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Immunogenicity and Efficacy against Lethal Aerosol Staphylococcal Enterotoxin B Challenge in Monkeys by Intramuscular and Respiratory Delivery of Proteosome-Toxoid Vaccines

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Staphylococcal enterotoxin B (SEB), a primary cause of food poisoning, is also a superantigen that can cause toxic shock after traumatic or surgical staphylococcal would infections or viral influenza-associated staphylococcal superinfections or when aerosolized for use as a potential biologic warfare threat agent. Intranasal or intramuscular (i.m.) immunization with formalinized SEB toxoid formulated with meningococcal outer membrane protein proteosomes has previously been shown to be immunogenic and protective against lethal respiratory or parenteral SEB challenge in murine models of SEB intoxication. Here, it is demonstrated that immunization of nonhuman primates with the proteosome-SEB toxoid vaccine is safe, immunogenic, and protective against lethal aerosol challenge with 15 50% lethal doses of SEB. Monkeys (10 per group) were primed i.m. and given booster injections by either the i.m. or intratracheal route without adverse side effects. Anamnestic anti-SEB serum immunoglobulin G (IgG) responses were elicited in all monkeys, but strong IgA responses in sera and bronchial secretions were elicited both pre- and post-SEB challenge only in monkeys given booster injections intratracheally. The proteosome-SEB toxoid vaccine was efficacious by both routes in protecting 100% of monkeys against severe symptomatology and death from aerosolized-SEB intoxication. These data confirm the safety, immunogenicity, and efficacy in monkeys of parenteral and respiratory vaccination with the proteosome-SEB toxoid, thereby supporting clinical trials of this vaccine in humans. The safety and enhancement of both bronchial and systemic IgA and IgG responses by the proteosome vaccine delivered by a respiratory route are also encouraging for the development of mucosally delivered proteosome vaccines to protect against SEB and other toxic or infectious respiratory pathogens.

Staphylococcal enterotoxin B (SEB), a toxin secreted by *Staphylococcus aureus*, is one of a family of staphylococcal enterotoxins that cause food poisoning manifested by vomiting and diarrhea (6, 38). SEB also causes a significant percentage of non-menstrual-associated cases of toxic shock syndrome in patients with a variety of staphylococcal infections, including surgical and postpartum wound infections, deep abscesses, burns, abrasions, insect bites, sinusitis, and influenza-associated superinfections of the respiratory tract (10, 23, 36, 45). Toxic shock syndrome is characterized by high fever, erythroderma, delayed desquamation, hypotension, shock, and other symptoms, including vomiting, diarrhea, severe myalgia, disorientation, mucous membrane changes, and abnormalities in renal and hepatic functions (10, 45). The case fatality rate for toxic shock syndrome from SEB can be 50% (10, 11), and in influenza-associated cases, mortality can reach 90% (36, 45). Accordingly, aerosolized SEB is considered a potential biologic warfare threat agent that can cause incapacitation, shock, and death in soldiers and civilians (15, 28).

The lethal toxic symptoms of SEB toxicosis are postulated to be due to the initiation of an overwhelming cytokine cascade subsequent to superantigenic binding and activation of mononuclear cells by SEB (12, 27, 36, 44). SEB initiates this outpouring of cytokines when it forms a complex with major histocompatibility complex II molecules on antigen-presenting cells and the T-cell Vβ region outside the antigen binding site of selected classes of T-cell receptors. Since SEB binding to T cells is independent of the specific antigen binding site, entire T-cell subsets (up to 20% of all T cells) can be stimulated with concomitant massive production of cytokines, resulting in shock and death (12, 27, 36, 44).

Immunization with vaccines containing SEB toxoid is predicated on the induction by the toxoid of antibodies that interact with the toxin so as to interfere with the toxin's capacity to initiate toxic events, such as those associated with superantigenic binding. In normal, untreated mice, SEB causes weight loss (26) and selected Vβ T-cell proliferation in vivo after systemic administration (12, 27). A formalinized SEB toxoid was successfully encapsulated in poly-lactide-co-glycolide microspheres by Eldridge et al., who used this nonlethal murine model of SEB toxicity to show that immunization with the microsphere-encapsulated toxoid, but not unencapsulated free toxoid, protected mice against the weight loss and T-cell changes induced by systemic administration of SEB (8). Since then, several murine models of lethal SEB toxicity have been developed (2, 7, 20, 31) in attempts to simulate the lethal effects of low doses of SEB in humans. Using D-galactosamine-sensitized murine models of lethal SEB toxicity, we have shown that either intramuscular (i.m.) or intranasal immunizations with formalinized-SEB-toxoid vaccines formulated with meningococcal outer membrane protein proteosomes protect against lethal challenge with SEB and that such vaccines afford protection against SEB challenge delivered by either the parenteral or respiratory route (20).

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Nonhuman primates are the only animals that are similar to humans in terms of both their clinical responses and lethal sensitivity to low doses of SEB (5, 6, 28, 30). While SEB ingestion causes vomiting and diarrhea (5, 6, 28), aerosol exposure of primates to SEB, like parenteral challenge (5, 6), results in gastrointestinal symptoms plus lethargy, shock, and death (15, 28, 40). The contribution of local lung immunity to protection against respiratory pathogens and the ability of mucosal immunization to facilitate such immunity have been well described for a number of diseases (3, 9, 24, 29, 37). Consequently, immunogenicity and efficacy against aerosol SEB challenge of the microencapsulated SEB toxoid vaccine of Eldridge et al. (that had been used in mice [8]) were tested in groups of monkeys (four per group) that were primed and given one booster injection with each of the nine possible combinations of i.m., oral (i.e., intragastric), and intratracheal (i.t.) routes (15, 40). The results of that study indicated that respiratory (i.t.) booster immunization was important for protection against aerosolized SEB since all four animals that were given booster injections i.t. after i.m. priming and four of the eight animals immunized either only i.t. or given booster injections i.t. after oral priming survived SEB challenge (15, 40). In contrast, 3 of 4 animals that were primed and given booster injections i.m. and all 12 animals in the three groups that received either two oral immunizations or one i.m. and one oral immunization (in either order) with the microsphere-SEB toxoid vaccine died of SEB intoxication (15, 40).

