

# Intranasal and Intramuscular Proteosome-Staphylococcal Enterotoxin B (SEB) Toxoid Vaccines: Immunogenicity and Efficacy against Lethal SEB Intoxication in Mice

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**Intranasal or intramuscular (i.m.) immunization of mice and i.m. immunization of rabbits with formalinized staphylococcal enterotoxin B (SEB) toxoid in saline elicited higher anti-SEB serum immunoglobulin G (IgG) titers when the toxoid was formulated with proteosomes. In addition, intranasal immunization of mice with this proteosome-toxoid vaccine elicited high levels of anti-SEB IgA in lung and intestinal secretions, whereas the toxoid without proteosomes did not. Two i.m. immunizations with proteosome-toxoid plus alum also induced higher murine serum responses than alum-adjuvanted toxoid without proteosomes. Furthermore, proteosome-toxoid delivered intranasally in saline or i.m. with either saline or alum afforded significant protection against lethal SEB challenge in two D-galactosamine-sensitized murine models of SEB intoxication, i.e., the previously described i.m. challenge model and a new respiratory challenge model of mucosal SEB exposure. Efficacy correlated with the induction of high serum levels of anti-SEB IgG. In contrast, intranasal or i.m. immunization with toxoid in saline without proteosomes was not significantly protective in either challenge model. Proteosome-toxoid plus alum given i.m. also elicited more significant protection against respiratory challenge than the alum-adjuvanted toxoid alone. The capacity of proteosomes to enhance both i.m. and intranasal immunogenicity and efficacy of SEB toxoid indicates that testing such proteosome-SEB toxoid vaccines in the nonhuman primate aerosol challenge model of SEB intoxication prior to immunogenicity trials in humans is warranted. These data expand the applicability of the proteosome mucosal vaccine delivery system to protein toxoids and suggest that respiratory delivery of proteosome vaccines may be practical for enhancement of both mucosal and systemic immunity against toxic or infectious diseases.**

The development of vaccine delivery systems and adjuvants to enhance parenteral or mucosal immunogenicity of microbial antigens and confer protection against infectious agents and toxins has received increased attention during the last decade (2, 3, 11, 13, 33, 50). We have previously shown that parenteral immunogenicity of peptides (25–27), gangliosides (23), and envelope proteins (20, 25) is enhanced by noncovalently complexing such antigens to meningococcal outer membrane protein proteosomes via hydrophobic moieties linked to the antigens. Proteosomes have also been shown to be an effective mucosal vaccine delivery system for induction of serum, lung, and intestinal immunoglobulin G (IgG) and IgA antibodies that recognize the O-polysaccharide antigens of *Shigella sonnei* or *Shigella flexneri* 2a lipopolysaccharides (LPS) following either intranasal or intragastric immunization (29, 39, 40). These mucosal proteosome-LPS vaccines are efficacious against challenge with infectious organisms in animal models of shigellosis protecting guinea pigs against keratoconjunctivitis shigellosa (Serény test) (40) and mice against lethal pneumonia (29). In addition, intranasal immunization with proteosomes hydrophobically complexed to lipopeptides containing selected influenza T-cell and B-cell epitopes reduces viral titers in lungs of mice challenged with influenza (21).

In this study, we examined the immunopotentiating effects of formulating a formalinized toxoid of staphylococcal enterotoxin B (SEB) with proteosomes. Mice were immunized intra-

muscularly (i.m.) or intranasally, and immunogenicity was evaluated by determining anti-SEB antibody levels in serum as well as in bronchial and intestinal lavage fluids. Vaccine efficacy was evaluated in mice lethally challenged with SEB delivered via either parenteral or respiratory routes. The previously described parenteral challenge model of lethal SEB intoxication in D-galactosamine (D-galn)-sensitized mice was used with only minor modification (36). In addition, to measure the ability of parenteral and intranasal delivery of SEB toxoid vaccines to protect against a mucosal challenge with this toxin, we developed a lethal respiratory SEB challenge model in D-galn-sensitized BALB/c mice in which SEB is administered to mice intranasally.

SEB belongs to a family of *Staphylococcus aureus* exotoxins (labeled SEA to SEE and SEG) that cause the majority of human food poisoning cases manifested by vomiting and diarrhea after ingestion (44, 46). SEB has also been recognized as a leading cause of human cases of nonmenstrual toxic shock syndrome that can accompany surgical or injurious wound infections and, more recently, viral infections of the respiratory tract in influenza patients (44, 46, 48). Toxic shock syndrome is characterized by high fever, hypotension, skin and mucous membrane changes, headache, vomiting, diarrhea and, in its most severe form, shock and death (37, 44). In nonhuman primates, SEB causes vomiting and diarrhea after ingestion (5) and lethal shock after systemic (5) or aerosol exposure (19, 32). Because of this potential for causing lethal shock in humans after aerosol exposure, there is concern that SEB could potentially be used as a biologic weapon (19, 32). This in vivo toxicity has been postulated to be related to the capacity of these

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molecules to act as superantigens that profoundly stimulate mononuclear cells to proliferate and secrete massive amounts of cytokines (30, 37, 44). Superantigens mitogenically activate entire subsets of T cells by forming a complex with major histocompatibility complex class II molecules and the V- $\beta$  region of selected classes of T-cell receptors. Since this binding is independent of the specific antigen binding site, up to 20% of all T cells can be stimulated with concomitant initiation of a cytokine cascade that is thought to be responsible for much of the described systemic toxicity (7, 30, 37), and in a recent study, interleukin-10 afforded measurable protection against SEB by regulating T-cell activation (4).

It was previously reported that formalized SEB toxoid was sufficiently immunogenic in mice to prevent manifestations of SEB toxicity only after i.m. immunization with the toxoid encapsulated in poly(DL-lactide-co-glycolide) (DL-PLG) microspheres (15). This study shows that proteosome formulation of formalized SEB toxoid improves the induction of anti-SEB antibodies with resultant increases in *in vivo* neutralization of toxin lethality. Moreover, formulating the toxoid with proteosomes allowed for successful intranasal delivery of the toxoid as measured by the enhanced induction of serum, lung, and intestinal antibodies as well as significant protection against lethal systemic or respiratory SEB toxin challenge.

#### MATERIALS AND METHODS

**Antigen isolation and vaccine formulation.** (i) **Proteosomes.** Outer membrane protein proteosome preparations were stored at  $-70^{\circ}\text{C}$  after purification, as described previously (29), from group B type 2 *Neisseria meningitidis* by extraction of phenol-killed bacterial paste with a solution of 6% Empigen BB (Albright and Wilson, Whitehaven, Cumbria, United Kingdom) in 1 M calcium chloride, followed by precipitation with ethanol, solubilization in Tris-buffered saline with EDTA and 1% Empigen BB, reprecipitation with ammonium sulfate, and then resolubilization in the Tris buffer with 1% Empigen BB.

