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Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies

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Microspheres composed of biocompatible, biodegradable poly(DL-lactide-co-glycolide) (DL-PLG) and staphylococcal enterotoxin B (SEB) toxoid were evaluated as a vaccine delivery system when subcutaneously injected into mice. As measured by circulating immunoglobulin G (IgG) antitoxin titers, the delivery of SEB toxoid via DL-PLG microspheres, 1 to 10 μm in diameter, induced an immune response which was approximately 500 times that seen with nonencapsulated toxoid. The kinetics, magnitude, and duration of the antitoxin response induced with microencapsulated toxoid were similar to those obtained when an equal toxoid dose was administered as an emulsion with complete Freund adjuvant. However, the microspheres did not induce the inflammation and granulomata formation seen with complete Freund adjuvant. The adjuvant activity of the microspheres was not dependent on the superantigenicity of SEB toxin and was equally effective at potentiating circulating IgG antitrinitrophenyl levels in response to microencapsulated trinitrophenyl-keyhole limpet hemocyanin. Empty DL-PLG microspheres were not mitogenic, and SEB toxoid injected as a mixture with empty DL-PLG microspheres was no more effective as an immunogen than toxoid alone. Antigen-containing microspheres 1 to 10 μm in diameter exhibited stronger adjuvant activity than those >10 μm, which correlated with the delivery of the 1- to 10-μm, but not the >10-μm, microspheres into the draining lymph nodes within macrophages. The antibody response induced through immunization with microencapsulated SEB toxoid was protective against the weight loss and splenic Vβ8$^+$ T-cell expansion induced by intravenous toxin administration. These results show that DL-PLG microsphere vaccine delivery systems, which are composed of pharmaceutically acceptable components, possess a strong adjuvant activity for their encapsulated antigens.

Recent scientific advances have provided information relevant to the design of vaccines for use against a wide variety of infectious agents (16). As a result, numerous vaccine antigens are being identified and produced in the form of subunits, synthetic peptides, and proteins expressed in a variety of vectors through recombinant genetics. Although these new antigens offer advantages in the selection of antigenic epitopes and safety, they are in many cases weakly immunogenic. This lack of immunogenicity has created an acute need to identify pharmaceutically acceptable delivery systems or adjuvants for these antigens. Among the potentiators of antibody responses under active investigation are those such as detoxified lipopolysaccharides (4, 25) and muramyl dipeptides (2, 5), which function through direct effects on lymphoreticular cells. Others, such as aluminum compounds (11, 37), nonionic block-polymer surfactants (40), liposomes (1, 28), oil emulsions (12), and controlled-release implants (23), function as antigen depots with directed delivery to draining lymph nodes. However, potentiators such as the lipopolysaccharides and muramyl dipeptides are nonspecific activators of the immune system, while the others suffer from problems of stability and/or consist of materials requiring extensive toxicologic evaluation. Currently, aluminum hydroxide and aluminum phosphate are the only adjuvants approved for use in humans, but they cannot be lyophilized and are not effective adjuvants for many vaccines and their use for booster immunizations has been questioned (3).

While examining the potential of poly(DL-lactide-co-glycolide) (DL-PLG) microspheres as an oral vaccine delivery system (7, 8), we noted a strong adjuvant activity when a microencapsulated toxoid of staphylococcal enterotoxin B (SEB) was administered by intraperitoneal injection. Several aspects of these microspheres make them attractive as an adjuvant for human vaccines. One important aspect is that DL-PLG is in the class of biodegradable and biocompatible copolymers from which resorbable sutures, resorbable surgical clips, and controlled-release implants are made (24). These copolymers are approved for, and have a history of safe use in, humans. When a vaccine is microencapsulated with DL-PLG, the vaccine is dispersed within the DL-PLG matrix of the microsphere in a dry state, thus providing extended shelf life without the need for stabilizers or a cold chain. After introduction into the body, DL-PLG induces only a minimal inflammatory response and biodegrades through the hydrolysis of its ester linkages to yield biocompatible lactic and glycolic acids (34). In the study presented here, we examined the immunopotentiating activity of DL-PLG microspheres with SEB toxoid when injected into solid tissue and have addressed the mechanism through which the toxin-neutralizing antibody response is heightened.

SEB is one of a group of enterotoxins produced by

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Staphylococcus aureus which cause food poisoning (32), and these toxins are mitogenic for the T cells of a variety of species (22). Recent studies have demonstrated that SEB directly binds to the major histocompatibility complex class II molecules of humans and mice and that binding to the surface of antigen-presenting cells via this mechanism is a prerequisite for polyclonal T cell stimulation (13, 31, 39). The T cells which proliferate in response to this combination of SEB and the major histocompatibility complex are restricted in their T cell receptor variable-region structure. In mice, those T cells bearing Vβ3, -7, and -8 are stimulated, while those bearing Vβ6 are not (39). The site where SEB binds to the T cell receptor appears to be on an exposed surface and not the region which conventionally engages antigenic peptides in the context of the major histocompatibility complex (18). Other manifestations of SEB toxicity in mice include weight loss and thymic atrophy, effects which have been linked to lymphokines released as a result of T cell activation (17). This unique mechanism of recognition and response by the immune system is the basis for the enterotoxins being termed superantigens.