These results in nonhuman primates, suggesting that the i.m. prime-i.t. booster regimen was advantageous (15, 40) together with the data demonstrating protection in murine models of SEB intoxication by intranasal or i.m. proteosome-SEB toxoid vaccines (20), formed the basis of the present nonhuman primate study examining the immunogenicity of the proteosome-SEB toxoid vaccine by using two route schedules and testing the efficacy of the vaccine against aerosolized-SEB challenge. Specifically, rhesus monkeys were primed i.m., given booster injections twice either i.m. or i.t., and then challenged with aerosolized SEB 1 month after the last immunization. Sera and bronchial lavage fluids (BAL) were collected after each immunization and several times after challenge to compare the effects of the different routes on antibody production at these sites and to examine correlations between serum and respiratory immunity and clinical outcome.

MATERIALS AND METHODS

Antigen isolation and vaccine formulation. (i) Proteosomes. Outer membrane protein proteosome preparations were stored at −70°C after purification, as previously described (20, 24), from group B type 2 *Neisseria meningitidis* by extraction of phenol-killed bacterial paste with a solution of 6% Empigen BB (EBB) (Albright and Wilson, Whitehaven, Cumbria, United Kingdom) in 1 M calcium chloride followed by precipitation with ethanol, solubilization in 1% EBB-Tris-EDTA-saline, precipitation with ammonium sulfate, and resolubilization in the 1% EBB buffer.

(ii) Toxoid. SEB (lot 14-30; obtained from Department of Toxinology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.), purified by the method of Schantz et al. (35), was treated with formalin for 30 days at 37°C and pH 7.5, as originally reported by Warren et al. (43), performed by Eldridge et al. (8), and described in detail by Lowell et al. (20). Briefly, in a biohazard hood, batches of 0.1 or 0.5 g of SEB were dissolved in 0.02 M sodium phosphate buffer (pH 7.35) at a concentration of 2.4 mg/ml. A dialysis bag (with Spectra/Por dialysis tubing with a molecular weight cutoff of 12,000 to 14,000) with a solution of 37% formalin–methanol (Sigma) which had been diluted sixfold in the pH 7.5 phosphate buffer was then emersed in the beaker with SEB and allowed to incubate overnight at room temperature. Then this dialysis bag was 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°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. Then the toxoid solution was centrifuged for 10 min at 4,000 rpm, dialyzed (with the tubing [12,000 to 14,000 Da] described above) against phosphate buffer (pH 7.5) for 4 days with daily buffer changes, and sterile filtered; the protein concentration was determined (20) and stored at 4°C until use. The safety of this toxoid in rabbits and mice, as determined by lack of lethality in rabbits (administering 2 mg i.m. to 4-kg rabbits) and in D-galactosamine-sensitized mice (at 100- and 500-μg i.m. doses) and lack of mitogenicity for murine lymphocytes, was previously reported (20).

(iii) Proteosome vaccine formulation. SEB toxoid was formulated with proteosomes by the technique previously described for noncovalent complexing of proteosomes to peptides (17, 18, 22) or lipopolysaccharides (24, 32), as detailed in the proteosome-SEB toxoid murine study (20). 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–0.15 M NaCl with 1% EBB and then the solution was dialyzed against 0.05 M Tris (pH 8.0) across a Spectra/Por 6 dialysis membrane (Spectrum Medical Industries, Los Angeles, Calif.) with a molecular weight cutoff of 1,000 for 8 to 10 days at 4°C with daily buffer changes. The immunogenicity and efficacy against SEB challenge in mice of the proteosome-SEB toxoid vaccine were determined prior to this study (13). Vaccines were stored at 4°C until use.

(iv) Alum adjuvant. For i.m. immunizations only, the proteosome-SEB toxoid vaccine was administered with alum as an adjuvant as previously described (20) by adding the appropriate volume of a preparation of 3% aluminum hydroxide (Alhydrogel; Superfos, Biosector a/s, Vedbaek, Denmark) to the vaccine in 0.5 M Tris normal saline buffer (pH 6.5 to 7.0) and incubating for at least 18 h at 4°C with occasional gentle mixing. The volume of aluminum hydroxide added was calculated so that each 0.5-ml dose of vaccine contained 2.17 mg of aluminum hydroxide with 0.76 mg of Al^{3+}, which is less than the maximum recommended dose of Al^{3+} allowable in humans (0.85 mg). Preparations were stored at 4°C.

Animal care and use. These experiments 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* (41), 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 Committees of our research institutes.

Animals. The twenty-two rhesus male and female monkeys (*Macaca mulatta*) used in this study weighed 5.5 to 11.2 kg and were in good health as shown by complete blood count, serum chemistry, and liver function enzyme analyses of two preimmunization blood samples. Sera were screened to exclude monkeys with simian immunodeficiency virus and those with substantial levels of preexisting anti-SEB antibodies.

Anesthesia. Telazol (6 mg/kg) was given i.m. in the thigh prior to all immunizations, collection of blood and BAL samples, and SEB challenge.

Immunizations. Two groups of monkeys, with 10 monkeys per group, received three doses of the proteosome-toxoid vaccine, spaced 4 weeks apart, by one of two route schedules. One group was immunized i.m. three times (IM/IM/IM), and the other group received an i.m. primary immunization followed by two i.t. immunizations (IM/IT/IT). For i.m. immunization, vaccine containing 100 μg of toxoid with 100 μg of proteosomes in 0.5 ml of buffered sterile saline was delivered with alum (as described above) in the caudal thigh muscle. The same thigh was used for primary and i.m. booster immunizations. For i.t. immunization, vaccine containing 250 μg of toxoid with 250 μg of proteosomes in 3 ml of buffered saline was delivered via a no. 5 French pediatric suction catheter placed through a pediatric endotracheal tube. Two control animals (to verify the lethality of SEB aerosol challenge) were given saline by the IM/IT/IT schedule.