(ii) **Toxoid.** SEB, lot 14-30, obtained from the Department of Toxinology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md., and prepared by the method of Schantz et al. (43), was treated with formalin for 30 days at  $37^{\circ}\text{C}$  and pH 7.5 as originally described by Warren et al. (51) and as performed by Eldridge et al. (15). Briefly, in a biohazard hood, batches of 0.1 or 0.5 g of SEB were dissolved at a concentration of 2.4 mg/ml in a beaker containing 0.02 M sodium phosphate buffer (pH 7.35). A dialysis bag consisting of Spectra/Por tubing with a molecular weight cutoff of 12,000 to 14,000 (Spectrum Medical Industries, Los Angeles, Calif.) containing a solution of 37% formalin-methanol (Sigma Chemical Co., St. Louis, Mo.) which had been diluted sixfold in the pH 7.5 phosphate buffer was then immersed in the beaker, which was incubated overnight at room temperature so that the formalin inside the dialysis bag could slowly dialyze out of the bag into the beaker which contained the SEB. The dialysis bag was then cut, the empty tubing was removed, and the resultant solution containing SEB at 2 mg/ml in 1% formalin was placed in a slowly shaking incubator at  $37^{\circ}\text{C}$ . The solution was adjusted to and maintained at pH 7.5 with 0.2 M sodium phosphate buffers (at pH 8 and pH 3.8) for 30 days. The toxoid solution was then centrifuged for 10 min at 4,000 rpm, dialyzed (12,000 to 14,000 molecular weight cutoff as described above) against phosphate buffer (pH 7.5) for 4 days with daily buffer changes, and sterile filtered; the protein concentration was determined (28), and the solution was stored at  $4^{\circ}\text{C}$ .

(iii) **Proteosome vaccine formulation.** SEB toxoid was formulated with proteosomes by use of the technique previously described for noncovalent complexing of proteosomes to peptides (25-27) or LPS (29, 39, 40). Briefly, equal amounts by weight of toxoid and proteosomes were combined at a concentration of 1 to 2 mg/ml in a buffer of 0.05 M Tris, EDTA, and 0.15 M NaCl with 1% Empigen BB, and the solution was dialyzed against 0.05 M Tris (pH 8.0) across a Spectra/Por 6 dialysis membrane (Spectrum Medical Industries) with a 1,000 molecular weight cutoff for 8 to 10 days at  $4^{\circ}\text{C}$  with daily buffer changes. Vaccines were stored at  $4^{\circ}\text{C}$ .

(iv) **Alum adjuvant.** For those i.m. immunizations in which SEB toxoid or proteosome-SEB toxoid had alum as an adjuvant, the appropriate volume of a preparation of 2% aluminum hydroxide (Alhydrogel; Superfos Biosector a/s, Vedbaek, Denmark) was allowed to incubate with either toxoid preparation in 0.5 M Tris normal saline buffer (pH 6.5 to 7.0) for at least 18 h at  $4^{\circ}\text{C}$  with occasional gentle mixing. The volume of aluminum hydroxide added was calculated so that each dose of vaccine contained aluminum hydroxide at a concentration of 4.34 mg/ml. Hence, rabbits, immunized with a volume of 0.5 ml received 2.17 mg of aluminum hydroxide with 0.76 mg of  $\text{Al}^{3+}$ , which is less than the maximum recommended dose of  $\text{Al}^{3+}$  allowable in humans, i.e., 0.85 mg.

Mice, immunized with a volume of 0.1 ml per dose, received 0.43 mg of aluminum hydroxide with 0.15 mg of  $\text{Al}^{3+}$  per dose. After formulation, both saline preparations and those with alum as an adjuvant were stored at  $4^{\circ}\text{C}$  for the duration of the experiment.

**Toxoid characterization and confirmation of detoxification.** (i) **PAGE.** Polyacrylamide gel electrophoresis (PAGE) analysis of a sodium dodecyl sulfate gel of the toxin showed one distinct band at 28,000 Da, whereas the toxoid showed two broad major bands with molecular weights of ca. 23,000 and 46,000 and minor bands with molecular weights of approximately 66,000 and 90,000.

(ii) **In vivo test of detoxification.** To verify the safety of the toxoid *in vivo*, the murine model of lethal sensitivity to parenteral SEB was used with only minor modification as follows. In the original description, 20  $\mu\text{g}$  of SEB was injected in the footpad of BALB/c mice simultaneously with the intraperitoneal injection of 20 mg of D-gal (36). Extensive preliminary experiments in our laboratory indicated that for i.m. injection in the thigh, 25  $\mu\text{g}$  of SEB admixed with 20 mg of D-gal was 100% lethal for BALB/c mice. To demonstrate toxoid detoxification, groups of 10 BALB/c mice were injected i.m. with 20 mg of D-gal admixed with either 100 or 500  $\mu\text{g}$  of toxoid, 4 and 20 times the 100% lethal dose ( $\text{LD}_{100}$ ) of SEB toxin, respectively. All mice injected with toxoid survived, whereas 10 control BALB/c mice injected with 25  $\mu\text{g}$  of SEB toxin admixed with 20 mg of D-gal died within 72 h. In addition, to confirm detoxification in the manner performed previously (14, 15), New Zealand White rabbits (ca. 4 kg) were injected i.m. with 0.5 mg of either SEB toxin or toxoid per kg of body weight. This dose of SEB has been shown to be 100% lethal in rabbits (22), and a control rabbit injected with SEB toxin manifested signs of SEB toxicity (22), including lethargy, shivering, and weight loss, necessitating sacrifice. In contrast, the two rabbits that received the toxoid did not lose weight or appear ill during the week of observation.

(iii) **In vitro test of detoxification using the mouse spleen lymphocyte proliferative assay.** Detoxification of the toxin was also demonstrated by verifying the lack of mitogenicity of the toxoid for murine splenic cells *in vitro* as described previously (15). Triplicate 200- $\mu\text{l}$  cultures each containing  $10^6$  mouse spleen cells were incubated in 96-well microtiter plates in RPMI medium (Gibco, Grand Island, N.Y.) with 10% fetal calf serum, glutamine, antibiotics, and either SEB toxin or toxoid at the indicated concentrations for 48 h and pulsed with [ $^3\text{H}$ ]thymidine for the last 18 h of culture. SEB toxin was mitogenic for mouse spleen cells at concentrations ranging from 0.37 to 10.0  $\mu\text{g}/\text{ml}$  with five- to ninefold stimulation indices (25,387 and 48,575 cpm of incorporated thymidine) at these doses, respectively, compared with unstimulated control cells. In contrast, the toxoid was nonmitogenic at all eight concentrations tested, which ranged from 0.04 to 100.0  $\mu\text{g}/\text{ml}$ .