MATERIALS AND METHODS

Mice. Specific-pathogen-free BALB/c mice, both male and female, were used throughout these experiments. They were bred and maintained in our barrier facilities at the University of Alabama at Birmingham. They were allowed food and water ad libitum and were entered into experimental protocols at 8 to 12 weeks of age.

Antigens. SEB, purified by the method of Schantz et al. (30), was provided for these studies by the Bacteriology Division of the U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Md. A single band of Coomassie blue staining material was detected after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A formalinized vaccine of SEB was prepared as described by Warren et al. (38). In brief, 1 g of toxin was dissolved in 0.1 M sodium phosphate buffer (pH 7.5) to a concentration of 2 mg/ml. Formaldehyde was added to this toxin solution to achieve a formaldehyde/toxin molar ratio of 4,300:1. The solution was slowly shaken in a 37°C controlled-environment incubator-shaker; the pH was monitored daily and maintained at 7.5 ± 0.1. After 30 days, the toxoid was concentrated and washed into borate-buffered saline (pH 8.4) (BBS) by use of a pressure-filtration cell (Amicon Corp., Danvers, Mass.) and sterilized by filtration. Conversion of the toxin to toxoid was confirmed by the absence of weight loss in 3- to 3.5-kg rabbits injected intramuscularly with 1 mg of toxoided material. Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were purchased from Calbiochem, La Jolla, Calif., and from Sigma, St. Louis, Mo., respectively. Trinitrophenyl (TNP)-KLH and TNP-BSA were prepared by the method of Rittenburg and Pratt (27). The degree of hapten substitution was estimated spectrophotometrically (26) and determined to be TNP_{861}-KLH and TNP_{21}-BSA.

Microspheres. SEB toxoid and TNP-KLH were microencapsulated in DL-PLG by an emulsion-based process which is a modification of a solvent extraction process which has been described in detail previously (6). In brief, concentrated aqueous solutions (300 μl; >30 mg/ml) of the antigens were mixed with 0.5 g of DL-PLG (Birmingham Polymers, Inc., Birmingham, Ala.) which had been dissolved in 4 g of reagent-grade methylene chloride (Eastman Kodak, Rochester, N.Y.). The polymer solution was then added, with mixing, to 60 ml of an 8% (vol/vol) aqueous solution of poly(vinyl alcohol) (Air Products and Chemicals, Allentown, Pa.) to produce an oil-in-water emulsion. The droplet size, and thus the size distribution of the microsphere product, was altered through adjustment of the stir rate during the emulsification process. The solvent was then removed from the microspheres by adding the emulsion to 3.5 liters of deionized water with stirring. The resulting microspheres were collected by centrifugation, washed twice in deionized water, and lyophilized. Size distributions of the preparations were obtained by use of a particle size analyzer (Malvern Instruments, Malvern, United Kingdom). Microspheres produced by this emulsion-based procedure have a Poisson distribution of sizes (7); the mean diameter, coefficient of variation (CV) of the diameters, and the extreme range of sizes (i.e., 1 to 10 μm or 10 to 110 μm) for each preparation are described in the text. The antigen content (core loading) was determined by dissolving a sample of the microspheres in methylene chloride, extracting the protein, determining the amount of protein by the BCA assay (Pierce Chemical Co., Rockford, Ill.), and calculating the percent antigen by weight. In vitro vaccine release kinetics were determined by a procedure which closely matches that described in the U.S. Pharmacopeia for dissolution testing of drugs. A sample of the microspheres was placed in a receiving fluid consisting of 0.5 M phosphate (pH 6.8), and the buffer was exchanged at 6 h, 24 h, and every 24 h thereafter until termination of the study. The amount of protein in the receiving fluids was quantified and related to the total protein in the sample of microspheres to determine the cumulative percent antigen release as a function of time. This assay does not necessarily provide an exact measure of antigen release in vivo; rather, it is used primarily to test for burst effects and to provide for batch-to-batch comparison. However, the in vitro release rate does provide data which are predictive of how individual batches release in vivo.

Immunizations. SEB toxoid was precipitated onto alum by slowly adding 1 ml of a 10% solution of $AlK(SO_4)_2 \cdot 12H_2O$ to 2.5 ml of a 1% solution of toxoid with stirring and adjusting the pH to 6.5 with NaOH (10). After 30 min, the suspension was harvested by centrifugation and the complete adsorption was confirmed by assaying the supernatant for protein. A water-in-oil emulsion consisting of equal volumes of toxoid in water (400 μg/ml) and complete Freund adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) was prepared with a probe sonicator. For parenteral administration, microspheres were suspended in phosphate-buffered saline (PBS) and soluble SEB toxoid and TNP-KLH were dissolved in PBS. Mice were immunized by subcutaneous injection of the adjuvanted or free antigens in a total volume of 0.25 ml. At the times indicated in the text, blood was collected in calibrated heparinized capillary pipettes by retro-orbital puncture under anesthesia and the plasma was harvested after centrifugation. All samples were frozen until assayed for antibody activity.