Biosamples. Handling of all monkey biosamples was performed with gloves and protective outerwear clothing.

(i) Serum samples. Blood samples were obtained from the femoral vein within 1 h prior to each immunization, 3 weeks after each immunization, 1 week after the third immunization, 1 h prior to SEB challenge, 2 and 16 weeks after SEB challenge, and either 6 or 10 weeks postchallenge. Blood samples were divided into appropriate tubes for blood chemistry and hematology analyses and serum antibody assays.

(ii) BAL samples. BAL samples were obtained 1 to 2 weeks prior to immunization and 3 weeks after each immunization. BAL samples were also obtained 1 week after the third immunization and 2, 6, and 10 weeks after SEB challenge. For BAL collection, sterile phosphate-buffered saline (PBS) (5 ml) was passed through a 3-mm-diameter endotracheal tube with a no. 5 French pediatric suction catheter and, using a surgical suction apparatus with a three-way stopcock, 1 to 1.5 ml of BAL was recovered in a collecting tube and stored on wet ice. To prevent antibody degradation, 0.25 ml of a solution of proteolytic enzyme inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 mM EDTA, 100 mM iodoacetamide) was added to the BAL, which was centrifuged to remove cell debris prior to storage in aliquots at −70°C.

Antigen-specific antibody assays. An enzyme-linked immunosorbent assay (ELISA) was used to measure sera and BAL for levels of immunoglobulin G (IgG) and IgA that recognized SEB toxin. The ELISA was performed as previously described (20) with minor modifications. Briefly, 96-well round-bottom microtiter plates (Immulon 2; Dynatech, Chantilly, Va.) were coated in a biosafety cabinet with SEB toxin (10 μg/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, plates were washed once with PBS containing 0.05% Tween (PBS-T)

and incubated with blocking solution containing 0.5% (each) casein and bovine serum albumin (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 sera or BAL 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 alkaline phosphatase-labeled goat anti-mouse IgG or IgA (Kierkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added and plates were incubated at room temperature overnight. After aspirating and washing twice with PBS-T, *p*-nitrophenylphosphate diluted in diethanolamine buffer (pH 9.8) was added and the plates were allowed to remain at room temperature for 20, 60, or 120 min for serum IgG, serum IgA, or BAL determinations, respectively, prior to determining the A_{450} with an ELISA plate reader (Molecular Devices, Menlo Park, Calif.). The antibody titers were expressed as the geometric mean of the greatest dilution of serum or BAL that elicited an optical density (OD) of greater than 1.0, 0.5, or 0.2 for serum IgG, serum IgA, or BAL samples, respectively. For serum antibody determinations, these values represent determinations within the linear part of the ELISA curve generated by twofold dilutions, while the BAL values represent determinations more closely reflective of endpoint titers. The antibody levels of all samples were determined individually, and error bars represent the standard errors of the means (SEM) for groups of monkeys receiving the same vaccine. Anti-SEB IgG and IgA levels in serum and BAL samples of sham-immunized control animals receiving only normal saline did not change significantly during the immunization period (data not shown) and were not different from the preimmunization levels of vaccinated animals.

Statistical analyses. The statistical significance of differences in ELISA immune responses was analyzed by the two-tailed, two-sample *t* test by using Minitab for Windows (version 10.5) after logarithmic conversion of ELISA titers.

Challenge with aerosolized SEB. (i) Study design. This study was designed to avoid challenging all monkeys with SEB simultaneously by dividing the 22 monkeys into two iterations of 11 monkeys each, spaced 2 weeks apart. Within each iteration, immunizations and challenges of five monkeys from each of the two vaccine groups and one control monkey were performed on the same days. The decision to proceed with challenging each immunized group in the second iteration was confirmed after it had been ascertained that at least two monkeys from that group in the first iteration were protected. In addition, in either iteration 1 week prior to challenging animals, serum anti-SEB antibody responses were measured by ELISA. Less-than-10-fold increases in serum geometric mean titers in an immunized group would have abrogated challenge of that group.

Thirty days after the third immunization, monkeys were challenged with a mean (and median) inhaled dose of aerosolized SEB of 15 (range, 10.9 to 18.7) 50% lethal doses (LD_{50}s). This dose was determined by using Guyton's formula (12a) by multiplying the amount of toxin per liter of aerosol by the number of liters of air inhaled during the exposure period (as indicated by the animal's tidal volume). The LD_{50} of aerosolized SEB for rhesus monkeys was based on data archived at U.S. Army Medical Research Institute of Infectious Diseases over 10 years in which at least 48 animals were challenged with aerosolized SEB (15, 28, 40).

(ii) Method of aerosol challenge. The procedures and principles for aerosolized-SEB challenge were similar to those for previous aerosol SEB challenges of monkeys (15, 28, 40). For aerosol challenge, in a class III hood, anesthetized monkeys were placed in a modification of the Henderson head-only exposure chamber (34). The monkeys in the chamber were exposed to SEB for 10 min with a subsequent 5-min flush with air. The toxin solution was aerosolized by a three-jet nebulizer driven by compressed air at 26 lb/in^2 gauge to generate an aerosol flow rate of 7.5 liters/min and disseminate the solution at a rate of 0.3 ml/min. Humidified air (8 liters/min) was mixed with the nebulized aerosol in a stainless steel tube on route to the exposure chamber to obtain a flow rate of 16 liters/min. The aerosol was sampled directly from the chamber for the total 10-min exposure period by using an all-glass impinger containing a 6.5-liters/min critical orifice to regulate the flow under a sustained vacuum of at least 15 in (1 in. = 2.54 cm) of Hg. Sampled toxin was impinged in 10 ml of collecting medium.