**Animal care and use.** The experiments performed strictly adhered to the 1985 Amendments to the Animal Welfare Act (7 U.S.C. 2131, et seq., Army regulation AR 70-18, and Public Law 99-198) and to the *Guide to the Care and Use of Laboratory Animals* (49) as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and adopted by the Laboratory Animal Care and Use Committee of our research institutes.

**Immunizations.** (i) **Parenteral immunization.** Female adult BALB/c or outbred CD-1 mice (Frederick Cancer Research Center, Frederick, Md.) were parenterally immunized i.m. two or three times as indicated at 3-week intervals with 100  $\mu\text{l}$  of one of the vaccine preparations containing 10 or 50  $\mu\text{g}$  of SEB toxoid. Rabbits were immunized i.m. at 0 and 3 weeks with vaccine preparations containing 100  $\mu\text{g}$  of toxoid in a volume of 0.5 ml.

(ii) **Intranasal immunization.** As described previously (29, 40), mice were mildly anesthetized with a mixture of xylazine and ketamine or with methoxyflurane and then allowed to inhale 25 to 35  $\mu\text{l}$  of vaccine or saline (for nonimmunized control animals) that was slowly instilled by micropipette into one or both nares. The toxoid or proteosome-toxoid vaccine preparations were formulated to deliver 50  $\mu\text{g}$  of toxoid per intranasal instillation. When 100- $\mu\text{g}$  doses per week of the toxoid vaccines were required, to limit the volume of vaccine instilled intranasally to 35  $\mu\text{l}$ , the 100- $\mu\text{g}$  dose was administered in two 50- $\mu\text{g}$  doses given either on the same day (1 to 4 h apart) or as a split dose given 2 days apart. In other experiments (data not shown), vaccines were concentrated with Centriprep tubes (Amicon, Beverly, Mass.) to achieve the required concentration of vaccine without necessitating a split dose. There were no significant differences in immunogenicity when the same microgram amount of toxoid was given in either one instillation or divided doses 1 to 4 h or split 2 days apart.

**Collection of sera and lung and intestinal secretions.** Blood was collected periodically as indicated from mice from the retro-orbital plexus and at the time of sacrifice and from rabbits from the ear vein. Mucosal secretions (lung and intestinal lavage fluids) were collected from mice and processed at the time of sacrifice as described previously (40) at weeks 4 to 6 or week 11, as indicated. Briefly, for bronchial lavage samples, immediately after sacrifice by  $\text{CO}_2$  suffocation, the lungs were surgically exposed, a cannula was inserted in the trachea and, by use of a three-way stopcock, two 1-ml lung lavage samples with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) were collected and combined. Intestinal lavage samples were then collected as described previously (40) with a 3-ml syringe to pass 2 ml of PBS containing 0.1% BSA, 50 mM EDTA, and 1 mg of soybean trypsin inhibitor per ml through a 20- to 25-cm section of small intestine. Lung and intestinal lavage fluids were vortexed and centrifuged to remove cell debris prior to storage at  $-70^{\circ}\text{C}$ .

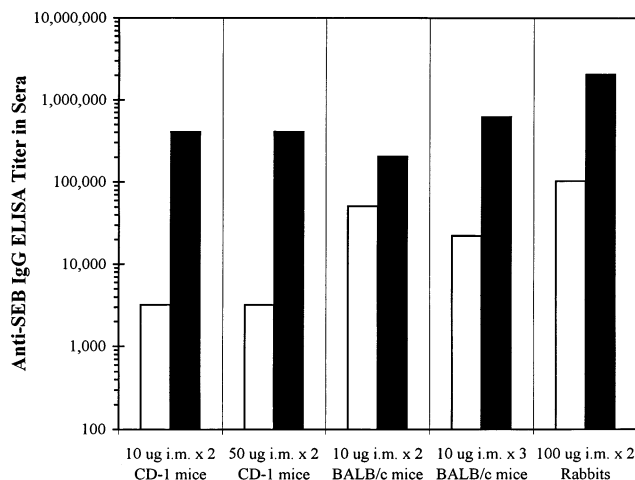


FIG. 1. Potentiation of serum IgG responses in mice and rabbits immunized i.m. by formulating SEB toxoid with proteosomes. Anti-SEB IgG titers were determined by ELISA of sera pooled from groups of CD-1 or BALB/c mice (five mice per group) or of individual serum samples from rabbits (three per group) after i.m. immunization with SEB toxoid formulated in saline with (■) or without (□) proteosomes. The data are expressed as GMTs of the highest dilutions of sera with OD values of  $>0.5$ . Sera were obtained from mice or rabbits 2 to 4 weeks after two or three immunizations as indicated with preparations containing the amounts of toxoid shown in the figure.

**Antigen-specific antibody assays.** An enzyme-linked immunosorbent assay (ELISA) was used to measure anti-SEB toxin IgG antibodies in sera and IgA antibodies in collected lung and intestinal lavage secretions as described previously (40) with minor modifications. Murine serum samples used in the ELISA were tested either individually or pooled from groups of five mice receiving the same preparation. All murine intestinal or bronchial lavage samples and rabbit sera were tested individually. Briefly, 96-well round-bottom microtiter plates (Immulon 2; Dynatech, Chantilly, Va.) were coated in a biosafety cabinet with SEB toxin (10  $\mu\text{g}/\text{ml}$ ) and incubated at 37°C for 1 h. All incubations were performed in a humid chamber. After aspiration of the toxin with a plate washer (Skatron, Inc., Sterling, Va.) operating in the biosafety cabinet, the plates were washed once with PBS containing 0.05% Tween (PBS-T) and incubated with blocking solution containing 0.5% each of casein and BSA (IgG and fatty acid free) for 60 to 90 min at 37°C. After aspirating the blocking solution and washing twice with PBS-T, duplicate samples of pooled or individual serum samples or individual bronchial or intestinal lavage fluids, serially diluted twofold in blocking solution, were added and the plates were incubated overnight at 37°C. After washing four times with PBS-T, affinity-purified horseradish peroxidase-labeled goat anti-mouse IgG or IgA (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) or alkaline phosphatase-labeled goat anti-rabbit IgG was added and the plates were incubated at room temperature overnight. After aspirating and washing twice with PBS-T, TMB solution (Bio-Rad) or *p*-nitrophenylphosphate diluted in diethanolamine buffer (pH 9.8) was added to mouse or rabbit samples, respectively, and the plates were kept at room temperature for 1 or 2 h for serum or lavage fluid determinations, respectively. The  $A_{630}$  or  $A_{450}$  was determined for horseradish peroxidase- or phosphatase-labeled plates, respectively, with a microtiter ELISA plate reader (Molecular Devices, Menlo Park, Calif.). The antibody titer is expressed as the geometric mean of the greatest dilution of serum or lavage fluids that elicited an optical density (OD) of  $\geq 0.5$  for sera and  $\geq 0.2$  for lavage samples. Error bars represent the standard errors of the means of samples assayed individually.

