Differential Immune Responses to Staphylococcal Enterotoxin B Mutations in a Hydrophobic Loop Dominating the Interface with Major Histocompatibility Complex Class II Receptors

Mary Alice Woody, Teresa Krakauer, Robert G. Ulrich, and Bradley G. Stiles

Bacterial superantigens, such as staphylococcal enterotoxin B (SEB), can trigger acute pathologic effects in humans. A hydrophobic loop on the surface of SEB and other bacterial superantigens, centered around a conserved leucine (L45) residue, is essential for binding to class II major histocompatibility complex molecules. Single residue changes of wild type SEB, designated Q43P, F44P, or L45R, resulted in nonlethal proteins at a dose equivalent to 30 murine LD₅₀ of SEB. Relative to SEB, the mutant proteins did not elevate serum concentrations of proinflammatory cytokines in mice and caused minimal proliferation of human lymphocytes. Anti-SEB titers of mice immunized with Q43P, F44P, L45R, or SEB were similar and protected 77%–100% of animals against a lethal SEB challenge. Levels of toxin-specific IgG1, IgG2a, IgG2b, and IgG3 in mice immunized with SEB, Q43P, or F44P were equivalent, but animals immunized with L45R had significantly elevated levels of IgG2a and IgG2b. Vaccines against staphylococcal superantigens should focus on this critical leucine residue.

Staphylococcus aureus, a member of the Micrococcaceae, primarily inhabits mammalian body surfaces [1]. It remains a major cause of disease, despite use of antibiotics and increased hygiene, because of numerous pathogenicity determinants and adaptability to adverse environmental conditions. S. aureus can produce several exotoxins that participate in pathogenesis, including an important group collectively referred to as the staphylococcal enterotoxins (SEs). The SEs are single-chain, 25- to 30-kDa proteins that possess an internal disulfide loop and are involved in the etiologies of some chronic disorders, since they can induce bacterial arthritis in rodents [20] and are implicated in human autoimmune disorders, such as rheumatoid arthritis [21, 22] and Kawasaki disease [23].
specifically inhibit the in vitro proliferation of human peripheral blood mononuclear cells (PBMC) by SEB [29].

In an attempt to refine the antigenic structure of a potential SEB vaccine, we examined the biologic activities of three mutant SEB molecules. Single amino acid substitutions were created by site-directed mutagenesis and included a proline for glutamine at position 43 or phenylalanine at position 44 (designated Q43P and F44P, respectively) and arginine for leucine at position 45 (L45R). The binding interface with a human MHC class II molecule, HLA-DR1, consists of two surfaces located in the N-terminal domain of SEB [30]. One contact surface is a pocket of polar side chains interacting with K39 of the DRα subunit, and the second surface is a hydrophobic loop. The hydrophobic loop consists of SEB residues 44–47, which make strong electrostatic interactions with DRα via backbone atoms, and a central leucine residue (L45), which is buried in hydrophobic contacts with the receptor. Because the L45R mutation greatly diminishes receptor binding [30], we speculated that minor disruptions of the surface hydrophobic loop by site-specific mutagenesis could be useful for generating an optimal SEB vaccine. By decreasing the binding of mutant SEB proteins to MHC class II molecules, we hoped to greatly reduce their biologic activities, yet retain common neutralizing epitopes found on the parent toxin. We investigated three mutant proteins of SEB to see if they provide protection against the toxin without eliciting unwanted side effects commonly associated with superantigens.

Materials and Methods

**SEB, mutant proteins, and lipopolysaccharide (LPS).** Purified SEB was purchased from Toxin Technology (Sarasota, FL). Mutant proteins of SEB (Q43P, F44P, and L45R) were derived by site-directed mutagenesis of the SEB toxin gene isolated from *S. aureus* 14458 [30]. The modified toxin gene, which resulted in 2 h, up to 12 h after the protein injection, and analyzed for IL-6 were determined by absorbance at 277 nm with an extinction coefficient (ε) of 14.4. Mutant protein concentrations were determined in a bicinechonic acid assay (Pierce Chemical, Rockford, IL) with SEB as a standard.

*E. coli* O55:B5 LPS was obtained from Difco (Detroit), reconstituted in sterile PBS, and stored at −50°C. ELISA. Sera from immunized mice were tested for anti-SEB titers in an ELISA. SEB, 5 μg/mL in carbonate buffer, was adsorbed overnight at 4°C on Immulon II microtiter plates (Dynatech Laboratories, Chantilly, VA). Unadsorbed sites were blocked for 1 h at 37°C with 5% skim milk solids in PBS. Wells were aspired, and mouse sera diluted in PBS containing 0.1% Tween 20 plus 2% skim milk solids were added for 1 h at 37°C. The wells were then emptied and washed with PBS containing 0.1% Tween 20, and sheep anti-mouse IgG conjugated to alkaline phosphatase (Sigma) was added for 1 h at 37°C. The plates were finally washed with PBS–0.1% Tween 20, and *p*-nitrophenyl phosphate substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added for 30 min at room temperature. Data represent the mean absorbance at 405 nm of triplicate readings ± SD.

In the competition ELISA, equal volumes of mouse anti-SEB serum (1:6400 dilution) and SEB or mutant protein were incubated for 1 h at 37°C before addition to SEB-coated wells. All other steps were as described above.