(iii) Clinical evaluation. Monkeys were evaluated every 1 to 2 h from 0700 to 2200 h and periodically overnight as required for 5 days, with subsequent evaluations twice daily through day 7, by a team of veterinarians blinded to the monkey's vaccine group. Animals were classified as having either mild, moderate, or severe illness on the basis of overall condition, temperament, and duration of illness (return to clinical normalcy), as well as specific symptoms of anorexia, diarrhea, emesis, coughing, depression, dyspnea, and shock.

RESULTS

Anti-SEB antibody responses in sera prior to SEB challenge. The proteosome-toxoid vaccine was safe by either the i.m. or i.t. route, as measured by lack of observed local or systemic reactions and absence of significant changes in clinical serum chemistry or hematology values. Figure 1 shows the anti-SEB IgA and IgG responses in sera of groups of monkeys (10 monkeys per group) before and after immunization with one, two, and three doses (at weeks 0, 4, and 8) of the proteosome-SEB toxoid vaccine. One group of monkeys was primed and boosted i.m. (IM/IM/IM), and the other group received one primary immunization i.m. followed by two respiratory booster immunizations i.t. (IM/IT/IT). High levels of anti-SEB IgG were induced by either route schedule (Fig. 1), whereas two monkeys sham immunized with saline did not show any significant antibody rises (data not shown). Anamnestic increases were evident after each booster immunization and were especially strong after the second i.t. booster immunization. There were no significant differences in the levels of anti-SEB IgG in sera induced by either route, indicating that either respiratory or i.m. immunization with SEB toxoid formulated with proteosomes is effective in boosting serum anti-SEB IgG.

The major difference in the serum responses of the two groups of monkeys was that anti-SEB IgA levels were significantly higher in sera obtained at each of the three time points from animals given booster immunizations twice via the respiratory route compared with those given booster injections i.m. ($P < 0.033$, $P < 0.016$, and $P < 0.015$ for sera from weeks 9, 11, and 12, respectively). The fact that strong increases in serum IgA levels were higher in animals given i.t. booster immunizations than in animals given i.m. booster injections only after two respiratory immunizations suggests that the enhancement of serum IgA responses is more effective after respiratory priming than after i.m. priming.

Anti-SEB antibody responses in BAL prior to SEB challenge. The advantage of giving booster immunizations via the respiratory route was most dramatically demonstrated by examination of the levels of anti-SEB IgA in BAL samples. As shown in Fig. 2, three i.m. immunizations did not elicit any bronchial IgA responses, whereas significant anti-SEB IgA responses were detected after only one respiratory booster immunization ($P < 0.018$ [compared with i.m. booster injection]). Exceptionally strong anamnestic IgA responses were found in BAL samples collected after the second respiratory booster immunization ($P < 0.0001$ [compared with i.m. booster injections]). After two immunizations, BAL IgG levels were similar whether the monkeys were given booster immunization i.t. or i.m. The influence of respiratory booster immunizations on BAL IgG responses was evident 1 week after the second i.t. booster immunization as the fourfold-higher levels found in animals given i.t. booster immunizations were significantly higher than those found in animals given i.m. booster injections ($P < 0.041$). One week before challenge, however, the threefold-higher levels of anti-SEB Ig6 in BAL in animals given i.t. booster immunizations were not significantly different from those in animals given i.m. booster injections ($P < 0.111$).

Vaccine efficacy against challenge with aerosolized SEB (Table 1). Immunization with the proteosome-SEB toxoid vaccine protected 100% of monkeys (10 of 10 in each of two groups) against severe illness and death from lethal aerosol challenge with a mean of 15 (range, 10.5 to 18.7) LD_{50}s of SEB (Table 1). To confirm the potency of SEB in the challenge, one monkey in each of two iterations was sham immunized with saline. These control animals died 21 to 43 h postchallenge after developing anorexia, vomiting, progressive depression, and shock. In contrast, 8 of 10 animals in each immunized group manifested mild illness and 2 animals per group showed moderate illness. No immunized animal developed severe illness consisting of dyspnea, progressive depression, or shock. Although 15 LD_{50}s of SEB characteristically induces vomiting and diarrhea in >80% of monkeys (30), no animals had diarrhea. Four animals given i.m. booster injections and three animals given i.t. booster immunizations had one or two episodes of emesis, while three animals given i.m. booster injections and two ani-