**SEB challenge assays of vaccine efficacy in vivo.** The SEB used in the challenges was lot 14-30 obtained from the U.S. Army Medical Research Institute of Infectious Diseases, prepared as described above by the method of Schantz et al. (43).

(i) **Parenteral (i.m.) challenge.** The previously described *D*-galn-sensitized murine model of lethal toxicity by parenteral SEB (36) was used (as described above) with only minor modification. Mice were injected in the thigh muscle with 100  $\mu\text{l}$  containing 20 mg of *D*-galn (Sigma), admixed with either 25  $\mu\text{g}$  of SEB (for BALB/c mice) or 100  $\mu\text{g}$  of SEB (for CD-1 mice). Following i.m. challenge (or respiratory challenge as described below), 65% of the mice that died succumbed within the first 24 h, an additional 30% died in the next 24 h, and only 5% died in the last 24 h.

(ii) **Respiratory (intranasal) challenge.** BALB/c mice were mildly anesthetized with halothane, injected with 20 mg of *D*-galn i.m., and challenged with SEB by placing 30 to 35  $\mu\text{l}$  containing 350  $\mu\text{g}$  of SEB in the nares with a micropipette. For both parenteral and intranasal challenges, mice were observed for 72 h, with

death as the end point, and control mice were given *D*-galn i.m. plus saline without SEB by the challenge route.

**Statistical analyses.** Analysis of statistical significance was determined by the Mann-Whitney test for ELISA immune responses by use of Minitab for Windows 10.5 and by the Fisher exact test for vaccine-induced protection by use of EpiInfo software.

## RESULTS

### Potentiation of serum anti-SEB toxin antibody responses in mice and rabbits immunized i.m. with proteosome-SEB toxoid.

To determine the ability of proteosomes to enhance the immunogenicity of SEB toxoid delivered i.m. without added adjuvants, mice and rabbits were primed and boosted one or two times at 3-week intervals with either the toxoid alone or the same amount of toxoid formulated with proteosomes. As shown in Fig. 1, proteosome formulation enhanced anti-SEB toxin serum IgG titers in all cases. Moreover, these increases were biologically significant since (as detailed below) the higher titers (i.e., greater than 102,400) afforded mice significant protection against lethal SEB challenge.

**Enhancement of anti-SEB serum IgG by intranasal immunization with proteosome-SEB toxoid (Fig. 2).** The capacity of proteosomes to confer intranasal immunogenicity on SEB toxoid without added adjuvants was tested with BALB/c mice immunized on weeks 0 and 3 with preparations containing 50 or 100  $\mu\text{g}$  of toxoid. The toxoid without proteosomes was very poorly immunogenic when given via the intranasal route at all three regimens tested, and although detectable antibodies were elicited in each of the mice, the serum geometric mean titer (GMT) of anti-toxin IgG was  $<120$  in each of the groups. In marked contrast, the proteosome-toxoid vaccine was able to induce a 3-log (1,000- to 4,000-fold) increase in anti-SEB se-

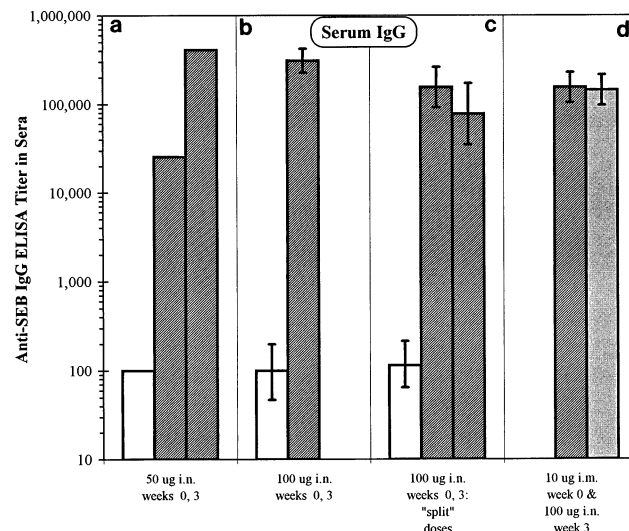


FIG. 2. Enhancement of serum IgG responses in mice by intranasal immunization with SEB toxoid formulated with proteosomes. Anti-SEB IgG titers were determined by ELISA of sera obtained from BALB/c mice. (a to c) Data from sera obtained at weeks 4 to 6 from mice that were primed and boosted intranasally with either SEB toxoid alone (□) or SEB toxoid formulated with proteosomes (■); (d) data collected either at weeks 4 to 6 (▨) or at week 11 (■) from groups of mice that were primed i.m. and boosted intranasally with SEB toxoid formulated with proteosomes. Each bar represents determinations from a group of five mice immunized at the same time. The panels with error bars (b to d) show GMTs and standard errors of the mean of the highest dilutions of individual serum samples of groups of five mice with OD values of  $>0.5$ . The panel without error bars (a) uses the same criteria for determinations, but the ELISA was performed on pooled serum samples containing equal aliquots from each of the five mice in the group. Vaccines were given by the schedules indicated at the bottom of the figure.



rum GMT with either 50- or 100- $\mu$ g doses. Comparable titers of ca. 100,000 to 300,000 were found when 100  $\mu$ g of the proteosome-toxoid vaccine on weeks 0 and 3 was divided into two 50- $\mu$ g doses administered on the same day or by use of the split dose schedule (in which the 100- $\mu$ g doses on weeks 0 and 3 were split by giving 50  $\mu$ g on days 0 and 3 and then 50  $\mu$ g on days 21 and 23). The serum IgG titers induced by either schedule with the proteosome-toxoid vaccine were significantly different from those induced by the toxoid alone, with a  $P$  of  $<0.012$ . The intranasal route was also effective for the proteosome-toxoid vaccine as a 100- $\mu$ g booster immunization series administered at week 3 (by use of the split dose schedule on days 21 and 23) after a 10- $\mu$ g i.m. primary immunization. Titers in such mice primed i.m. and boosted intranasally with the proteosome-toxoid vaccine remained level on week 11 (Fig. 2d).