Antigen-specific antibody assays. Radioimmunometric assays (RIA) of toxin-specific antibodies were performed in Immulon strips (Dynatech Laboratories, Inc., Chantilly, Va.) coated with toxin (1 μg/ml) in BBS overnight at 4°C. Control strips were left uncoated, but all were blocked for 2 h at 25°C with 1% BSA in BBS, which was also used as the diluent for all samples and ^{125}I-labeled reagents. Various twofold dilutions of test samples were added to washed triplicate wells and incubated for 6 h at 25°C. After the wells were washed, 100,000 cpm of ^{125}I-labeled affinity-purified goat antimouse immunoglobulin G (IgG) heavy-chain-specific antibody (Southern Biotechnology Associates, Birming-

ham, Ala.) was added per well and incubated overnight at 4°C. After the removal of unbound antibodies by washing, the bound ^{125}I-antibodies were detected in a gamma spectrometer (Gamma 5500; Beckman Instruments, Irvine, Calif.). The results are presented as the reciprocal of the greatest sample dilution producing a signal significantly different from that of the group-matched prebleed at the same dilution (endpoint titration).

Quantitative enzyme-linked immunosorbent assays (ELISA) for TNP-specific IgM and IgG_1 were carried out in rigid 96-well assay plates (Pro-Bind; Becton Dickinson, Lincoln Park, N.J.) coated overnight with TNP-BSA at 1 μg/ml in BBS. All washing steps employed PBS containing 0.05% Tween 20, and the diluent for all samples and reagents was PBS-Tween with 1% BSA. After blocking, serial twofold dilutions of the plasma samples, in triplicate, were added and incubated at 25°C for 6 h. The TNP-binding antibodies were detected by sequential incubation with biotin goat anti-mouse IgM or IgG_1 heavy-chain-specific antibodies (overnight, 4°C; Southern Biotechnology Associates), horseradish peroxidase-streptavidin (2 h, 25°C), and the substrate 2,2′-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) at 0.3 mg/ml in citrate buffer (pH 4.0) containing 0.0003% H_2O_2. IgM and IgG_1 anti-TNP calibration curves were prepared by using dilutions of purified monoclonal antibodies 1B7.11 and 1F3-C1.10, respectively, which were purified in our laboratories (cell lines obtained from American Type Culture Collection, Rockville, Md.). The developed color was read after 15 min at 415 nm on a model EL312 kinetics reader (Bio-Tech Instruments, Inc., Winooski, Vt.), and the calibration curves and interpolations of unknowns were obtained by computer by using a log/log program (Δ Soft; BioMetallics, Inc., Princeton, N.J.).

Proliferative assay. Mice were killed by cervical dislocation, their spleens were aseptically removed, and single-cell suspensions were prepared by mechanically dispersing the tissue through sterile wire mesh into RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.). After two washes in RPMI medium, the cells were resuspended in RPMI medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, sodium bicarbonate, gentamicin, and 2% fetal calf serum. The cells were cultured (10^6/200 μl) for 48 h in 96-well, flat-bottomed plates (Falcon; Becton Dickinson) alone or with various dilutions of SEB toxin or toxoid in quadruplicate. The cultures were pulsed with 0.5 μCi of [^{3}H]thymidine for the final 18 h and then harvested onto glass fiber filters with a MASH II cell harvester (Microbiological Associates, Walkersville, Md.). DNA-incorporated [^{3}H]thymidine was measured by use of a scintillation counter (LS 8000; Beckman).

Analysis of Vβ expression. Spleen cells were isolated from normal mice and mice which had intravenously received various doses of SEB toxin or toxoid by mechanical dispersion as outlined above. After the erythrocytes were removed by lysis with ammonium chloride buffer (20), the cells were washed in PBS and resuspended in PBS containing 5% fetal calf serum and 0.1% NaN_3. Samples of 10^6 cells in 100 μl were incubated with saturating concentrations of a fluorescein isothiocyanate-conjugated monoclonal anti-CD3 (145-2C11) antibody in combination with either biotin–anti-Vβ8.1,8.2,8.3 (F23.1) or biotin–anti-Vβ6 (RR4) monoclonal antibodies (14, 15, 33). The cells reactive with the biotinylated antibodies were stained with phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates) and analyzed by use of a FACStar^{plus} flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

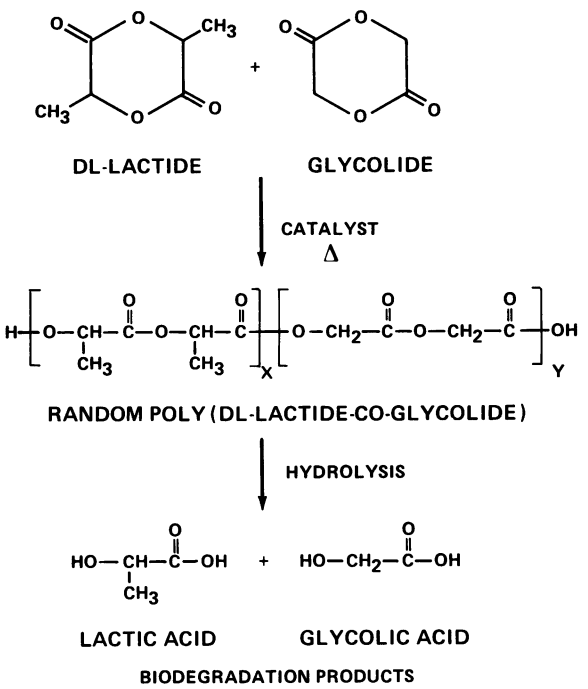

FIG. 1. Synthesis, structure, and biodegradation products of DL-PLG. Polymerization of DL-lactide and glycolide, the respective cyclic dimers of lactic and glycolic acids, into the high-molecular-weight DL-PLG through ionic, ring-opening, addition polymerization. Upon exposure to water, this aliphatic polyester degrades through hydrolysis of ester linkages to yield the biocompatible products lactic and glycolic acids.