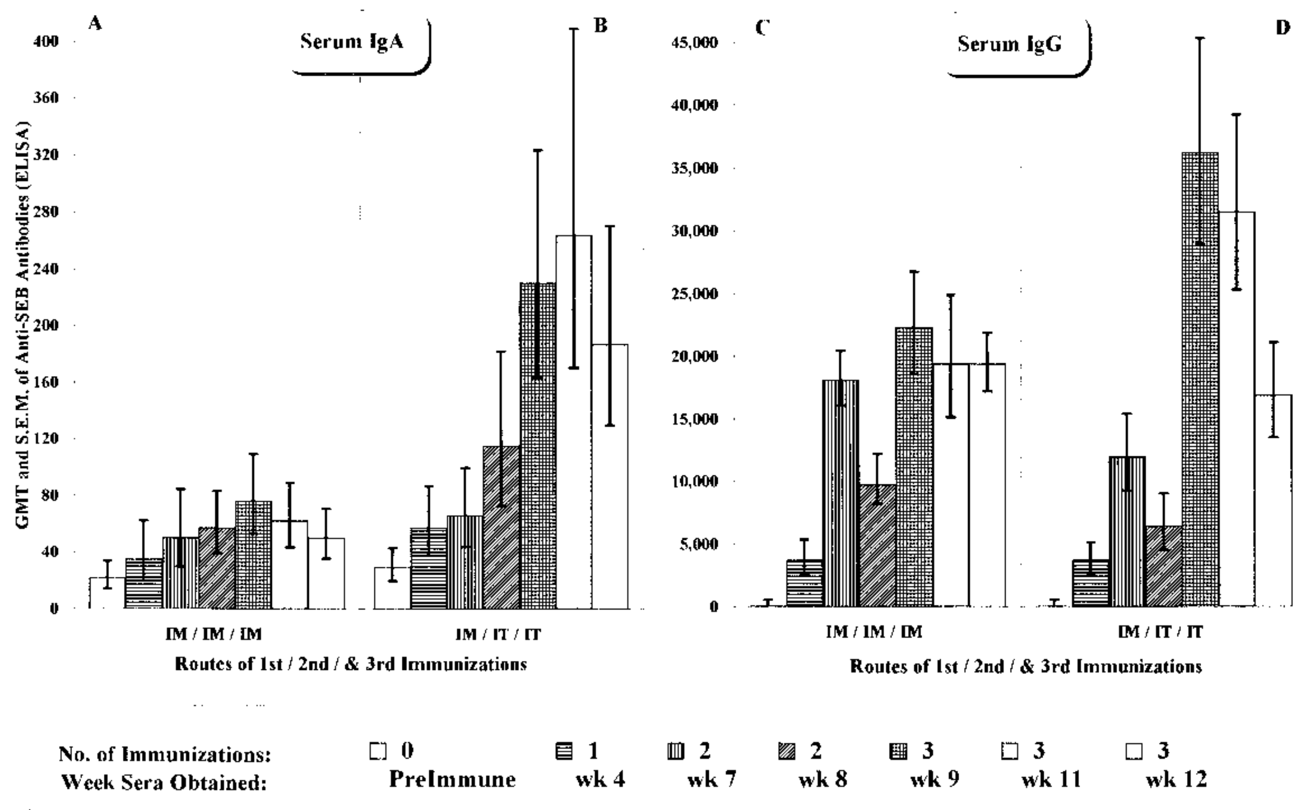

FIG. 1. Serum IgA (A and B) and IgG (C and D) responses prior to SEB challenge in rhesus monkeys primed i.m. and given booster immunizations either i.m. (A and C) or i.t. (B and D) with proteosome-SEB toxoid vaccine. Anti-SEB IgG and IgA titers prior to challenge with SEB, as determined by ELISA, in individual sera obtained from monkeys (10 per group) prior to immunization and after one, two, and three immunizations with proteosome-SEB toxoid. The primary immunization for all monkeys was given i.m.; two booster immunizations were administered i.m. to one group of 10 animals and i.t. to the other group. i.t. immunizations contained 250 μg (each) of toxoid and proteosomes delivered in 3 ml of buffered saline. i.m. immunizations contained 100 μg (each) of toxoid and proteosomes and were administered with alum in a volume of 0.5 ml. The data are titers showing the geometric mean (GMT) of the highest dilutions of sera with OD values of greater than 1.0 and 0.5 for IgG and IgA, respectively. Error bars indicate the SEM.

mals given i.t. booster injections had bouts of coughing. There were no significant differences in either clinical signs or duration of illness in monkeys immunized only i.m. compared with those given i.t. booster immunizations, indicating that for the proteosome-toxoid vaccine either route effectively protects against both lethality and severe illness elicited by aerosol SEB challenge given 30 days after the last immunization.

Serum anti-SEB antibody responses after SEB challenge. Both groups of animals responded to the aerosol challenge with higher serum IgA and IgG levels (Fig. 3) than were present prior to challenge at week 12 (Fig. 1). The animals given i.t. booster immunizations, however, showed stronger anamnestic responses in sera to aerosolized SEB than did animals immunized only i.m. (Fig. 3). Serum IgA responses were higher postchallenge in monkeys given i.t. booster immunizations than in those given i.m. booster injections ($P < 0.0541$ and $P < 0.0281$ at 2 and 6 to 10 weeks, respectively). In addition, serum IgG responses after challenge were significantly better in monkeys given i.t. booster immunizations than in monkeys given i.m. booster injections ($P < 0.0034$ and $P < 0.0001$ at 10 and 16 weeks, respectively).

Anti-SEB antibody responses in BAL samples after SEB challenge. After aerosolized-SEB challenge, monkeys given booster immunizations by the respiratory route also developed enhanced responses in their BAL samples compared with those of monkeys immunized only i.m. (Fig. 4). Bronchial anti-SEB IgA levels in i.m.-immunized animals were exceedingly low, indicating that three i.m. immunizations do not prime for bronchial IgA responses even after a large aerosol challenge. In marked contrast, monkeys given booster immunizations by the respiratory route showed significantly increased anti-SEB IgA levels in BAL samples obtained at each time postchallenge (weeks 2, 6, and 10) (Fig. 4) compared with the postchallenge levels of monkeys immunized only i.m. ($P < 0.017$, $P < 0.0001$, and $P < 0.0001$ at these times, respectively). Postchallenge bronchial IgG levels were significantly higher in animals given i.t. booster injections only in BAL samples collected 6 weeks postchallenge (Fig. 4).

