CONCISE COMMUNICATIONS

# Cross-Reactive Antibodies Prevent the Lethal Effects of *Staphylococcus aureus* Superantigens

Sina Bavari, Robert G. Ulrich, and Ross D. LeClaire

*US Army Medical Research Institute of Infectious Diseases, Frederick, Maryland*

**The exotoxins produced by *Staphylococcus aureus*, staphylococcal enterotoxins (SE) A–E and toxic shock syndrome toxin (TSST)–1, which are associated with serious diseases, including food poisoning and toxic shock syndrome, are termed superantigens (SAgs). To examine whether common antigenic epitopes were present and whether vaccination with 1 bacterial SAg could protect against challenge with a different SE or TSST-1, mice were vaccinated with SEA, SEB, SEC1, or TSST-1 individually or in combination. Mice injected with a single toxin developed high antibody titers against other SAgs. Marked improvement in survival was observed when immunized mice were challenged with a heterologous toxin. Mice vaccinated with a mixture of toxins were fully protected against 1 or multiple toxin challenges, indicating no interference effects of multivalent vaccinations. More importantly, higher titers were found against each SAg with the multivalent vaccination than with injection with a single SAg. Thus, immunizations with 1 SAg can induce cross-protective antibodies to heterologous SAgs, and multicomponent vaccination can enhance antibody responses against each bacterial SAg.**

Bacterial superantigens (SAgs) produced by group A streptococci and *Staphylococcus aureus* are ligands for major histocompatibility complex (MHC) class II molecules and interact with T cell antigen receptors (TCRs) [1, 2]. The binary complex of SAg and MHC class II receptor circumvents the usual requirement for peptide-specific recognition by TCR. The responding T cells express particular subsets of the variable (V) $\beta$-gene products of TCR. For example, staphylococcal enterotoxin (SE) A preferentially stimulates human T cells with V$\beta$1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9.1, and 23.1, whereas SEB activates T cells carrying V$\beta$3 [3].

Food poisoning caused by enteric exposure to SEs results in vomiting and diarrhea; however, nonenteric exposure to bacterial superantigens causes the more severe toxic shock syndrome. In addition, SAgs are associated with a number of diseases, including bacterial arthritis, and these toxins may be an important component of autoimmune disorders [2].

A comparison of amino acid sequences suggests that SEs and streptococcal pyrogenic exotoxins (SPEs) fall into 4 homology groups. The first consists of SEA, SED, and SEE. The second consists of SEB and SEC 1–3. The third group includes SPE-A, which is more related to SEB and the SECs than SEA. SPE-C and toxic shock syndrome toxin (TSST)–1 form the fourth group and have very little primary amino acid sequence homology (<15%) to each other and to the other bacterial SAgs. Although distantly related, SAgs appear to have limited key primary amino acid sequences in common and similar 3-dimensional folds [2]. For example, diverse SAgs (e.g., SEB and TSST-1) are closely related in 3-dimensional structures and exhibit homologous protein folds composed largely of $\beta$-strands within the 2 distinct globular domains, and all except SPE-C contact MHC class II molecules via their amino-terminal domain [2, 4].

We have demonstrated that proteins engineered by a site-directed mutagenesis strategy aimed at disrupting MHC class II or TCR-binding surfaces of SAgs can produce effective vaccines for SEA and SEB [5, 6]. These vaccines elicited protective antibodies, and immunized animals were protected against a subsequent challenge of wild-type SAg. The studies presented here were designed to examine whether vaccination with 1 bacterial SAg could protect against a challenge from a different SE or TSST-1. In addition, we investigated the efficacy of a multivalent vaccine approach against SEs and TSST-1.

## Materials and Methods

*Mice.* Pathogen-free BALB/c (H-2d) or C57BL/6 (H-2b) mice, 10–12 weeks old, were obtained from the Frederick Cancer Research and Development Center (Frederick, MD). Mice were maintained under pathogen-free conditions and fed laboratory chow and water ad libitum.

*Bacterial SAgs and endotoxin.* Recombinant SEA and SEB

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Reprints or correspondence: Dr. Sina Bavari, Dept. of Cell Biology and Biochemistry, USAMRIID, 1425 Porter St., Frederick, MD 21702-5011 (sina.bavari@amedd.army.mil).

