 32 weeks onwards. Microparticles with a single entrapped antigen (TT) induced better antibody responses than microparticles with two antigens (TT and diphtheria toxoid) entrapped simultaneously. A combination vaccine consisting of TT adsorbed to alum and also entrapped in microparticles gave the best antibody responses. In an inhibition assay designed to determine the relative levels of binding of antisera to the antigens, the sera from the microparticle- and the alum-immunized animals showed comparable levels of binding. In addition, in a passive-challenge study with mice, TT adsorbed to alum and TT entrapped in microparticles provided equal levels of protection against a lethal challenge with tetanus toxin. An intradermal-challenge study was also performed with rabbits, which showed similar levels of protection in sera from alum- and microparticle-immunized animals at 4, 12, and 32 weeks after immunization.

Tetanus is a fatal bacterial disease whose clinical manifestations occur as a consequence of the release of a toxin by *Clostridium tetani*. The presence of toxin-neutralizing antibodies induced by active immunization with tetanus toxoid (TT) remains the most potent method of preventing this disease. TT has been used as a vaccine in humans since 1924, when Ramon (27) and Glenny and Hopkins (14) carried out formaldehyde detoxification of tetanus and diphtheria toxins, which destroyed their toxicities but retained their immunogenic properties.

Through the children's vaccine initiative, researchers have identified several areas in which the efficacies and cost-effectiveness of vaccines, including those against diphtheria, pertussis, and tetanus, can be improved (19, 31). The development of a single-dose vaccine for TT, which would eliminate the requirement for multiple injections and, therefore, prevent dropouts among subjects needing to be immunized, is currently a major focus for the World Health Organization. A single-shot vaccine would generate greater compliance from patients and would be more cost-effective.

The polylactide-co-glycolides (PLG) are biodegradable and biocompatible polymers which are nonimmunogenic and have a long history of safe use in humans as sutures and as controlled-release delivery systems (16, 26, 35–37). PLG polymers, e.g., Zoladex (Zeneca), Decapeptyl (Ipsen Biotech), and Prostap SR (Lederle), which are licensed for use in humans in Europe and the United States (6, 13), have been used to prepare controlled-release delivery systems. Consequently, there is growing interest in the development of microparticle formulations as vaccine-delivery systems (1–4, 11, 21, 23–25, 32). Microparticles have already been shown to be effective delivery systems for vaccine antigens and have been shown to induce potent immune responses (1–3, 23). In addition, several groups have evaluated the potential of controlled-release microparticles as single-dose vaccines (4, 11, 21, 24, 25, 32). The use of PLG microparticles as vaccine-delivery systems offers a number of potential advantages. The rate of release of antigens from PLG microparticles can be controlled by manipulating parameters such as polymer composition, molecular mass, and crystallinity (10, 16, 26). Therefore, the duration of release of the antigen can be varied from a few weeks to months. In addition, variation of the microparticle size can control particle uptake by antigen-presenting cells (34). Smaller microparticles (diameter, <10 μm) can be taken up by macrophages to promote antigen presentation, while larger microparticles (diameter, >10 μm) are not taken up by macrophages (11, 24, 25, 32) and are more effective as controlled-release delivery systems. Consequently, two or more batches of microparticles with different rates of antigen release may be prepared and combined to provide a single-dose vaccine.

Several groups in the past few years have reported preliminary observations of the effects of controlled-release vaccines based on microparticles (3, 4, 21). The issue of the stability of TT within the microparticles and during the process of microencapsulation has also been addressed recently (29, 30). Several formulation excipients and antigen-modification steps to improve the stability of TT within PLG microparticles have been described. However, none of the studies reported so far have evaluated protection from lethal toxin challenge after immunization with TT in microparticles. In our study, microparticles with entrapped TT were evaluated for immunogenicity in Sprague-Dawley rats. In addition, the protective efficacies and the neutralizing capacities of the antibodies generated by

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the controlled-release microparticles containing TT were also evaluated.