DISCUSSION

The data presented here demonstrate that the proteosome-SEB toxoid vaccine is safe and efficacious, inducing protective immunity in 100% of nonhuman primates (20 monkeys) against lethal aerosol challenge with SEB 1 month after the last immunization. The safety of the proteosome-SEB toxoid vaccine confirms previous reports concerning the safety of SEB toxoid detoxified according to the recommendations of Eldridge et al. (8, 15, 20, 40) based on the original protocol of Schantz et al. (35). Indeed, there were no hypersensitivity reactions such as those previously reported after the challenge of monkeys immunized with SEB toxoid produced in the 1970s (5). The efficacy shown in the present study confirms that the array of multiorgan system failure and shock induced by su-

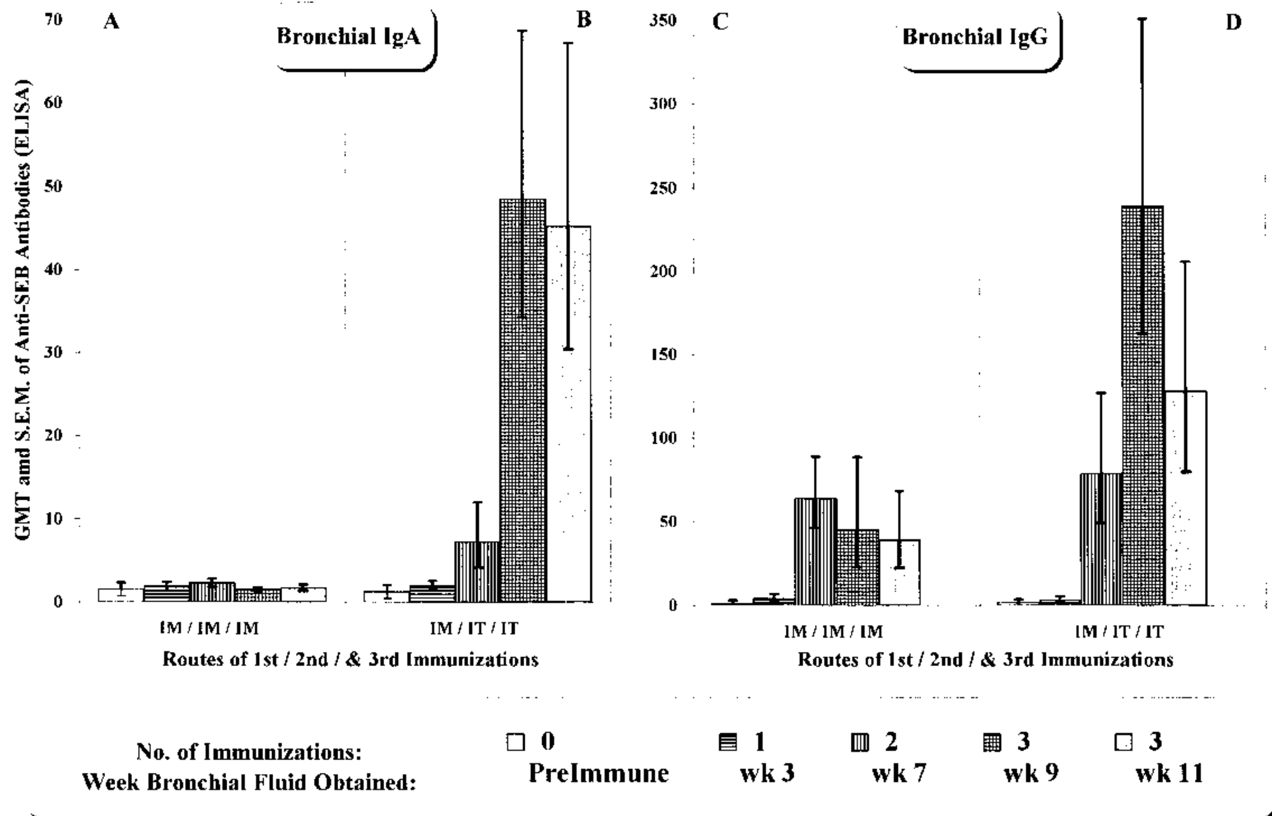

FIG. 2. IgA (A and B) and IgG (C and D) responses in BAL samples prior to SEB challenge in rhesus monkeys primed i.m. and given booster immunizations either i.m. (A and C) or i.t. (B and D) with proteosome-SEB toxoid vaccine. Anti-SEB IgG and IgA levels prior to challenge with SEB, as determined by ELISA, in individual BAL samples obtained from monkeys (10 per group) prior to immunization and after one, two, and three immunizations with the proteosome-SEB toxoid vaccine, administered as described in the legend to Fig. 1. The data are titers showing the geometric mean (GMT) of the highest dilutions of BAL samples with OD values of greater than 0.2. Error bars indicate the SEM.

perantigen toxins like SEB can be prevented by immunizing with proteosome-toxoid vaccines that elicit antibodies that recognize the toxin.

Priming i.m. with the proteosome-SEB toxoid vaccine with alum and giving booster immunizations twice either i.m. with alum or via the intratracheal respiratory route in saline elicited comparable serum anti-SEB IgG responses prior to SEB challenge, thereby indicating a level of immunity sufficient to protect against aerosol challenge after immunization with the proteosome-toxoid vaccine. These results are entirely consistent

TABLE 1. Efficacy of proteosome-SEB toxoid vaccine against lethal challenge with aerosolized SEB

Vaccine	Schedule[a]	Mean SEB challenge dose (LD_{50})[b]	Clinical illness					No. of survivors/total no. of animals (%)
			Overall severity	No. of animals	Range of duration (h)	No. of animals with symptom/total no. of animals[c]		
						Emesis	Cough	
Proteosome-toxoid	i.m., i.m., i.m.	14.1 (10.5–16.7)	Mild	8	20–96	3/8	1/8	10/10 (100)
			Moderate	2	72–168	1/2	2/2	
			Severe	0				
Proteosome-toxoid	i.m., i.t., i.t.	15.1 (13.3–18.7)	Mild	8	45–70	1/8	0/8	10/10 (100)
			Moderate	2	96–120	2/2	2/2	
			Severe	0				
Saline control	i.m., i.t., i.t.	12.0 (10.9–13.0)	Mild	0				0/2 (0)
			Moderate	0				
			Severe	2	21–43	2/2	0/2	

[a] i.m. doses were administered after adsorbing the vaccine to alum; i.t. doses were administered in saline without alum. Each schedule consists of the first (prime) and second and third (booster) immunization routes.
[b] Parenthetical data are ranges.
[c] Emesis consisted of one or two bouts and occurred 3 to 6 h after challenge. Coughing consisted of one to five bouts and occurred 27 to 46 h after SEB challenge. Of the five animals that coughed, four also experienced emesis.