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were prepared in our laboratory as described elsewhere [5]. SEC1 and TSST-1 were purchased from Toxin Technology (Sarasota, FL). Each toxin was determined to be >95% pure by electrophoresis on 5%–20% gradient SDS-polyacrylamide gels and by Western blot analysis. SEC1 and TSST-1 were determined to be devoid of SEA and SEB by use of Western blotting with homologous or heterologous monoclonal antibodies. Each toxin contained <10 endotoxin U/200 μL, as determined by limulus lysate assay. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was obtained from Difco Laboratories (Detroit) and reconstituted with PBS. Aliquots were stored at −70°C for future use.

*Determination of $LD_{50}$s for SEA, SEB, SEC1, and TSST-1.* The dose-response effects of SEA, SEB, SEC1, and TSST-1 potentiated by LPS were compared in BALB/c mice. Mice in groups of 10 were given various concentrations of SE or TSST-1 followed 3 h later by a nonlethal, potentiating dose of LPS (75 μg). The dose-response regression for probit analysis was calculated by use of data pooled from at least 3 experiments with a 3-fold geometric dose progression for 8–10 doses of each toxin. The calculated $LD_{50}$ of the toxins were 34.2, 0.3, 18.1, and 1.3 μg/mouse for SEA, SEB, SEC1, and TSST-1, respectively.

*Serum antibody titers.* Serum antibody titers were determined as described elsewhere [5]. The mean duplicate absorbance of each treatment group was obtained, and data are presented as the inverse of the highest dilution that produced an absorbance reading 3 times above the negative control wells (antigen or serum was omitted from the negative control wells).

*Vaccination protocol and passive protection.* Prior to vaccination, mice were bled, and their serum antibody titers against SEs and TSST-1 were determined to be ≤1 : 50. For vaccination protocol, mice were injected intraperitoneally with 5 or 10 μg of bacterial SAg in 100 μL of Ribi adjuvant (Ribi Immunochem Research, Hamilton, MT) or adjuvant alone and boosted at 2 and 4 weeks in the same manner as described for the primary injection. Ten days after the last injection, blood was collected from tail veins, and serum was separated. Mice were challenged 2 weeks after the second boost with 5 $LD_{50}$/mouse of SEA, SEB, SEC1, and TSST-1 or with a combination of all 4 toxins and LPS (75 μg) as described elsewhere [5]. Challenge controls were adjuvant-injected or naive mice injected with both toxin and LPS (all mice died) or with either agent (no death was observed). For passive transfer studies, serum was obtained from mice prior to vaccination and 14 days after the last vaccination. Pooled sera (500 μL) were incubated (1 h/37°C) with a lethal dose of toxin, and mice ($n = 8$) were injected as described above. Deaths were recorded after 4 days.

*Lymphocyte proliferation assay.* Mice were given 3 doses of adjuvant or toxin as described above. Splenic mononuclear cells from vaccinated mice were obtained 14 days after the last injection. The cells were resuspended in culture medium containing 5% fetal bovine serum, and 100 μL ($5 \times 10^5$ cells) of the cell suspension was added to triplicate wells of 96-well flat bottom plates. The mononuclear cells were cultured (37°C, 5% $CO_2$) for 3 days in the presence of 1 or 10 μg of SE or TSST-1. The cells were pulsed with 1 μCi/well of [$^3$H]thymidine (Amersham, Arlington Heights, IL) for 16 h, and incorporated radioactivity was measured by liquid scintillation. The data are presented as percentage of adjuvant control, where % control = (experimental [SAg vaccinated] cpm)/(adjuvant cpm) × 100.

## Results

*Vaccination with 1 SAg protects against distantly related SAgs.* To address whether vaccination with 1 bacterial SAg could elicit cross-reactive antibody against a different SE or TSST-1, groups of 10 mice were injected 3 times (10 μg/injection) with SEA, SEB, SEC1, or TSST-1 in adjuvant. The immunogenicity of the 4 antigens was examined at the end of the experiment by measuring serum antibody titers to the SAg and against other closely related (SEB and SEC1) or diverse SAgs, such as SEB and TSST-1 (figure 1). As expected, sera of mice that were vaccinated with SEA, SEB, SEC1, or TSST-1 had the highest titers against the homologous toxin; however, low-to-moderate antibody titers were found against heterologous toxins (figure 1*A*–1*D*). A more relevant parameter by which to gauge the results is the percentage of vaccinated mice that attained protective levels of antibodies and survived the homologous or heterologous toxin challenge. All mice that were vaccinated with SEA were protected against lethal dose of SEA. A persistent cross-protective antibody was observed in 30% of mice vaccinated with SEA and challenged with SEB, SEC1, or TSST-1.