MATERIALS AND METHODS

Materials. The PLG polymers were obtained from Boehringer Ingelheim. The Resomers used in this study were RG505 (PLG lactide/glycolide ratio, 50:50 [hereinafter PLG50/50]; molecular mass, 65 kDa), RG858 (PLG lactide/glycolide ratio, 85:15 [hereinafter PLG85/15]; molecular mass, 108 kDa), and R208 (PLG lactide/glycolide ratio, 100:0 [hereinafter PLG100/0]; molecular mass, 138 kDa). The reagents for the enzyme-linked immunosorbent assay (ELISA) and the inhibition assay were obtained from KPL, Inc., Gaithersburg, Md. The U.S. standard tetanus toxin was obtained from the Center for Biologics Evaluation and Research (CBER) at the National Institutes of Health (NIH). All other reagents were obtained from Sigma Chemicals, St. Louis, Mo., and were used as shipped. Aluminum hydroxide gel (alhydrogel) was purchased from Superfos, Vedbaek, Denmark.

Antigen and dose. Diphtheria toxoid (DT) and TT were obtained from the Michigan Department of Public Health. The concentration of TT was 1,500 limits of flocculation (Lfs)/ml and that of DT was 2,200 Lfs/ml. The doses selected for TT immunization in this study were 15 Lfs in a single injection (with TT entrapped in microparticles) and 5 Lfs in each of three injections (with TT adsorbed to alum). The dose for TT was based on the currently used antigen dose for this vaccine in the United States.

Antigen characterization. The antigens were characterized by determining their protein contents by bicinchoninic acid (BCA) assay and by estimating their molecular masses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard prestained markers. A 10% gel was used for the electrophoresis under standard operating conditions. These parameters were also used later for estimating antigen integrity and the extent of aggregation after microencapsulation.

Preparation of microparticles. Microparticles were prepared by a solvent-evaporation technique as previously reported (17, 18). Briefly, the larger microparticles with a 2%, wt/wt, TT loading level were prepared by diluting the antigen solution to 2 ml and emulsifying the solution at high speed with a silverson homogenizer and 10 ml of a 10%, wt/vol, polymer solution in methylene chloride. The primary emulsion was then added to 50 ml of distilled water containing polyvinyl alcohol (10%, wt/vol). This resulted in the formation of a w/o/w emulsion, which was stirred at 1,000 rpm for 12 h at room temperature and from which the methylene chloride was allowed to evaporate. The resulting microparticles were filtered, washed twice in distilled water, lyophilized, and dried in a desiccator.

Smaller-sized microparticles were prepared in a similar manner, but the polymer concentration and stirring speeds were varied to obtain uniform microparticles with diameters of <10 μm. Both of the preparation methods for small and large microparticles had been standardized earlier with ovalbumin (20). Microparticles were also prepared in the same manner with both of the antigens (TT and DT) simultaneously entrapped within the same particles. An equimolar ratio of DT and TT was used to obtain a total theoretical loading level of 2%, wt/wt.

Particle size and surface morphology. The size distribution of the microparticles was determined with a particle size analyzer (Malvern Instruments, Malvern, United Kingdom). The surface morphology was determined by scanning electron microscopy (35 JEOL microscope) with a 100 Å gold-palladium coating.

Antigen loading levels. The loading level of the antigen within the biodegradable microparticles was determined by dissolving 20 mg of the microparticles in 2 ml of 5% SDS–0.1 M sodium hydroxide solution at room temperature. The amount of vaccine was determined by BCA protein assay.

Assessment of antigen integrity. The antigen integrity following microencapsulation was evaluated by SDS-PAGE and immunoblotting. The encapsulated antigen was released from PLG microparticles over 3 weeks at 37°C in phosphate-buffered saline (50 mM, pH 7.4) and concentrated by Amicon ultrafiltration before being evaluated by Western blot analysis. Hyperimmune serum to TT raised in BALB/c mice was used to bind to the antigens. Antigen samples were analyzed before microencapsulation and after release from the microparticles with a 12% gel in the Mini-Protean system from Bio-Rad. The standard immunoblot assay kit (Bio-Rad catalog no. 170-6463) was used. The blots were visualized with 4-chloro-1-naphthol.