RESULTS

Potentiation of the anti-SEB toxin response by microencapsulation of SEB toxoid. DL-PLG is a biodegradable and biocompatible polyester. Its properties make it suitable as an excipient for microspheres that can be used as a parenteral vaccine delivery system (35). More specifically, this copolymer exhibits excellent tissue compatibility (36) and biodegrades through random, nonenzymatic hydrolysis of ester linkages to yield lactic and glycolic acids, both normal metabolic compounds (Fig. 1).

To determine the degree to which the circulating antibody response to SEB can be potentiated when delivered via microspheres, mice were subcutaneously immunized with 50 μg of microencapsulated SEB toxoid vaccine. These microspheres were made from DL-PLG composed of equimolar ratios of DL-lactide and glycolide (50:50 DL-PLG), contained 1.76% (wt/wt) toxoid, and ranged in size from 1 to 10 μm (mean diameter, 2.6 μm; CV = 0.58). For comparison, groups of mice were immunized with the same dose of toxoid in PBS (50 μg shown to be maximally immunogenic in preliminary titrations) and two adjuvanted forms, one precipitated on alum and one emulsified in CFA. Plasma samples were collected at 10-day intervals, and their IgG antitoxin levels were determined by endpoint titration in an RIA employing solid-phase-adsorbed toxin (Fig. 2). Relative to the nonadjuvanted toxoid, the alum-precipitated form of the vaccine induced an eightfold increase in the peak IgG titer (3,200 versus 25,600) and sustained the antibody response. In comparison, the peak plasma IgG antitoxin titer induced by the toxoid delivered either in microspheres or CFA was

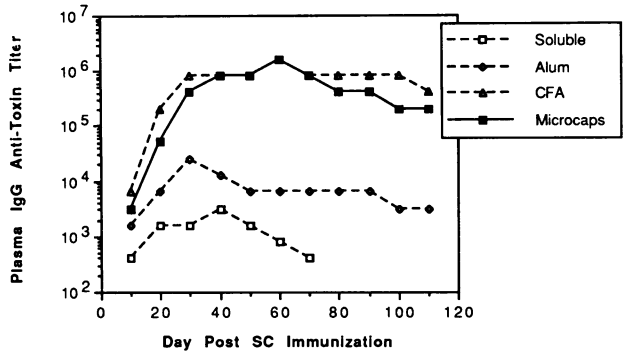

FIG. 2. Enhancement of the antibody response to SEB toxin through immunization with microencapsulated SEB toxoid. Groups of six mice were subcutaneously immunized with 50 μg of SEB toxoid in PBS, precipitated on alum, emulsified in CFA, or encapsulated in 1- to 10-μm microspheres (50:50 DL-PLG; 1.76% [wt/wt] SEB toxoid). Plasma samples were obtained at 10-day intervals, and the IgG antitoxin titer was determined by endpoint titration in an RIA with solid-phase-adsorbed SEB toxin.

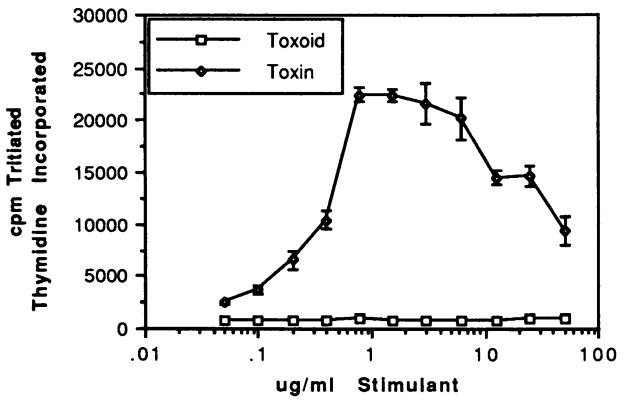

FIG. 3. Dose relationship of the proliferative response of mouse spleen cells to SEB toxin or toxoid. Quadruplicate 200-μl cultures, each containing 10^6 mouse spleen cells, were incubated with the indicated concentration of stimulant for 48 h and pulsed with 0.5 mCi of [^{3}H]thymidine for the last 18 h of culture. Results are presented as counts per minute (mean ± standard deviation) per culture.

more than 500 times the response to the free toxoid (3,200 versus 1,638,400). Although the CFA-adjuvanted vaccine appeared to be marginally better at maintaining a very high titer at later time points, it induced the formation of a large granulomatous lesion at the site of injection. In contrast, the microencapsulated toxoid caused no palpable lesion and no detectable inflammatory response.

SEB toxoid is not a superantigen. One possible factor contributing to the strong adjuvancy observed when SEB toxoid was administered in microspheres could be that this immunogen retains one or more of the stimulatory properties of the toxin. Among the activities associated with the administration of SEB toxin to mice are mitogenic activation of T cells (22) bearing particular Vβ segments (39) and weight loss after in vivo administration (17). However, each of these activities was found to have been abolished by toxoiding.