Mice that were injected with either SEB or SEC1 were completely protected when challenged with either SEB or SEC1. In the same groups of vaccinees, lower levels of antibody titers were elicited against SEA and TSST-1. Among SEB- or SEC1-vaccinated mice, 30% survived when challenged with SEA; however, 40% and 30% of mice survived when challenged with TSST-1, respectively. When mice were vaccinated with TSST-1, 80% survived the TSST-1 challenge. In the same group of vaccinees, 30%, 20%, and 30% of mice survived when challenged with SEA, SEB, or SEC1, respectively. Mice injected with adjuvant had little to no detectable antibodies against any bacterial SAg and died when challenged with a single SAg (figure 1*E*).

The results described above suggest that vaccination with 1 bacterial SAg elicited cross-protective antibodies against other SAgs and subsequently diminished their toxicity. An alternative explanation is that induction of clonal anergy of specific subsets of T cells could have protective effects. Administration of SAg can induce peripheral deletion of specific T cells and suppress proliferative responses to the same SAg in vitro [7]. However, rechallenge of mice with SEB failed to induce T cell anergy [8]. Indeed, serum concentrations of interleukin-2 and -4 and tumor necrosis factor in rechallenged mice were similar to those observed during the primary injection [9, 10]. In addition, responses to heterologous SAg would be expected to be only minimally affected, because different subsets of Vβ T cells would be involved. Moreover, coadministration of adjuvant with the SAgs most likely induced higher levels of inflammatory cytokines that rescued SAg-stimulated T cells from programmed cell death [11]. To further examine this phenomenon, splenic T lymphocyte responses of mice injected 3 times with SEA mixed with adjuvant or adjuvant only were measured after