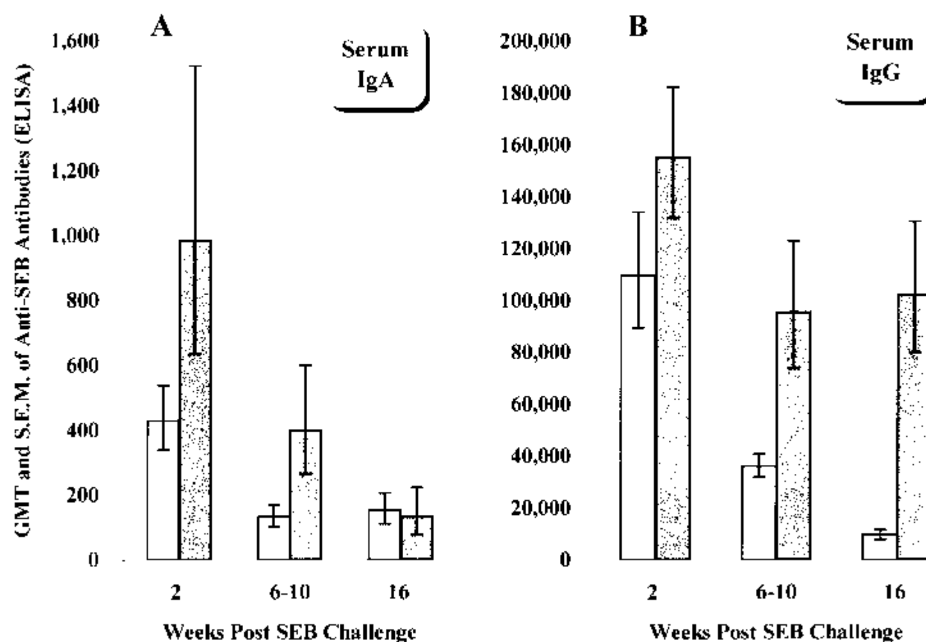

FIG. 3. Serum IgA (A) and IgG (B) responses after SEB challenge in rhesus monkeys primed i.m. and given booster immunizations either i.m. (open bars) or i.t. (solid bars) with proteosome-SEB toxoid vaccine. Anti-SEB IgG and IgA titers, as determined by ELISA, in individual sera obtained from groups of monkeys after challenge with aerosolized SEB. All 10 monkeys in each group were sampled, except at 16 weeks postchallenge when serum samples were obtained from one group of 5 monkeys. Monkeys were immunized as described in the legend to Fig. 1 with proteosome-SEB toxoid. The data are titers showing the geometric mean (GMT) of the highest dilutions of sera with OD values of greater than 1.0 and 0.5 for IgG and IgA, respectively. Error bars indicate the SEM.

with the data from a murine study which showed that either i.m. or intranasal immunization with the proteosome-toxoid vaccine could confer significant protection against either parenteral or respiratory SEB challenge in the D-galactosamine-sensitized murine model of SEB intoxication (20). These data also support the thesis that parenteral immunization alone can be protective even if the offending pathogen initially invades the host via a mucosal port of entry (33). Nevertheless, it is clear that i.t. booster immunizations provided immune responses that were not present in monkeys immunized only i.m. Specifically, prior to challenge, IgA responses in sera (Fig. 1) and, perhaps more importantly, in BAL samples (Fig. 2) (and, to a lesser extent, IgG levels in BAL samples) were significantly higher after respiratory booster immunizations compared with those after only i.m. immunizations. These differences were also reflected in postchallenge anamnestic antibody responses since monkeys given i.t. booster immunizations developed higher and longer-lasting serum IgA and IgG immunity (Fig. 3) and bronchial IgA immunity (Fig. 4) in response to aerosol SEB challenge. These data indicate that immune recall responses to aerosol challenge is greater in monkeys immunized via a respiratory route.

The prechallenge respiratory immunity provided by i.t. immunizations with the proteosome-SEB vaccine may be important for optimal protection, for example, when challenged 70 days after the last immunization instead of 30 days postimmunization as in the present study. Similarly, the enhanced IgA recall responses after SEB challenge in animals given i.t. booster immunizations may be advantageous for protection when a longer interval has elapsed between the last booster immunization and the day of aerosol challenge. This may have occurred in the monkeys immunized with the microsphere-encapsulated SEB toxoid and challenged 70 days after booster immunization since four of four monkeys primed i.m. and given one i.t. booster immunization with that vaccine were protected, whereas only one of four animals survived aerosol challenge after immunization by the i.m. route alone despite high serum IgG titers (15, 40). The importance of respiratory antibodies for protection against aerosol challenge was also emphasized in that study since monkeys with the highest antitoxin IgA and IgG levels in BAL samples tended to be protected, whereas those with the lowest bronchial antibody levels, including several animals with high serum IgG titers, were more susceptible to intoxication (15, 40). Furthermore, in a more recent confirmatory experiment at our institutes in which monkeys were immunized with the microsphere-SEB toxoid vaccine according to the same regimen used in the first study, i.t. booster immunizations protected six of nine monkeys, whereas only one of five monkeys immunized solely i.m. survived aerosol challenge 70 days after the last immunization (4a). These results emphasize that respiratory immunization can provide protective immunity not afforded by immunizing only i.m. The ability of the proteosome-toxoid vaccines used here to protect 70 days after i.m. and/or respiratory immunization requires further study. Nevertheless, the high antibody titers present at the time of challenge 30 days postimmunization in the present work suggest sufficient immunity for protection would still be present several months postimmunization.