Animals. Sprague-Dawley rats aged 6 to 8 weeks and weighing about 60 to 80 g were used for the study and were maintained in standard housing with a normal diet at HRP Inc., Denver, Pa. Each group consisted of eight animals in all of the studies. CD1 mice weighing 20 to 24 g each were used in the passive-challenge experiments and were housed at the small-animal facility at United Biomedical, Inc. The rabbits used in the intradermal-challenge study were housed at the National Vaccine and Serum Institute (NVSI) animal facility in Beijing, China.

Groups of rats were immunized with TT encapsulated in microparticles suspended in normal saline by the intramuscular route. The injection (500 μl) was administered at one site in a hind leg. TT adsorbed to alum was also administered in the same injection volume to different groups of rats.

Immunogenicities of individual microparticles containing TT. Two groups of eight rats were immunized with 15 Lfs of TT in either PLG50/50 (microparticle diameter, <10 μm) or PLG100/0 (microparticle diameter, >10 μm). The immunogenicities of these TT-microparticles were compared to the immunogenicities for the TT-alum-immunized animal group. The animals were bled through the retro-orbital plexus at periodic intervals for 1 year.

Immunogenicities of a combination of different types of microparticles containing TT. One group received microparticles prepared from three polymers, PLG50/50, PLG85/15, and PLG100/0, containing a total dose of 15 Lfs of TT. A second group received the same three types of microparticles, but these contained both DT (30 Lfs) and TT (15 Lfs) entrapped in them. A third group received 15 Lfs of TT, half of which was adsorbed to alum and half of which was entrapped in microparticles prepared from PLG50/50 and PLG85/15 (5 Lfs in each component). Both the PLG50/50 and the PLG85/15 microparticles were small in size (diameter, <10 μm). A fourth group received three individual doses of TT (5 Lfs each) on alum at monthly intervals. The animals were bled through the retro-orbital plexus at periodic intervals for 1 year.

Immunoassays. (i) ELISAs to detect IgG in serum. Sensitive enzyme immunoassays were developed to monitor serum anti-TT antibody titers in rats. These assays were direct ELISAs and utilized a goat anti-rat immunoglobulin G (IgG)–peroxidase conjugate and tetramethylbenzidine (TMB) as a substrate. The optical densities (ODs) of the plates were read at 450 nm. The reported antibody titers are serum $\log_{10}$ antibody titers for all time points.

(ii) Inhibition assay. Briefly, 96-well flat-bottom Nunc immunoplates were coated with 1 μg of TT per well and incubated overnight at 4°C. The plates were washed with washing buffer three times and incubated with bovine serum albumin blocking buffer for 2 h. Dilutions of antigen-antibody mixtures that contained anti-TT serum at a dilution capable of providing 50% binding in the presence of different concentrations of free tetanus toxin were added to each well, and the plates were incubated at 37°C for 2 h. After the plates were washed three times, 50 μl of horseradish peroxidase-conjugated goat anti-rat Ig (KPL 04-1612) was added to each well and the plates were incubated for another 2 h. The plates were washed again three times. Finally, the ODs of the plates were read at 450 nm after addition of the TMB substrate and TMB stop solution at requisite intervals. The percent inhibition of binding was calculated by the following equation: percent inhibition = (OD test value − OD background value)/(OD maximum − OD background value) where OD maximum represents the value obtained in the absence of competing toxin.

Evaluation of protection against toxin challenge in mice. Five groups of eight CD1 mice were used. Three groups were injected subcutaneously with a 0.5-ml mixture of test sera and standard tetanus toxin (CBER, NIH) in normal saline after incubation at 37°C for 1 h. The three serum samples evaluated were obtained from blood samples taken at 12 weeks from rats immunized with microparticle formulations (TT entrapped in microparticles and TT entrapped in microparticles and adsorbed to alum) and from the control group of rats immunized three times with TT adsorbed to alum. Two additional control groups were administered the following: a mixture of standard toxin with no serum for one group and standard toxin mixed with a standard antitoxin (CBER, NIH) for the other group. The 0.5-ml volumes of mixtures were administered following incubation at 37°C for 1 h. All groups were monitored for mortality for 96 h.