Cultured spleen cells proliferated in response to SEB toxin in a dose-dependent manner, with maximal [^{3}H]thymidine incorporation observed at a concentration of 0.8 μg/ml (Fig. 3). Parallel cultures into which the toxoid was titrated showed no proliferation above background at any dose up to and including 50 μg/ml. In additional experiments, it was found that neither empty microspheres nor microspheres containing SEB toxoid exhibited mitogenic activity in cultures of mouse spleen cells across a wide range of doses and times (data not shown).

Marrack et al. (17) recently demonstrated that mice injected intraperitoneally with SEB toxin lose weight as a function of the dose administered. These results were confirmed and extended to intravenously administered SEB toxin. A dose of 10 μg of toxin in 0.2 ml of PBS administered into the lateral tail vein reproducibly induced an 8 to 16% reduction in total body mass over a period of 2 to 3 days (Fig. 4). In contrast, 1,000 μg of toxoid administered in the same manner was without effect.

In addition, two-color flow cytometry was used to analyze the T-cell receptor Vβ expression of the splenic T cells 3 days after the intravenous administration of 10 μg of toxin or 1,000 μg of toxoid. At this time point, mice which had received toxoid exhibited a T-cell receptor Vβ distribution identical to that of normal BALB/c mice, with ~31% and ~14% of the CD3$^+$ spleen cells expressing Vβ8 and Vβ6, respectively. However, the spleens of the mice which had been treated with 10 μg of toxin were hypercellular, and the toxin-sensitive Vβ8 subpopulation had expanded to ~61% of the CD3$^+$ cells while the toxin-insensitive Vβ6 T-cell subpopulation had decreased proportionately.

Potentiation of the anti-TNP antibody response by microencapsulation of TNP-KLH. To ensure that the adjuvant activity imparted by microencapsulation was not unique to the SEB system, microencapsulated TNP-KLH was evaluated as an immunogen. Groups of mice were subcutaneously immunized with a maximally immunogenic dose (50 μg) of TNP-KLH in PBS or an identical dose in microspheres (mean diameter, 5.0 μm; CV = 0.49) suspended in PBS. Preimmunization and postimmunization plasma samples were evaluated for their levels of IgM and IgG$_1$ (the predominant IgG subclass in this response) anti-TNP antibodies in isotype-specific ELISA which employed solid-phase TNP-

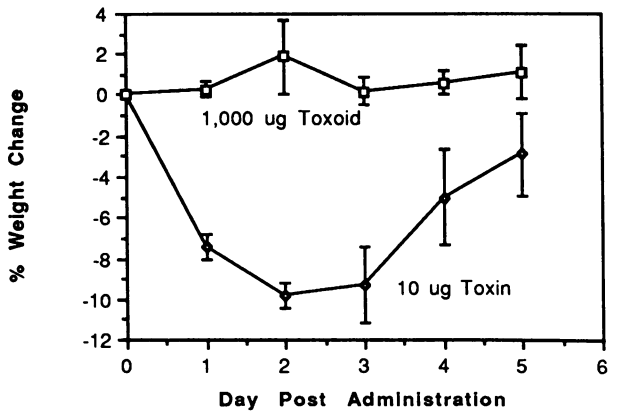

FIG. 4. Weight change as a function of time after the administration of SEB toxin or toxoid. Groups of six mice were intravenously injected with either 10 μg of SEB toxin or 1,000 μg of SEB toxoid in 0.2 ml of PBS on day zero. The results are presented as the percent weight change (mean ± standard deviation) calculated relative to the weight at day zero for each mouse.

TABLE 1. Effect of microencapsulation on the levels of anti-TNP antibodies induced by immunization with TNP-KLH

Immunogen	Plasma TNP-specific antibodies (μg/ml)[a]							
	Day 10		Day 20		Day 30		Day 40	
	IgM	IgG_1	IgM	IgG_1	IgM	IgG_1	IgM	IgG_1
TNP-KLH[b]	3.29 ± 0.16	5.64 ± 0.21	2.96 ± 0.09	3.44 ± 0.14	3.01 ± 0.28	2.93 ± 0.16	2.41 ± 0.34	1.28 ± 0.04
TNP-KLH microspheres[c]	2.73 ± 0.11	14.63 ± 0.93	3.37 ± 0.21	40.66 ± 2.91	3.19 ± 0.16	142.31 ± 13.40	2.40 ± 0.34	130.11 ± 8.79

[a] Anti-TNP antibodies determined in ELISA standardized to the TNP-specific monoclonal antibodies 1F3-C1.5 (IgM) and 1B7.11 (IgG_1). Prebleed levels of IgM and IgG_1 anti-TNP were <0.2 and <0.1 μg/ml, respectively, in both immunization groups.
[b] Subcutaneous immunization with 50 μg of TNP_{861}-KLH in 0.5 ml of PBS.
[c] Subcutaneous immunization with 9.26 mg of microspheres (50:50 DL-PLG; 1- to 10-μm diameter; 0.54% [wt/wt] TNP_{861}-KLH) containing 50 μg of TNP-KLH.