Several efficacy studies of vaccines in which animals were challenged with live bacteria or viruses have indicated that the local immunity in respiratory or gastrointestinal tracts elicited by mucosal immunization can provide enhanced protection against lethal or infectious challenge (3, 9, 24, 29, 37). The importance of this concept is emphasized by the fact that 95% of pathogens infect via respiratory, gastrointestinal, or genitourinary mucosal tracts, and interdicting such infections at the portals of entry may be critical to interfering with the establishment of generalized systemic infections or toxinemia in addition to preventing localized mucosal diseases (39, 42). We

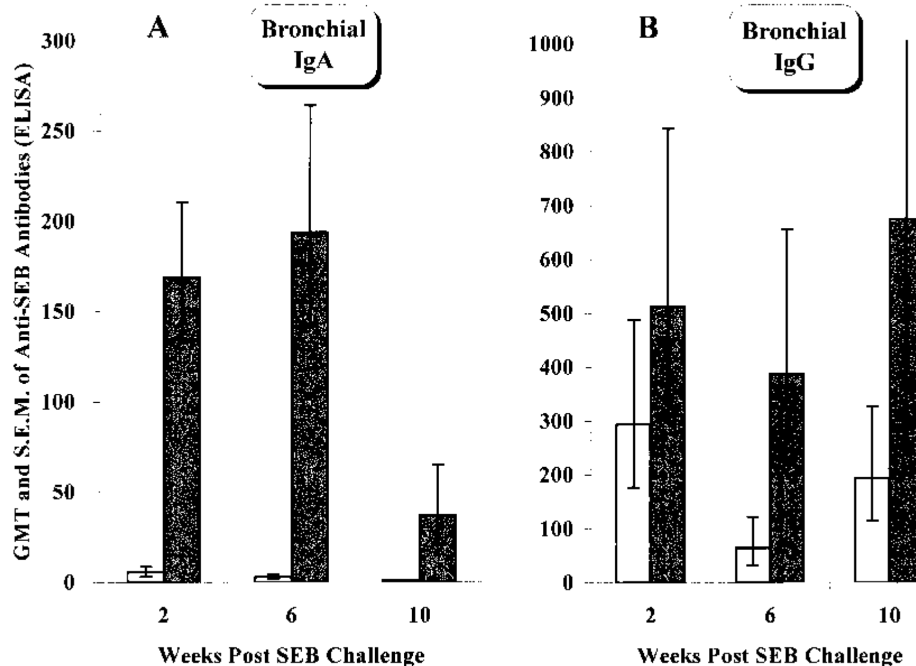

FIG. 4. IgA (A) and IgG (B) responses in BAL samples after SEB challenge in rhesus monkeys primed i.m. and given booster injections either i.m. (open bars) or i.t. (solid bars) with proteosome-SEB toxoid vaccine. Anti-SEB IgG and IgA titers, as determined by ELISA, in individual BAL samples obtained from monkeys (10 per group) after challenge with aerosolized SEB. Monkeys were immunized as described in the legend to Fig. 1 with proteosome-SEB toxoid. The data are titers showing the geometric mean (GMT) of the highest dilutions of BAL samples with OD values of greater than 0.2. Error bars indicate the SEM.

have developed the proteosome mucosal vaccine delivery system to elicit mucosal and systemic immunity against such diseases and have shown that intranasal and intragastric proteosome vaccines are immunogenic and protective in animal models of disease using shigella lipopolysaccharides (24, 32), influenza peptides (16), and SEB toxoid (20) antigens in small animals. Mucosal immunogenicity of proteosome vaccines containing SEB or ricin peptides (1) or human immunodeficiency virus envelope protein (21) has also been demonstrated. Other vaccine delivery and adjuvant systems that have been used for respiratory delivery include microspheres (15, 37, 40), liposomes (3, 4, 9), cochleates (25), emulsomes (21), and cholera toxin analogs (13, 14).

The demonstration here that proteosome vaccines in saline induce strong bronchial and serum IgG and IgA responses that are evident after two respiratory booster immunizations and are further increased by aerosol exposure to the pathogenic antigen confirms that mucosal vaccinations with proteosome vaccines are effective in monkeys as well as in mice (16, 20, 24) and guinea pigs (32). The recent demonstration of the mucosal immunogenicity of proteosome-shigella LPS vaccines in monkeys immunized by the intragastric, i.t., or intranasal (using either nose drops or a metered dose spray) route also supports the effectiveness of the proteosome vaccine system for mucosal administration in nonhuman primates (19). In the shigella vaccine study of monkeys (19) and in the SEB toxoid (21) and human immunodeficiency virus gp160 (20) experiments with mice, the proteosome vaccines were effective when both primary and booster immunizations were given by respiratory routes. Unfortunately, because of financial and logistic constraints, respiratory immunogenicity without primary i.m. immunization was not tested in monkeys in this study. Nevertheless, the importance of two respiratory immunizations for optimal mucosal responses is indicated by the fact that strong serum and bronchial IgA responses were most apparent after the second i.t. immunization.

In conclusion, whether or not mucosal immunization is an absolute requirement for optimal protection against specific disease, the ability to successfully deliver vaccines via respiratory and gastrointestinal routes will be welcomed by vaccinologists and clinicians alike because of the greater patient compliance and ease of needle-free administration of such vaccines for worldwide applications. The demonstrated clinical safety in humans (46) and facility of large-scale production of the outer membrane proteins of proteosome vaccines are promising for the development of parenteral and mucosal proteosome vaccines for human use. Indeed, clinical trials of nasal and oral proteosome-shigella LPS vaccines in humans are in progress. The protection against aerosol challenge by i.m. priming and i.m. or i.t. booster immunizations of proteosome-SEB toxoid vaccines as shown here support the advanced development of such vaccines to protect against shock and death from this potential biologic warfare threat agent.

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