Evaluation of protection against toxin challenge in rabbits. Test sera (0.1 ml) and the specific toxin (tetanus toxin, 0.1 ml), after incubation at 37°C for 1 h, were injected intradermally into the shaven skins of rabbits, and the erythematous areas around the site of injection were measured for each group. Positive and negative controls of the U.S. standard tetanus toxin and antitoxins (NVSI) were also evaluated to obtain a correlation between reference standards and the test samples. Data were recorded in international units. The test sera were evaluated based on the sizes of the skin reaction areas, which were the same size as or smaller than those of the group receiving the standard antitoxin dose (22).

RESULTS

Antigen integrity and microparticle characterization. The antigenic integrity of DT and TT before and after microencapsulation was unaltered, as shown by Western blot analysis of the antigens released from the microparticles over several days (results not shown).

SDS-PAGE analysis of both DT and TT showed molecular masses of about 150 kDa for TT and 62 kDa for DT (results not shown). The method used to prepare the microparticles for TT and DT resulted in uniform batches of microparticles. The mean diameters for the smaller-sized microparticles ranged from 0.35 to 0.88 μm, whereas the mean diameters for the larger-sized microparticles ranged from 26.0 to 37.0 μm. The loading efficiency for TT in both the small and the large microparticles varied from 80 to 94% as determined by BCA assay. The surface morphologies of both small- and large-sized microparticles were highly regular and smooth, as seen under scanning electron microscopy.

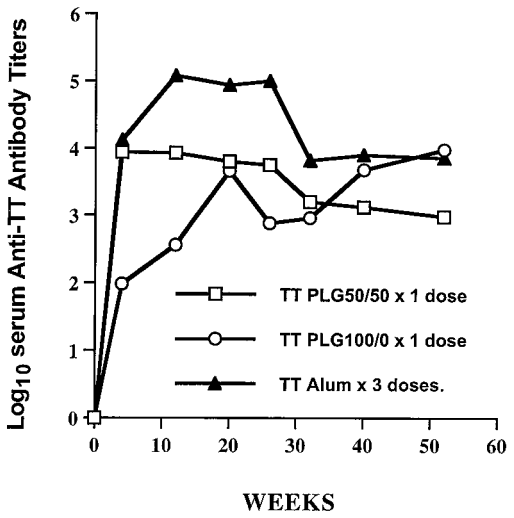

FIG. 1. Antibody responses in rats following immunization with controlled-release TT-microparticles prepared from two polymers, PLG50/50 and PLG100/0. The alum group was injected three times at 0, 4, and 8 weeks. Groups of rats were immunized with TT in microparticles suspended in normal saline by the intramuscular route. The injection (500 μl) was administered at one site in a hind leg. Alum was also administered in the same injection volume to different groups of rats. The titers are $\log_{10}$ antibody titers in pooled sera.

Evaluation of the immunogenicity of single-polymer TT microparticles. The objective of this study was to determine the immunogenicity of microparticles prepared from a single polymer with entrapped TT. The objective was to evaluate the feasibility of making a controlled-release TT vaccine with a single polymer only.

Figure 1 shows the anti-TT antibody responses induced with microparticles prepared with single polymers. Microparticles prepared with PLG50/50 showed an early peak of antibody, followed by a rapid decline. In contrast, microparticles prepared from PLG100/0 showed a delayed peak of antibody response. In both cases, the single-polymer microparticles were less effective than three doses of TT on alum.

Evaluation of the immunogenicities of combined microparticles. Based on the results from the above-described experiments, it was clear that a single-polymer formulation would not provide an effective controlled-release formulation. Therefore, it was decided to evaluate a combination of three polymer types administered simultaneously. The objective of this study was to evaluate the immunogenicities of candidate formulations of TT in microparticles and to compare the responses obtained to those obtained with three doses of TT on alum at monthly intervals. Each microparticle formulation for TT consisted of a combination of microparticles prepared from three different polymers (PLG50/50, PLG85/15, and PLG100/0). All the microparticle groups in this study were immunized with small microparticles (diameter, <10 μm) prepared from PLG50/50 and larger microparticles (diameter, >10 μm) prepared from PLG85/15 and PLG100/0.

The group of rats which were immunized with TT combined with microparticles showed sustained levels of anti-TT antibodies, which were comparable to the levels induced by TT adsorbed to alum from week 26 onwards (Fig. 2). However, the anti-TT antibody response induced by microparticles containing two antigens (TT and DT) was not as high as that induced by the single antigen. The group receiving the alum-microparticle combination as a single injection gave the best response in comparison to that of the three-injection alum control.