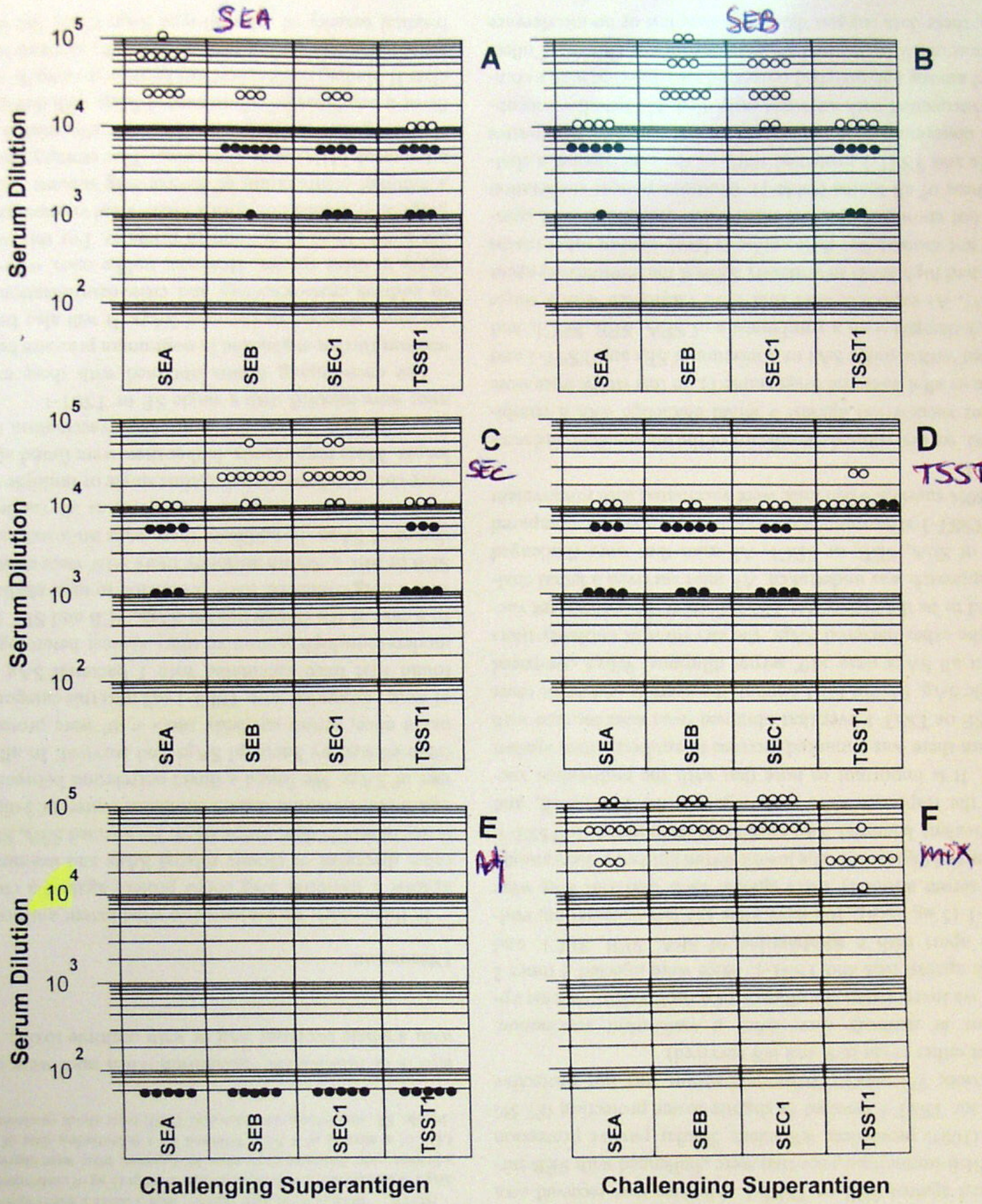


**Figure 1.** Antibodies against 1 bacterial superantigen (SAg) protects against heterologous SAgs. BALB/c mice in groups of 10 were injected with 3 doses of staphylococcal enterotoxin (SE) A *(A)*, SEB *(B)*, SEC1 *(C)*, toxic shock syndrome toxin-1 (TSST-1) *(D)*, adjuvant alone *(E)*, or mixture of 4 SAgs *(F)* every 14 days. Ten days after last injection, sera were collected and analyzed for IgG binding to SEA, SEB, SEC1, or TSST-1 by ELISA. Each circle represents end-point titers of single mouse and is mean absorbance of triplicate wells. At 14 days after 3d injection, mice were challenged with 5 $LD_{50}$ of SEA, SEB, SEC1, or TSST-1 toxins and 3 h later with potentiating dose of lipopolysaccharide (LPS). Mice were observed for 96 h after challenge: ●, dead mice; ○, live mice. Controls included age-matched mice that were challenged with LPS alone (no lethality observed), LPS and SAg (all died), 1 SAg alone, or combination of all SAgs (no lethality observed).

in vitro challenge with SEA, SEB, SEC1, and TSST-1. Although responses to homologous toxin were slightly diminished (73% of adjuvant control), robust proliferation against SEB, SEC1, and TSST-1 were observed (112%, 94%, and 132% of adjuvant control, respectively).

To formally demonstrate the essential role of antibody in protection against these SAgs, we passively transferred into naive mice preimmune sera or sera from mice that were immunized against SEB or TSST-1. All mice that received sera from SEB-immunized mice that were challenged with SEB survived (100% protection; 8/8 mice). Similar passive protection study for TSST-1 resulted in slightly lower protection (87.5% protection; 7/8 mice). Preimmune serum was not protective against either toxin (0/8 and 0/8 survived).

*Boost in antibody titers after a multivalent vaccination.* Next, we investigated the efficacy of a multivalent vaccine approach against SEs and TSST-1. Mice were injected 3 times 2 weeks apart with a combination of SEA, SEB, SEC1, and TSST-1 (5 $\mu$g each). Ten days after the last vaccination, end-point serum antibody titers against each bacterial SAg were determined (figure 1*F*). The lowest serum antibody titers among quadrivalent bacterial SAg vaccine corresponded to TSST-1, while the responses were most vigorous for SEA, SEB, and SEC1. It is important to note that with the multivalent vaccination there was a marked increase in antibody titers against each SE or TSST-1 over that obtained from mice injected with a single SAg. Except for 1 animal, the average end-point titers against all SAgs were $>10^4$ serum dilutions. When compared with the other bacterial SAgs, the elevation in antibody titers seemed to be the highest for TSST-1 when the multivalent vaccine approach was undertaken. All mice survived a lethal challenge of SEA, SEB, or SEC1. All mice that were challenged with TSST-1 after multivalent vaccination survived, compared with 80% survival when mice were vaccinated with monovalent TSST-1.