BSA (Table 1). The free and microencapsulated TNP-KLHs were equivalent in their capacity to induce an IgM response, with each causing the appearance of slightly more than 3 μg of IgM anti-TNP per ml of plasma. However, at all time points, the microencapsulated antigen induced a significantly higher level of IgG_1 anti-TNP. On days 30 and 40, the levels of plasma IgG_1 anti-TNP induced by the microencapsulated TNP-KLH were ~50 and ~100 times that stimulated by the nonencapsulated antigen, respectively. When tested in a TNP-specific endpoint titration assay analogous to that used to evaluate anti-SEB toxin responses, the day-30 and day-40 plasma samples from the mice immunized with microencapsulated TNP-KLH were found to have an IgG_1 anti-TNP titer of 819,600 (data not shown). Thus, the IgG responses induced to SEB toxin and TNP by immunization with microencapsulated SEB toxoid and TNP-KLH, respectively, are of approximately the same magnitude. These results show that the potentiation of antibody responses through delivery in microspheres is not restricted to SEB and may be extended to other protein antigens.

Mechanism of immune enhancement by microspheres. Immunologic adjuvants generally act through the direct activation of lymphoid cells or through a depot effect which may be combined with directed delivery of immunogen to antigen-presenting cells. To differentiate between these possible mechanisms, experiments were performed to define the physical requirements for microsphere adjuvancy.

The necessity for the antigen to be physically contained within the microspheres was addressed in experiments in which mice were subcutaneously immunized with 50 μg of SEB toxoid alone, toxoid within microspheres, or as a mixture of toxoid with empty microspheres. Determination of the plasma IgG antitoxin response induced by each of these immunization methods revealed that the toxoid mixed with empty microspheres was no more effective than the toxoid alone, while the toxoid delivered inside the microspheres induced a significantly potentiated antibody response (Table 2). The absence of a bystander effect, which is seen with the lipopolysaccharides and muramyl dipeptide, indicates that microsphere adjuvancy is not predominantly a function of lymphoid cell activation by this delivery vehicle.

In addition, it has been consistently observed that the size of the microspheres has a profound effect on the degree to which the antibody response is potentiated and the time at which it is initiated. These effects are best illustrated under conditions of a limiting antigen dose. Mice immunized subcutaneously with 10 μg of SEB toxoid encapsulated in 1- to 10-μm (mean diameter, 3.5 μm; CV = 0.36) microspheres mounted a more rapid, and a substantially more vigorous, IgG antitoxin response than did mice immunized with the same dose of toxoid in 10- to 110-μm (mean diameter, 54.5 μm; CV = 0.38) microspheres (Fig. 5). A likely explanation for these effects involves the manner in which these microspheres of different sizes deliver antigen into the draining lymphatics. We have observed with several preparations of fluorescent DL-PLG microspheres that a significant proportion of those microspheres that are less than approximately 10 μm in diameter are phagocytized and transported by macrophages into the draining lymph nodes. In contrast, microspheres that are greater than approximately 10 μm in diameter remain localized at the site of injection (data not shown). Taken together, these data suggest that the extremely strong adjuvant activity of 1- to 10-μm microspheres is due to their efficient loading of antigen into accessory cells which direct the delivery of the microencapsulated antigen into the draining lymph nodes.

SEB toxoid administered in 1- to 10-μm microspheres induces high levels of toxin-neutralizing antibodies. To determine whether the antibodies induced through immunization with SEB toxoid in 1- to 10-μm microspheres (mean diameter, 4.3 μm; CV = 0.33) were capable of neutralizing SEB toxin, an in vivo neutralization assay was designed on the basis of the expansion in the Vβ8-bearing T-cell subpopulation observed after the administration of SEB toxin. Forty-eight hours after the intravenous injection of 5 μg of toxin into unimmunized mice, the proportion of the splenic $CD3^+$ cells bearing Vβ8 had increased from ~33 to ~51%, while the proportion of the toxin-insensitive Vβ6 subpopulation fell from ~14 to ~10% (Table 3). Immunization with 50 μg of free toxoid 30 days prior to challenge (time of the peak plasma IgG antitoxin response; titer of 3,200) provided partial protection against a 5-μg dose of toxin but none when

TABLE 2. IgG anti-SEB toxin antibody response elicited by administration of microencapsulated SEB toxoid or free SEB toxoid as a mixture with empty microspheres

Immunogen form	Plasma IgG antitoxin titer[a]			
	Day 10	Day 25	Day 35	Day 50
Toxoid[b]	400	3,200	1,600	400
Toxoid in microspheres[c]	3,200	102,400	409,800	1,638,400
Toxoid plus empty microspheres[d]	100	1,600	1,600	50

[a] Titer determined by endpoint titration in an RIA with soilid-phase-adsorbed SEB toxin.
[b] SEB toxoid (50 μg) in 0.5 ml of PBS injected subcutaneously.
[c] Microspheres (2.8 mg) (50:50 DL-PLG; 1- to 10-μm diameter; 1.76% [wt/wt] SEB toxoid) containing 50 μg of SEB toxoid in 0.5 ml of PBS injected subcutaneously.
[d] SEB toxoid (50 μg) plus 2.8 mg of placebo microspheres (50:50 DL-PLG; 1- to 8-μm diameter) in 0.5 ml of PBS injected subcutaneously.