Inhibition assays. To assess the specificities of the anti-TT antibodies generated by the microparticle formulations and to compare these specificities with those of the alum-immunized rats, we developed an inhibition assay based on the one previously reported by Men et al. (21). In this assay, anti-TT serum was incubated with free toxin, which was recognized by the antibodies. This antigen-antibody complex was then incubated in an ELISA plate with TT adsorbed to it, so that the excess serum could bind to the TT. Therefore, a reduction in OD values in the subsequent ELISA would be observed only if the serum had successfully bound to the free toxin. Hence, binding of the free toxin to the test antiserum would limit the availability of antibodies to bind to the TT adsorbed to the plate. The percent inhibition of binding to TT was evaluated at various TT concentrations. The binding curves obtained (results not shown) in the inhibition assays with sera from rats immunized with TT in microparticles and those obtained for the groups immunized with three doses of TT-alum were similar, suggesting that the sera from both groups had the same inhibition profiles.

Evaluation of protection against toxin challenge in mice. A passive-challenge study was performed with mice to evaluate the presence of neutralizing antibodies to TT. The passive-challenge protocol has been described by researchers at CBER, NIH (22), and has previously been used by other groups (15, 28).

Table 1 shows the results from the passive-challenge study with mice. Both the negative and the positive control groups (groups 1 and 2, respectively) gave the expected results, with total mortality in the first group and total protection in the second group. The week 12 sera from rats immunized with three doses of alum (group 3) or microparticle formulations (TT entrapped in microparticles [group 4] and TT entrapped in microparticles and adsorbed to alum [group 5]) also showed complete protection from the lethal toxin challenge.

Evaluation of protection against toxin challenge in rabbits. An alternative passive-challenge assay (15, 22, 28) which uti-

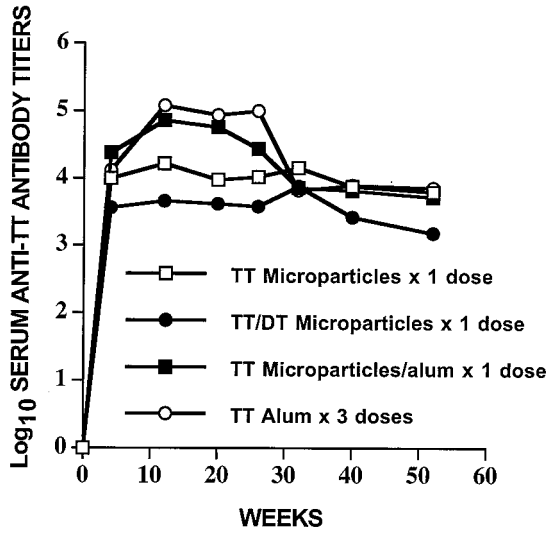

FIG. 2. Antibody responses in rats following immunization with controlled-release TT-microparticles prepared by combining three polymer types, PLG50/50, PLG85/15, and PLG100/0. One group received a combination of two antigens (TT and DT) within the same microparticles. Another group received a combination of TT adsorbed to alum and TT entrapped in microparticles as a single injection. The last group was injected three times at 0, 4, and 8 weeks with divided doses of TT on alum. The titers are $\log_{10}$ antibody titers in pooled sera.

TABLE 1. Results of passive-challenge study of mice with TT sera and standard toxin[a]

Group	Sample injected	No. of animals dead in 96 h/total no. of animals	Time of death (no. of animals)
1	Std. toxin plus normal saline	6/6	24 h (5) and 48 h (1)
2	Std. toxin plus Std. antitoxin plus normal saline	0/6	NA
3	Std. toxin plus test serum (TT-alum) plus normal saline	0/6	NA
4	Std. toxin plus test serum (TT-microparticles) plus normal saline	0/6	NA
5	Std. toxin plus test serum (TT-microparticles and -alum) plus normal saline	0/6	NA