**Enhancement of anti-SEB IgA in bronchial secretions by intranasal immunization with proteosome-SEB toxoid (Fig. 3A).** An advantage of intranasal immunization is the ability to induce anti-SEB IgA in respiratory secretions. Intranasal immunization with 50 or 100  $\mu$ g of toxoid without proteosomes (by use of either the same-day or split dose regimen) did not consistently induce measurable amounts of anti-SEB IgA even in undiluted bronchial lavage fluid: responses were detected in only 1 mouse receiving 50  $\mu$ g of toxoid and in 3 of the 10 mice receiving 100  $\mu$ g of toxoid alone. In contrast, intranasal immunization with two 50- $\mu$ g doses of toxoid formulated with proteosomes induced anti-SEB IgA in bronchial lavage fluid dilutions with GMTs of 11 to 28 in 100% of the 10 mice examined; these levels were significantly different from the toxoid-alone values, with a  $P$  of  $<0.020$ . Furthermore, intranasal immunization with 100  $\mu$ g of the proteosome-toxoid at weeks 0 and 3 (with 100  $\mu$ g on days 0 and 21 or by use of the split dose schedule with 50  $\mu$ g on days 0, 2, 21, and 23) induced responses in 100% of the mice with 3-log (1,000- to 2,000-fold) increases in the GMT of anti-SEB IgA in bronchial fluids ( $P < 0.012$  compared with toxoid alone). Replacing the primary proteosome-toxoid intranasal immunization series with a 10- $\mu$ g i.m. dose and boosting intranasally with the split 100- $\mu$ g dose of this vaccine on week 3 (50  $\mu$ g on days 21 and 23) resulted in bronchial IgA responses with a GMT of bronchial fluid dilutions of  $>2,000$ ; these values were similar to the levels induced when both the primary and secondary immunizations were intranasal. Bronchial anti-SEB IgA responses in mice primed i.m. and boosted intranasally with proteosome-toxoid were still significantly elevated 8 weeks after the boost (sera obtained on week 11) (Fig. 3A, panel d).

**Induction of anti-SEB IgA in intestinal secretions by intranasal immunization with proteosome-SEB toxoid (Fig. 3B).** Intranasal immunization with the proteosome-toxoid also improved anti-SEB IgA in intestinal lavage fluids. The minimal level of intestinal IgA detected in each of the mice immunized twice intranasally with 50  $\mu$ g of toxoid alone was significantly improved by fivefold ( $P < 0.028$ ) by immunization with 50  $\mu$ g of toxoid formulated with proteosomes (Fig. 3B, panel a). As shown in Fig. 3B, panels b and c, intranasal immunization with toxoid alone at an increased dose of 100  $\mu$ g at weeks 0 and 3 (administered either on days 0 and 21 or by use of the split dose regimen, giving 50  $\mu$ g on days 0 and 2 and 50  $\mu$ g on days 21 and 23) also elicited responses in intestinal lavage fluids in all of the mice, and the geometric mean intestinal anti-SEB IgA ELISA titers in both groups were 5. Although the GMT of anti-SEB IgA increased to 16 in intestinal fluids of mice immunized intranasally with 100  $\mu$ g of proteosome-toxoid on days 0 and 21, this increase was not significant at the  $P$  of 0.05 level compared with the results of immunizing with the toxoid

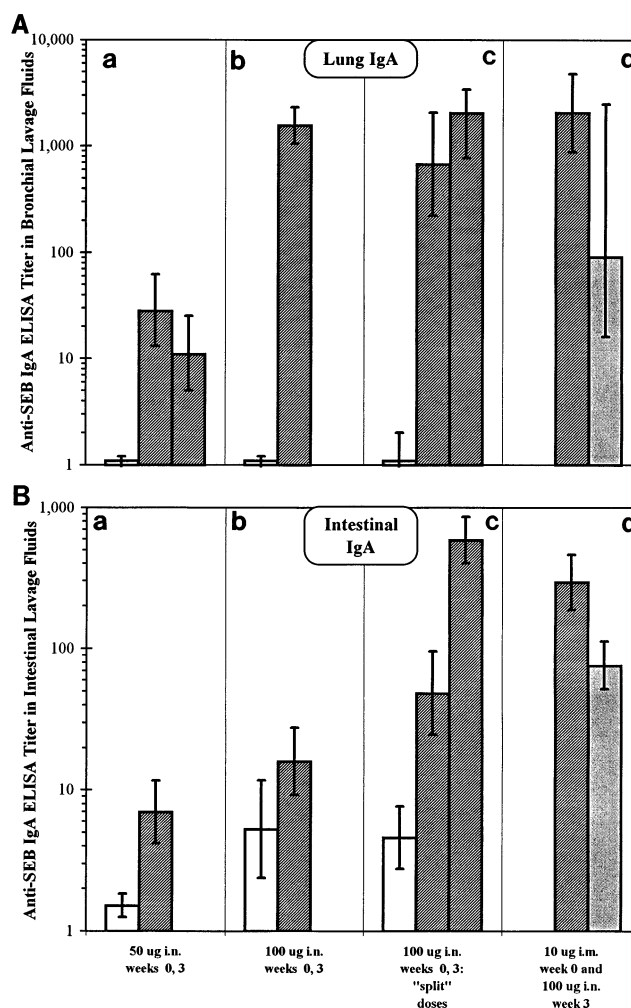


FIG. 3. Enhancement of bronchial (A) and intestinal (B) IgA responses in mice by intranasal immunization with SEB toxoid formulated with proteosomes. Anti-SEB IgA titers were determined by ELISA in bronchial and intestinal lavage fluids obtained from BALB/c mice. (A and B, panels a to c) Data from samples obtained at weeks 4 to 6 from mice that were primed and boosted intranasally with either SEB toxoid alone ( $\square$ ) or SEB toxoid formulated with proteosomes ( $\text{hatched}$ ); (A and B, panels d) data from fluids collected at weeks 4 to 6 or week 11 ( $\blacksquare$ ) from groups of mice that were primed i.m. and boosted intranasally with SEB toxoid formulated with proteosomes. Each bar represents determinations from a group of five mice immunized at the same time. Results are expressed as GMTs plus and standard errors of the means of the highest dilutions of individual lavage samples with ELISA OD values of  $>0.2$ . Vaccines were given by the schedules indicated at the bottom of the figure.

alone (Fig. 3B, panel b). Nevertheless, in two groups of mice immunized intranasally with 100  $\mu$ g of the toxoid formulated with proteosomes by the split dose schedule, all mice responded, resulting in 11- and 128-fold increases in the GMT of intestinal anti-SEB IgA compared with that resulting from immunizing with the toxoid without proteosomes, and these increases were significant at  $P$  values of  $<0.016$  and  $<0.012$ , respectively (Fig. 4c). High levels of anti-SEB IgA were also found in intestinal fluids collected at weeks 4 to 6 and at week 11 from mice primed i.m. with 10  $\mu$ g of the proteosome-toxoid vaccine and then boosted intranasally with 100  $\mu$ g (split in two 50- $\mu$ g divided doses) of this vaccine at week 3 (Fig. 3B, panel d).