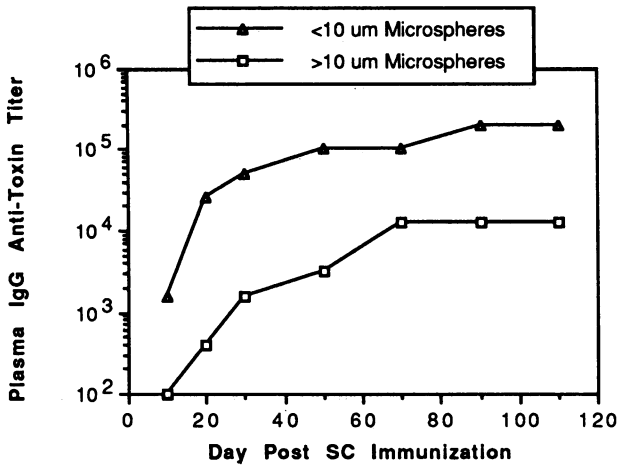

FIG. 5. Antibody response to SEB toxin induced through immunization with SEB toxoid encapsulated in 1- to 10-μm (<10 μm) or 10- to 110-μm (>10 μm) DL-PLG microspheres. Groups of five mice were subcutaneously immunized with 10 μg of SEB toxoid encapsulated in 1- to 10-μm (85:15 DL-PLG; 0.65% [wt/wt] SEB toxoid) or 10- to 110-μm (85:15 DL-PLG; 1.03% [wt/wt] SEB toxoid) microspheres. Plasma samples were obtained at 10-day intervals, and the IgG antitoxin titer was determined by endpoint titration in an RIA with solid-phase-adsorbed SEB toxin.

a 10-μg challenge was employed. In contrast, mice immunized with 50 μg of microencapsulated toxoid 140 days prior to challenge (IgG antitoxin titer of 204,800) showed no significant increase in their Vβ8 cells when intraveneously challenged with a dose as high as 25 μg of toxin (Table 3) nor did they lose weight. Therefore, it appears that immunization with a microencapsulated SEB toxoid vaccine results in a substantial potentiation and extension of the anti-SEB toxin response without adversely affecting the neutralizing character of that response.

DISCUSSION

After subcutaneous injection, 1- to 10-μm DL-PLG microspheres containing SEB toxoid induced an IgG antitoxin response which arose with similar kinetics and reached a peak level comparable to that induced by toxoid emulsified in CFA (Fig. 2). This level of response was approximately 100 times that obtained when alum, presently the only adjuvant licensed for human use, was employed. In other experiments, SEB toxoid microspheres made with 50:50 DL-PLG excipient maintained the IgG antitoxin level at a minimum of 25% of the peak response for greater than 250 days, a time well in excess of complete degradation of the copolymer. Thus, the antibody response to microencapsulated SEB toxoid appears to have the advantages in magnitude and duration of the antibody response obtained with CFA. In addition, the microspheres are well tolerated and do not have the problems of inflammation and granulomata formation which are common to many of the oil-based vehicles used as antigen depots.

Several lines of evidence suggest that the adjuvant activity of the 1- to 10-μm microspheres resides in their ability to load a relatively large amount of antigen directly into phagocytic accessory cells, followed by the directed migration of these antigen-containing cells into the draining lymph nodes. First, the microspheres appear to have no intrinsic mitogenicity and little, if any, ability to enhance the antibody response to an antigen unless it is incorporated within the copolymer matrix (Table 2). Second, microspheres which

TABLE 3. In vivo neutralization of SEB toxin-induced T-cell proliferation by prior immunization with encapsulated SEB toxoid

Mouse	Immunization	Intravenous challenge	% Splenic lymphocytes on day 2 postchallenge[a]		
			$CD3^+$	$V\beta8^+/CD3^+$	$V\beta6^+/CD3^+$
1	None	None	41.9	33.2	14.1
2			40.0	34.5	14.3
3			40.5	33.6	14.4
4	None	5 μg of toxin	42.7	49.4	10.4
5			41.8	53.9	10.3
6			35.5	52.2	11.2
7	50 μg of toxoid[b]	10 μg of toxin	43.2	49.1	NT[c]
8			44.3	45.0	
9			41.5	48.5	
10	50 μg of toxoid[b]	5 μg of toxin	48.3	40.9	NT
11			46.1	41.7	
12	50 μg of microencapsulated toxoid[d]	25 μg of toxin	43.4	33.8	NT
13			38.5	31.1	
14	50 μg of microencapsulated toxoid[d]	5 μg of toxin	39.4	29.9	NT
15			44.1	29.3	
16			41.6	28.1	

[a] Splenic T cells and T-cell subsets were quantified by flow cytometry after staining with fluorescein isothiocyanate anti-CD3 (monoclonal antibody 145-2C11) and biotin–anti-Vβ8 (monoclonal antibody F23.1) or biotin–anti-Vβ6 (monoclonal antibody RR4) plus phycoerythrin-avidin.

[b] Subcutaneous immunization with 50 μg of SEB toxoid in 0.5 ml of PBS, 30 days prior to toxin challenge.

[c] NT, Not tested.