[a] Three groups of six mice were injected subcutaneously with a 0.5-ml mixture of test sera and standard tetanus toxin (CBER, NIH) in normal saline after incubation at 37°C for 1 h. The three serum samples evaluated were obtained at 12 weeks from rats immunized with microparticle formulations (TT entrapped in microparticles [group 4] and TT entrapped in microparticles and adsorbed to alum [group 5]) and from the control group of rats immunized three times with TT adsorbed to alum (group 3). Two additional control groups of six mice each were administered the following: a mixture of standard toxin with no sera in one group and standard toxin mixed with a standard antitoxin (CBER, NIH) in another group. The 0.5-ml volumes of mixtures were administered intravenously following incubation at 37°C for 1 h. All groups were monitored for mortality for 96 h. The number of dead animals is reported for each group. Std., standard; NA, not applicable.

lizes local skin reactivity in rabbits as a means of determining the presence of TT-neutralizing units was performed at the NVSI. Serum samples taken at 0, 4, 12, and 32 weeks from microparticle- and alum-immunized rats were evaluated in this assay.

Table 2 shows the intradermal-toxin-challenge results from experiments with rabbits and the TT sera. The results are calculated in international units and compared with results from an experiment with a standard reference antitoxin. By measuring the erythematous areas around the site of injection with the test serum-toxin mixture and by running the standard antitoxin-toxin mixture as a reference, the units of antitoxin in the test sera were calculated. The results showed the presence of increased levels of toxin-neutralizing antibody titers for TT. The levels of neutralizing anti-TT antibodies were reduced at week 32. However, the same trend of a reduction in titer over time was also seen for the groups immunized with TT adsorbed to alum.

DISCUSSION

Reproducible batches of both small- and large-sized microparticles containing entrapped TT were prepared by the solvent-evaporation technique. The mean diameter for the smaller microparticles was about 500 nm, the ideal size for phagocytosis by antigen-presenting cells (34). The larger microparticles had an average diameter of about 25 μm, which made them too large to be phagocytosed. Therefore, they were ideal for the controlled release of antigens over prolonged periods. The Western blots of TT and DT before and after microencapsulation looked comparable (results not shown), suggesting that antigen integrity had been maintained throughout the microencapsulation process. This is an important finding, especially with regard to TT, which has been reported to aggregate upon exposure to moisture and organic solvents (29, 30). It was thought likely that aggregation of TT in microparticles would result in lower levels of TT-neutralizing antibodies and in a reduction in protective efficacy. However, the results obtained here showed that microencapsulated TT generated toxin-neutralizing antibodies and protection from a lethal toxin challenge in two animal models. Moreover, the level of protection induced was comparable to that induced by three individual doses of TT adsorbed to alum.

The use of a single polymer to prepare the microencapsulated vaccine would be an attractive approach for the development of a controlled-release vaccine. Consequently, single-polymer microparticles were evaluated for immunogenicity in rats. However, the results obtained indicated that microparticles prepared from a single polymer were not as effective as formulations prepared from three different polymers for TT (Fig. 2). Nevertheless, the single-polymer microparticles were potently immunogenic and demonstrated controlled-release properties for the encapsulated antigens. As expected, the PLG50/50 microparticles gave an early peak of antibody response while the PLG100/0 microparticles gave a delayed peak of antibody response. These observations are entirely consistent with the degradation profiles of the polymers. PLG50/50 is a quickly eroding polymer and tends to release the antigen more rapidly than PLG100/0, which is a more hydrophobic polymer and degrades more slowly. Hence, the optimal approach for the development of a single-dose vaccine appears to involve the combination of microparticles prepared from several different polymers.

More-potent antibody responses were induced with a single antigen in the microparticles rather than with two antigens in the same microparticles. One possible explanation for this observation is the presence of antigenic competition between the two antigens within the same microparticles, resulting in poorer antigen presentation. It has previously been reported that the presence of more than one antigen in a multicomponent vaccine may result in reduced immunogenicity for all the antigens (5, 9, 12).