**Serum anti-SEB IgG responses in mice and rabbits immunized i.m. with alum-adjuvanted preparations of SEB toxoid or proteosome-toxoid (Fig. 4).** When SEB toxoid without pro-

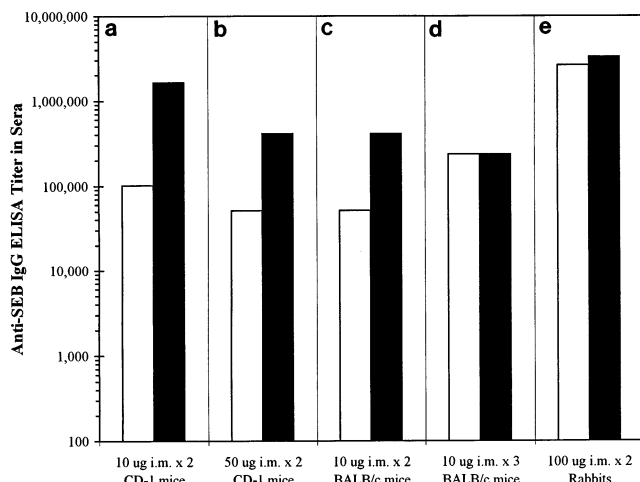


FIG. 4. Serum anti-SEB IgG responses in mice and rabbits immunized i.m. preparations of SEB toxoid or proteosome-toxoid, with alum as an adjuvant. Anti-SEB IgG titers were determined by ELISA of sera pooled from groups of CD-1 or BALB/c mice (five mice per group) or in individual serum samples from rabbits (three per group) after i.m. immunization with SEB toxoid formulated with (■) or without (□) proteosomes and with alum as an adjuvant. The data are expressed as titers showing the geometric mean of the highest dilutions of sera with OD values of  $>0.5$ . Sera were obtained from mice or rabbits 2 to 4 weeks after two or three immunizations as indicated with preparations containing the amounts of toxoid shown at the bottom of the figure.

teosomes contained alum as an adjuvant, reciprocal anti-SEB serum IgG titers of 50,000 to 100,000 were induced in CD-1 or BALB/c mice after two i.m. immunizations containing either 50 or 100  $\mu\text{g}$  of toxoid. These levels induced by the toxoid-alum without proteosomes were improved 8- to 16-fold by immunizing with the proteosome-toxoid-alum vaccine. Thus, immunizing with proteosome-toxoid either with (Fig. 4) or without (Fig. 1) alum elicited comparable anti-SEB titers in the range that was more consistently within the level associated with that required for protection in the SEB challenge model (see details below). When three i.m. immunizations were used in mice or two 100- $\mu\text{g}$  doses were given i.m. to rabbits, with alum as an adjuvant, both the toxoid and proteosome-toxoid preparations were highly effective in eliciting similar anti-SEB serum IgG titers (Fig. 4).

**Efficacy of i.m. or intranasal proteosome-SEB toxoid vaccines against lethal systemic (i.m.) challenge with SEB toxin.** To evaluate the efficacy against parenteral SEB challenge, 5 to 15 BALB/c mice were challenged i.m. (in groups of 5 to 10 per group) 3 to 4 weeks after the last i.m. or intranasal immunization with the toxoid or proteosome-toxoid vaccines. As shown in Table 1, immunization with toxoid without proteosomes i.m. (with or without alum) or intranasally (without alum) did not significantly protect against lethal systemic SEB challenge. Immunization with control vaccines (four groups, 10 mice per group) containing proteosomes plus peptides (that were not relevant to SEB protection) instead of the toxoid did not confer any protection, demonstrating that proteosomes could not protect nonspecifically (data not shown). In contrast, each of the four different route-formulation combinations of the proteosome-toxoid vaccine, namely, immunizing twice i.m. with or without alum, twice intranasally (in saline), or as an intranasal boost after an i.m. prime, resulted in significant protection against i.m. challenge.

These results were not unexpected since the vaccines that afforded the greatest level of protection also induced the strongest immune responses as described in Fig. 1 to 4. This corre-

lation between immunogenicity and efficacy was also examined by measuring the anti-SEB antibody responses in sera obtained 1 week prior to challenge from 20 individual BALB/c mice immunized i.m. with either the toxoid or proteosome-toxoid vaccine formulated in either saline or with alum. Regardless of the SEB toxoid vaccine received, 11 of the 14 mice (79%) with anti-SEB ELISA titers greater than 102,400 survived, whereas 5 of the 6 mice (83%) with titers of 102,000 or less died, and this difference was statistically significant ( $P < 0.019$ ).

To evaluate i.m. vaccine efficacy in outbred (CD-1) mice which are naturally more resistant than BALB/c mice to SEB toxicity, the i.m. challenge dose of SEB toxin (administered with 20 mg of D-galn) was increased from 25  $\mu\text{g}$  (the LD<sub>100</sub> in BALB/c mice) to 100  $\mu\text{g}$ . Although 500  $\mu\text{g}$  of SEB was 90% lethal in the CD-1 mice, 100% lethality was not reproducibly achieved in unvaccinated mice of this strain in several experiments; therefore, the vaccine efficacy experiment was performed with a 100- $\mu\text{g}$  challenge dose which resulted in 56% lethality in unvaccinated mice. In this experiment (Table 2), i.m. immunization with either the saline or the alum preparations of the proteosome-toxoid vaccine induced 100% protection ( $P < 0.006$  for either vaccine), thereby confirming the significant protection determined for these vaccine preparations in the BALB/c mice described above (Table 1). Moreover, even though these CD-1 mice were more naturally resistant to SEB lethality in the D-galn model, immunization with saline or alum preparations of the toxoid without proteosomes did not induce significant protection in the outbred mice compared with that of the saline-immunized CD-1 controls. The 67 and 55% levels of survival found with the toxoid vaccine given in saline or alum, respectively, were not significantly different from the 44% level of survival attained by saline sham-immunized mice; hence, the resultant level of protection of these

TABLE 1. Protection against systemic (i.m.) challenge with SEB in D-galn BALB/c mouse model after i.m. or intranasal immunization with SEB toxoid vaccines formulated with proteosomes and/or alum

Vaccine <sup>a</sup>	Route	Adjuvant	No. of dead mice	Total no. of mice	% Protection <sup>b</sup>	P value <sup>c</sup>
Saline control	i.m.	None	15	15	0	
	Nasal	None	15	15	0	
Toxoid	i.m.	None	13	15	13	$<0.242$ (NS)
	Nasal	None	10	10	0	NS
Proteosome-toxoid	i.m.	None	7	15	53	$<0.002$
	Nasal	None	7	15	53	$<0.002$
	i.m. prime, nasal boost	None	2	10	80	$<0.0004$
Toxoid	i.m.	Alum	3	5	40	$<0.096$ (NS)
Proteosome-toxoid	i.m.	Alum	2	5	60	$<0.022$

<sup>a</sup> Groups of five mice were immunized on weeks 0 and 3 with 10  $\mu\text{g}$  i.m. or 50 or 100  $\mu\text{g}$  intranasally. The 100- $\mu\text{g}$  doses were divided into two 50- $\mu\text{g}$  doses spaced 2 days apart.