[d] Subcutaneous immunization with 4.8 mg of microspheres (50:50 DL-PLG; 1- to 10-μm diameter, 1.05% [wt/wt] SEB toxoid) containing 50 mg of SEB toxoid in 0.5 μl of PBS, 140 days prior to challenge.

allow the encapsulated antigen to escape by leaching within the first 24 h after injection are virtually devoid of immunopotentiating activity (data not shown). Third, microspheres with a diameter small enough to allow ready phagocytosis by macrophages (less than approximately 10 μm) consistently exhibit a substantially higher degree of immunopotentiation than do those with a diameter which precludes phagocytic engulfment (Fig. 5). Further, the adjuvancy of the 1- to 10-μm microspheres correlates with their translocation within macrophages into the lymph nodes draining the injection site. Fourth, microspheres that are greater than approximately 10 μm in diameter remain at the site of deposition until the bulk (homogeneous) hydrolysis of the DL-PLG has advanced to the point that the copolymer matrix structurally fails and the fragments are then engulfed. By this time, a portion of the microencapsulated antigen has been released from the microspheres, but that which is phagocytized with the copolymer fragments is likely to account for the lesser, although strong, adjuvancy of the larger microspheres. These data are consistent with the view that the antigen released from the microspheres into the extracellular milieu is no more effective than antigen injected in solution and that the predominant mechanism of immune enhancement is a directed intracellular delivery of antigen to accessory cells.

The ability of a microsphere vaccine delivery system to potentiate the immune response to TNP-KLH (Table 1) demonstrates that the immunogen need not possess unique activating properties, such as those of a superantigen. However, the nature of the antigen does determine the extent to which microencapsulation can enhance the antibody response. We have observed that antibody responses to purified polysaccharides are not consistently potentiated by microencapsulation and are never increased to the degree seen with proteins. This observation is not unexpected in light of the manner in which polysaccharide antigens are recognized by the immune system and is most likely a consequence of the inability of these T-cell-independent antigens to stimulate T-cell help regardless of how they are delivered. However, immunization with a microencapsulated type 3 pneumococcal polysaccharide-protein carrier conjugate induces dramatically potentiated IgG anti-type 3 responses (18a). Thus, the adjuvant activity can be extended to carbohydrates presented in a T-cell-dependent form and suggests that this delivery system results in the efficient induction of helper T cells.

Limited information is available with respect to the neutralization of SEB toxin by antibodies. This is due in large part to the absence of an appropriate small-animal model. The recent observations of Marrack et al. (17) that intraperitoneal administration of the toxin to mice resulted in reproducible weight loss, thymic atrophy, and expansion of the Vβ8-bearing subset of mesenteric lymph node T cells suggested that the mouse could serve as an in vivo model for SEB toxin neutralization. When injected intravenously, it was found that as little as 5 μg of toxin induced both weight loss and a profound, highly reproducible expansion of the splenic Vβ8 T cells over a period of several days. On the basis of these findings, inhibition of the increase in the proportion of T cells expressing Vβ8 at 48 h post-intravenous toxin administration was found to be a relatively rapid, simple, and reproducible in vivo assay for toxin neutralization.

If challenged on day 30 when their IgG antitoxin titer (3,200) had peaked (Table 3), mice immunized with a maximally immunogenic dose (50 μg) of soluble toxoid were found to be partially protected against the Vβ8 T-cell expansion induced by a 5-μg dose of toxin. When the toxin challenge was increased to 10 μg, no protection was observed. In contrast, mice immunized with an equal dose of microencapsulated toxoid 140 days prior to challenge still exhibited an antitoxin titer of 204,800 and were completely protected from both 5- and 25-μg doses of toxin. These data demonstrate that both the magnitude and the duration of the protective, neutralizing antibody response to SEB toxin are potentiated through delivery of the toxoid vaccine in DL-PLG microspheres and suggest that this delivery system may find wide application in vaccination.

The studies presented here involve one application of biodegradable and biocompatible DL-PLG microspheres to the delivery of protein antigens. The overall goal in our investigations with microencapsulated antigens is to provide a simple, safe, and broadly applicable vaccine delivery system with adjuvant activity for use in humans. For this reason, microspheres made with DL-PLG were selected as the system offering the best combination of characteristics. DL-PLG is within the class of copolymers that have been used for implantable and injectable controlled-release, drug delivery systems (9, 21, 24, 29), four of which are currently approved for human use in Europe and two of which are approved for human use in the United States. This copolymer induces only a minimal inflammatory response upon introduction into the body and degrades through simple hydrolysis into biocompatible products which are normal body constituents (36). Further, the rate at which DL-PLG biodegrades is a function of the ratio of lactide to glycolide (19). Although not addressed in this study, this property of the DL-PLGs permits the production of vaccine-containing microspheres which initiate vaccine release at various intervals. Thus, microspheres of different lactide-to-glycolide ratios, when blended prior to administration, allow a single injection to release one or more programmed booster doses at predicted intervals (unpublished data).

Our previous work in this area has involved the use of microspheres for the delivery of antigens into mucosal lymphoid tissues, such as the Peyer's patches in the gut, for the purpose of inducing a disseminated secretory IgA response. No currently employed vaccination method is routinely effective at producing a secretory antibody response, which generally requires direct immunization of a mucosal surface. In an oral approach to the induction of secretory antibodies, the microspheres provide several advantages. First, the DL-PLG excipient of the microspheres protects the vaccine from degradation by the low pH of the stomach and from the proteolytic enzymes and bile salts in the gastrointestinal tract. Second, the microspheres are readily phagocytized by the M cells overlying the Peyer's patches, delivering intact antigen into this IgA-inductive tissue and effectively inducing a disseminated mucosal immune response (7, 8). Among the reasons for the success of orally administered microencapsulated antigens is the strong adjuvant activity of the microspheres, which has been addressed in the present study.

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