The addition of a portion of the total dose of antigen on alum in combination with the microparticles gave the best results with respect to induction of peak antibody titers and

TABLE 2. Results of intradermal-challenge study with rabbits[a]

Injection formulation	Neutralization titer (IU) at wk:			
	0	4	12	32
TT-PLG50/50 microparticles injected once	0.100	25	160	25
TT-microparticles (three polymers) injected once	0.100	40	150	55
TT-microparticles and TT-alum injected once	0.100	13	100	13
TT-alum injected three times	0.01	35	NA	30

[a] Test sera (0.1 ml) from groups administered TT entrapped in PLG50/50 microparticles; TT entrapped in PLG50/50, PLG85/15, and PLG100/0 microparticles and adsorbed to alum; and TT adsorbed to alum (administered in three injections) were incubated with the toxin (tetanus toxin, 0.1 ml) at 37°C for 1 h. These mixtures were injected intradermally into the shaven skins of rabbits, and the erythematous areas around the site of injection were measured for each group. Positive and negative controls of the U.S. standard tetanus toxin and antitoxins (NVSI) were also evaluated to obtain a correlation between reference standards and the test samples. Data were recorded in international units. The test sera were evaluated based on the skin reaction area, which was equal to or smaller than that of the group receiving the standard antitoxin dose. The week 12 sample for the group administered TT-alum was insufficient to run the assay (NA, not applicable).

duration of the response (Fig. 2). The addition of an alum component appeared to prime the immune system better than microparticles alone. Therefore, the addition of a portion of the total dose of antigen on alum appears to offer an advantage over all the other formulations evaluated. For a prophylactic vaccine, it is desirable to achieve high and long-lasting antibody titers for optimum protection in humans. In this regard, we feel that the alum-microparticle formulation is a better choice as it meets both of the requirements of high initial antibody titers and use in a single injection.

We have seen previously with hepatitis B surface antigen that splitting the total alum-microparticle formulation between two sites in an animal, such that one site received the alum component and the other received the microparticles alone in saline, did not produce any difference in the antibody response (33). Therefore, the alum-microparticle formulation can be given as a single injection at a single site only.

The inhibition assay results indicated that the sera from both the microparticle- and the alum-immunized animals bound to the native toxin to the same extent. This suggested that the microparticle formulations generated qualitatively similar antibody responses to the alum formulations. The ability of the serum to bind with the toxin is critical, since following infection, these antibodies are required to neutralize the toxin in vivo to provide protective immunity. These results also suggest that microencapsulation does not result in complete denaturation of TT and that microencapsulated toxoids can induce toxin-specific antibodies.

The passive-challenge study with mice and tetanus toxin (Table 1) showed that the microparticle formulation with entrapped TT induced protective immunity against toxin challenge. Hence, the ability of TT in microparticles to provide protection after a lethal toxin challenge is reported for the first time. These observations suggest that microencapsulated TT induces toxin-neutralizing antibodies in amounts comparable to those induced by the traditional immunization schedule with the TT adsorbed to alum.

The intradermal-challenge study with rabbits (Table 2), which was performed with TT in microparticles, showed that neutralizing titers peaked at around 12 weeks and then diminished until week 32. These results suggested that the antigen being released from the microparticles at later time points may have had reduced capacities to induce neutralizing antibodies. This could be due to a reduction in the quality of antigen being released due to aggregation, following incubation in vivo at 37°C. Nevertheless, a similar observation was also made for the alum-immunized group, which showed a reduction in toxin-neutralizing capacity over time. Earlier studies with TT also compared the immunogenicity of TT in microparticles with that in formulations with alum as the adjuvant (3, 4, 21). However, the TT antibody responses observed in the studies described in this paper appear to be higher than those reported earlier (3, 4, 21). This is probably a consequence of the optimal selection of polymeric components for microparticle preparation and the preparation of microparticles with optimal size and release characteristics. The induction of toxin-neutralizing antibodies with microencapsulated toxoids and the induction of passive protection against toxin challenge have not previously been reported.

Controlled-release TT-microparticles prepared by the same method have undergone detailed toxicological evaluation in rats (7, 8) and have been shown to be free from any major side effects. These formulations have also been shown to be free from embryotoxicity and teratogenic effects in wistar rats. These encouraging results demonstrate the safety of biodegradable microparticles for human use.

In conclusion, we have demonstrated the viability of the development of a single-shot vaccine as an important pediatric vaccine. The optimal formulation evaluated was a combination of alum and microparticles administered at a single site. Further evaluations of these multicomponent microparticle formulations are recommended for larger animal models.

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