<sup>b</sup> Challenge with 25  $\mu\text{g}$  of SEB plus 20 mg of D-galn i.m. was 4 weeks after the last immunization. Percent protection was calculated by the following previously described (29) formula: [(% death in controls) - (% death in vaccinees)  $\times$  100]/% death in controls.

<sup>c</sup> P values were calculated by the Fisher exact test (one tailed) comparing survival of vaccine immunized mice with survival of sham-immunized mice given saline. NS, not significant at the 0.05 level.

TABLE 2. Protection against systemic (i.m.) challenge with SEB in D-galn CD-1 mouse model after i.m. immunization with SEB toxoid vaccines formulated with proteosomes and/or alum

Vaccine <sup>a</sup>	Route	Adjuvant	No. of dead mice	Total no. of mice	% Protection <sup>b</sup>	P value <sup>c</sup>
Saline control	i.m.	None	10	18	0	
Toxoid	i.m.	None	3	9	40	<0.250 (NS)
	i.m.	Alum	4	9	20	<0.446 (NS)
Proteosome-toxoid	i.m.	None	0	9	100	<0.006
	i.m.	Alum	0	9	100	<0.006

<sup>a</sup> Groups of four to five mice were immunized i.m. on weeks 0 and 3 with 10 or 50 µg of vaccine; there was no significant difference in the serum antitoxin IgG titers between the groups receiving 10 or 50 µg of the same vaccine.

<sup>b</sup> Challenge with 100 µg of SEB plus 20 mg of D-galn was 4 weeks after the last immunization. Percent protection was calculated with the formula described in Table 1, footnote b.

<sup>c</sup> P values were calculated by the Fisher exact test (one tailed) comparing survival of vaccine-immunized mice with survival of sham-immunized mice given saline. NS, not significant at the 0.05 level.

vaccines was 40% ( $P < 0.250$ ) and 20% ( $P < 0.446$ ), respectively (calculated by the formula described in Table 2). These results were also consistent with the lack of significant protection against i.m. challenge induced by the saline and alum preparations of the toxoid alone in BALB/c mice. Furthermore, antitoxin IgG titers of pooled sera from groups of CD-1 mice immunized with proteosome-toxoid either in saline or with alum (9 to 10 mice per vaccine type) were greater than 102,400, whereas titers of mice immunized with the toxoid without proteosomes in either saline or alum were equal to or less than 102,400. This observation is consistent with the correlation between serum antitoxin IgG titer and the survival against SEB challenge described for BALB/c mice (see details above).

#### Efficacy against lethal respiratory challenge with SEB toxin induced by i.m. or intranasal proteosome-SEB toxoid vaccines.

Since respiratory exposure to SEB is toxic to monkeys and humans and since such exposure reflects the potential biologic warfare threat of SEB, a murine model of lethal toxicity following respiratory challenge with SEB that could assess the ability of the parenteral or mucosal toxoid and proteosome-toxoid vaccines to protect against such challenge was developed. After extensive preliminary experiments, it was found that for respiratory intoxication, 350 µg of SEB in 35 µl given intranasally to BALB/c mice immediately after i.m. injection of 20 mg of D-galn was the most reliable technique to induce 100% lethality.

The immunization protocols used in these experiments were as described above for the vaccine efficacy study performed with i.m. SEB challenge. As shown in Table 3, neither i.m. nor intranasal immunization with the toxoid alone protected any mouse against respiratory challenge with SEB. In contrast, either i.m. or nasal immunization with the proteosome-toxoid vaccine elicited significant protection of 70% ( $P < 0.005$ ) and 40% ( $P < 0.044$ ), respectively (Table 3). Furthermore, i.m. immunization with the proteosome-toxoid vaccine with alum as an adjuvant elicited 90% protection ( $P < 0.0001$ ), whereas immunization with the alum preparation of the toxoid without proteosomes induced 40% protection ( $P < 0.044$ ) (Table 3). Surprisingly, priming i.m. and boosting intranasally with the proteosome-toxoid vaccine elicited only 10% protection ( $P < 0.434$ ) against this respiratory challenge (Table 3), whereas this

combination protected 80% of the mice against i.m. challenge (Table 1).

## DISCUSSION

This study demonstrates that formulation of SEB toxoid with proteosomes improves antitoxin immunity induced by either i.m. or intranasal immunization without additional adjuvants. It was previously reported by Eldridge et al. (15) that neutralizing immunity could be induced by immunizing mice subcutaneously with formalinized SEB toxoid encapsulated in poly(DL-lactide-co-glycolide) microspheres whereas unencapsulated toxoid was less immunogenic and induced markedly less neutralization as measured by the lack of increase in splenic Vβ8 T cells after intravenous challenge. In its molecular weight as well as its lack of mitogenicity for murine cells, the toxoid used here was similar to the toxoid prepared by Eldridge et al. (15) and was unlike the formalinized toxoid prepared at pH 7.5 in the early 1970s, which was greater than 90% polymeric, had a molecular weight of  $>10^6$  (51), and also was mitogenic (47). This report is consistent with the microsphere study in confirming the inability of formalinized toxoid without adjuvant to induce sufficient antibodies to neutralize SEB toxicity in vivo and extends this observation to both parenteral and respiratory challenge with lethal amounts of SEB in D-galn models. Toxoid without proteosomes with alum as an adjuvant also did not induce consistent protection against parenteral challenge. In contrast, protection against either parenteral or respiratory SEB challenge after i.m. immunization was most significant when the toxoid was formulated with proteosomes regardless of whether alum was added. These data demonstrate that the determinants on the toxoid that are important for induction of neutralizing antibodies are readily accessible to recognition by the immune system after formulation of the toxoid with proteosomes. The results reported here showing