Next, we determined the efficacy of the monovalent and multivalent vaccination against a lethal challenge with a combination of all 4 bacterial SAgs (table 1). In this study, mice were injected with a single SAg or a mixture of SEs and TSST-1 and then challenged with a combination of SEA, SEB, SEC1, and TSST-1. As expected, mice that were vaccinated with a single toxin had high levels of antibody against the immunizing agent (data not shown), but their antibody levels against other toxins were not enough to protect them when challenged with combinations of all toxins (table 1). Multicomponent vaccination of SEs and TSST-1 protected 100% of the mice against a challenge containing SEA, SEB, SEC1, and TSST-1. All control mice vaccinated with adjuvant only died. No lethality was observed among age-matched untreated mice injected with a combination of all toxins or LPS alone (data not shown). Collectively, these data suggest that there were few or no interference effects of multivalent vaccination when mice were challenged with a single bacterial SAg or with multiple toxins.

**Table 1.** Multivalent vaccination results in protection against all of component superantigen (SAg) challenge.

| Vaccine | Challenge | Live/dead |
|---|---|---|
| Adjuvant | SEA, SEB, SEC1, and TSST-1 | 0/10 |
| SEA | SEA, SEB, SEC1, and TSST-1 | 0/10 |
| SEB | SEA, SEB, SEC1, and TSST-1 | 1/10 |
| SEC1 | SEA, SEB, SEC1, and TSST-1 | 2/10 |
| TSST-1 | SEA, SEB, SEC1, and TSST-1 | 0/10 |
| SEA, SEB, SEC1, and TSST-1 | SEA, SEB, SEC1, and TSST-1 | 10/0 |

NOTE. Mice ($n = 10$) were injected with 3 doses 2 weeks apart: with single SAg (10 $\mu$g/injection), with combination of SAgs (5 $\mu$g of each/injection), or with adjuvant only. Fourteen days after 3d injection, mice were challenged with 5 $LD_{50}$ of a mixture of 4 SAgs followed by a potentiating dose of lipopolysaccharide. SE, staphylococcal endotoxin; TSST, toxic shock syndrome toxin.

## Discussion

In this report, we evaluated to what extent antibodies raised against 1 bacterial SAg could protect against a challenge of other divergent or closely related SAgs and examined the efficacy of multivalent vaccination. We selected SEA, SEB, SEC1, and TSST-1 because these 4 exotoxins represent 3 distinct families of SAgs. We found a direct correlation between antibody titers elicited by bacterial SAgs and survival. In all SE-vaccinated mice, serum antibody titers $\geq 10^4$ were protective; 80% of mice vaccinated with TSST-1 fell into this category. We also found that mice vaccinated with 1 bacterial SAg developed moderate-to-high protective titers against heterologous SAgs. In a case of the closely related SAgs, SEB and SEC1, injection with 1 SAg conferred 100% protection to mice challenged with SEB or SEC1. Serum antibody titers $\geq 10^4$ were elicited against divergent SAgs. In addition, there were no interference effects after vaccination with the 4 antigens, as all vaccinated mice were completely protected against single or multiple toxin challenges. More importantly, higher titers were found against each SE or TSST-1 during the multivalent vaccination than when mice were injected with a single SE or TSST-1.

The encouraging results obtained with these experiments warrant further evaluation in nonhuman primates because they are more sensitive to bacterial SAgs. It will also be necessary to address cross-reactivity and cross-neutralization issues directly in these species. However, unlike mice, wild-type SAgs are highly toxic to nonhuman primates. For this purpose, we propose immunization with a multivalent vaccine comprised of a minimal combination of diverse SAg subunit vaccines with attenuated MHC class II binding. This strategy should be effective against the majority of structurally related SAgs. We favor a combination of attenuated SAgs with disrupted MHC class II binding contact surfaces because genetically altered bacterial SAgs that lacked binding to TCRs retained some of the residual toxicity of the wild-type SAgs [5, 6]. We believe that

a combination of these attenuated bacterial SAg vaccines may be useful for prevention of acute toxic shock syndrome or chronic pathologies that may occur as a consequence of single or multiple toxigenic bacterial infections. Moreover, bacterial SAg vaccines may be a means to modulate the immune system in novel ways.

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