TABLE 3. Protection against mucosal (respiratory) challenge with SEB in D-galn BALB/c mouse model after i.m. or intranasal immunization with SEB toxoid vaccines formulated with proteosomes and/or alum

Vaccine <sup>a</sup>	Route	Adjuvant	No. of dead mice	Total no. of mice	% Protection <sup>b</sup>	P value <sup>c</sup>
Saline control	i.m.	None	10	10	0	
	Nasal	None	10	10	0	
Toxoid	i.m.	None	10	10	0	NS
	Nasal	None	10	10	0	NS
Proteosome-toxoid	i.m.	None	3	10	70	<0.005
	Nasal	None	6	10	40	<0.044
	i.m. prime, nasal boost	None	9	10	10	<0.501 (NS)
Toxoid	i.m.	Alum	6	10	40	<0.044
Proteosome-toxoid	i.m.	Alum	1	10	90	<0.0001

<sup>a</sup> Groups of five mice were immunized on weeks 0 and 3 with 10 µg i.m. or 50 or 100 µg intranasally. The 100-µg doses were divided into two 50-µg doses spaced 2 days apart.

<sup>b</sup> Challenge with 350 µg of SEB intranasally plus 20 mg of D-galn i.m. was 4 weeks after the last immunization. Percent protection was calculated by the formula described in Table 1, footnote b.

<sup>c</sup> P values were calculated by the Fisher exact test (one tailed) comparing survival of vaccine-immunized mice with survival of sham-immunized mice given saline. NS, not significant at the 0.05 level.



that higher antitoxin levels are associated with enhanced protection strongly suggest that pathogenic superantigenic binding of SEB to target cells can be neutralized by the acquisition of anti-SEB antibodies such as those induced by immunization with the formalinized SEB toxoid formulated with proteosomes.

The most striking effects of proteosome formulation on immunogenicity and efficacy of the toxoid were found after intranasal immunization. It was reported previously that proteosomes confer mucosal immunogenicity to LPS (29, 39, 40) and peptides (21); such antigens are also exceedingly poor immunogens when given i.m. without proteosomes or other immunopotentiating carriers and adjuvants. Although SEB toxoid is a protein antigen that can induce serum antibodies when given i.m. without adjuvants, when given intranasally, the toxoid alone was unable to induce lung IgA while intestinal IgA and serum IgG levels were exceedingly low. This dichotomy between parenteral and mucosal immunogenicity is not unique to SEB since protein antigens without an adjuvant are commonly ineffective when administered via mucosal routes even when they are highly immunogenic when injected parenterally (35). In contrast, formulation of the toxoid with proteosomes elicited 3-log increases in lung and serum antibody levels and 2-log increases in intestinal antitoxin levels compared with those after intranasal immunization with the same amount of toxoid without proteosomes.

These data support previous reports (21, 24, 29, 39, 40) that proteosomes are one of a limited number of immunopotentiating vaccine delivery systems capable of enhancing mucosal immunogenicity. The ability of other mucosal adjuvants to potentiate immune responses has been related in part to their capacity to facilitate mucosal uptake for recognition by local antigen-processing cells. Thus, binding to mucosal cell surface GM<sub>1</sub> ganglioside is thought to contribute strongly to the mucosal immunopotentiality induced by cholera toxin (8, 18), cholera toxin B subunit (8, 9, 17, 42, 53), and the recombinant analogs of related pertussis and *Escherichia coli* heat-labile toxins (10, 41). Since meningococci naturally colonize respiratory mucosa, we suggest that the neisserial outer membrane proteins of proteosomes may potentiate mucosal immunogenicity by facilitating binding to mucosal cell surface ligands such as those on M cells thought to be responsible for the selective uptake and transport of bacteria to subepithelial antigen-presenting cells (38).

The size and particulate nature of poly(DL-lactide-co-glycolide) microspheres, which have been used for mucosal delivery of SEB toxoid (14, 19, 48) and of *E. coli* (12, 34), *Bordetella pertussis* (45), and SIV antigens (31), have been cited as important for enhanced cellular uptake and adjuvanticity. For example, larger microspheres have poor uptake and are less effective than those with diameters of <10 μm (14, 15). The hydrophobic, membrane-like physical structure of liposomes which are generally <2 μm in diameter is also thought to promote uptake and antigen processing (1–3, 16). By light-scattering and electron microscopic analyses, proteosome vesicles and membrane fragments are generally 0.04 to 0.1 μm in diameter. This size as well as the vesicular and hydrophobic membranous nature of proteosomes may also contribute to optimal uptake and processing by mucosal cells. In addition, the immunopotentiating capacity of proteosomes may be related to their ability to mitogenically stimulate B cells (25) since a prerequisite for such cell activation is recognition and binding to the cell. In this scenario, immune recognition and processing of antigens bound to proteosomes is facilitated by the enhanced capacity of such cells to recognize and bind to the proteosomes that deliver the antigens for immune process-

ing. The recent demonstration that neisserial outer membrane proteins recognize and upregulate major histocompatibility complex class II and B7.2 costimulatory ligands on B cells (52) and perhaps other antigen-presenting cells and the report (6) that alveolar macrophages present antigen inefficiently to CD4<sup>+</sup> T cells because of defective expression of B7 costimulatory cell surface molecules suggest that proteosomes may perform an immunologically active role in enhancing respiratory immunogenicity.

For several antigen delivery systems, respiratory immunization can be advantageous for both serum and mucosal immune responses. In proteosome-shigella LPS vaccine studies, 10-fold-less vaccine could elicit similar or stronger serum, intestinal, and lung antibody responses and protection when intranasal rather than intragastric immunization was used (29, 40). Polysaccharide antigens encapsulated in liposomes were also effective at lower doses when delivered intranasally rather than orally (1), and intranasal immunization with influenza glycoproteins in liposomes was more protective and induced higher nasal antibody levels than i.m. immunization (16). Furthermore, microencapsulated *B. pertussis* antigen was immunogenic and protective intranasally but not orally (45). Similarly, although oral immunogenicity of microencapsulated SEB toxoid has been reported in mice (14), intratracheal immunization (especially boosting) of monkeys with this vaccine was immunogenic and could protect against aerosol challenge whereas oral immunization was poorly immunogenic and nonprotective (19, 48).

The data in this murine study that significant protection against either parenteral or respiratory challenge is elicited by either intranasal or i.m. immunization with the proteosome-toxoid vaccine are supported by recent results indicating that i.m. and respiratory (intratracheal) delivery of proteosome-toxoid vaccines protected 100% of monkeys (10 per group) against lethal aerosol SEB challenge (24). These data are encouraging for the advanced development of proteosome vaccines delivered intranasally and/or i.m. to protect against respiratory, mucosal, or systemic exposure to SEB or other toxic or infectious pathogens.

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