Anti-SEB immunoglobulins in pooled sera of immunized and naive mice were isotyped with a panel of rabbit anti-mouse immunoglobulin sera (Bio-Rad Laboratories, Hercules, CA), as described by the manufacturer. Wells were coated with SEB or a 1:100 dilution of normal mouse serum (NMS) in carbonate buffer. Serum diluent was added to the NMS-coated wells, while the SEB-coated wells were treated with NMS or immune mouse sera diluted 1:100.

**Mice lethality assay.** BALB/c mice (18–20 g) were obtained from the Animal Production Division of Cancer Treatment, National Cancer Institute (Frederick, MD), and housed in a pathogen-free rodent colony, as determined by serologic and histologic analysis by the veterinary and pathology staff of USAMRIID. The lethality assay consisted of a 10-μg intraperitoneal (ip) dose of SEB/mouse, equivalent to 30 LD50 [31], or mutant protein, which was followed 4 h later by 75 μg of LPS. Deaths were recorded over a 72-h period. Controls were injected with either LPS or SEB alone.

**Serum cytokines.** Mice were each injected with 5 μg of SEB or mutant protein plus 75 μg of LPS, as described for the lethality assay. Control groups received either 5 μg of SEB or 75 μg of LPS. Sera were pooled from 4 terminally bled mice/group every 2 h, up to 12 h after the protein injection, and analyzed for IL-6 and TNF in an ELISA (Genzyme, Boston). IFN-γ concentrations were determined in a bioassay with recombinant IFN-γ as a reference (BioSources International, Camarillo, CA) [32].

**Immunization.** Preimmune sera were collected from mice before the first antigen injection. Antigen (3 μg/mouse) was combined with alum adjuvant (Pierce Chemical), and each mouse was injected ip with 200 μL. One control group received alum only. Subsequent injections were given 14 and 28 days later, and sera were collected on days 21 and 35. Anti-SEB titers for each mouse were determined by absorbance at 405 nm of triplicate readings ± SD.
Table 1. Lethal effects of SEB, Q43P, F44P, and L45R in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. live/total</th>
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<tbody>
<tr>
<td>10 μg of Q43P</td>
<td>9/10</td>
</tr>
<tr>
<td>30 μg of Q43P</td>
<td>5/5</td>
</tr>
<tr>
<td>10 μg of F44P</td>
<td>10/10</td>
</tr>
<tr>
<td>10 μg of L45R</td>
<td>10/10</td>
</tr>
<tr>
<td>10 μg of SEB</td>
<td>0/15</td>
</tr>
<tr>
<td>LPS only</td>
<td>14/15</td>
</tr>
</tbody>
</table>

NOTE. Treatment: dose of mutant protein or SEB, plus 75 μg of lipopolysaccharide (LPS), per mouse. Controls were injected with 75 μg of LPS only.

Results

Toxicity of SEB mutant proteins. Three mutant proteins of SEB (Q43P, F44P, and L45R), defective in binding to MHC class II molecules [30] (unpublished data), were initially screened for lethal effects in mice, thus identifying those suitable for further investigation as vaccine candidates. Mice survived a 10-μg ip injection of any mutant protein, which is in contrast ($P < .005$) with an equivalent dose of SEB (30 LD$_{50}$) [31] that was 100% lethal (table 1). The single death observed with the 10-μg dose of Q43P was anomalous because all animals survived when given a 3-fold higher quantity of Q43P.

Serum cytokine levels. High levels of particular cytokines, such as IFN-γ, IL-6, and TNF, are often associated with the toxicity of SEB and other superantigens [9, 10, 14, 33]. A potential superantigen vaccine should produce a markedly different cytokine pattern than the native toxin; therefore, we compared the serum levels of IFN-γ, IL-6, and TNF induced by the mutant proteins with those induced by SEB. Mice injected with SEB plus LPS had significantly elevated serum levels of IFN-γ (4-h reading, $P < .001$), IL-6 (2- and 4-h readings, $P < .02$), and TNF (2-h reading, $P < .007$) compared with animals injected with any mutant protein plus LPS (figure 1). Background levels of IFN-γ were produced in mice given the mutant proteins plus LPS, LPS alone, or only SEB (figure 1A). Q43P, F44P, and L45R induced similar amounts of IL-6 (figure 1B) and TNF (figure 1C) in LPS-treated mice that were comparable to those found in controls given LPS alone.

Proliferation of human PBMC induced by SEB and mutant proteins. Q43P, F44P, and L45R were very ineffective, unlike SEB, at inducing proliferation of human PBMC (figure 2). These findings are consistent with the toxicity and serum cytokine data collected from the mouse experiments. SEB caused substantial lymphocyte proliferation at protein concentrations as low as 0.1 ng/mL, but the mutant proteins (0.1–1000 ng/mL) elicited no more than 20% of the proliferation stimulated by equivalent amounts of SEB ($P < .016$). The data revealed that Q43P, F44P, and L45R did not appear significantly different ($P > .05$) from each other at concentrations ranging from 1 to 10,000 ng/mL.

Serologic similarity of mutant proteins and SEB. A single amino acid substitution could disrupt important epitopes and therefore reduce the effectiveness of a mutant protein as an immunogen. The serologic similarities of Q43P, F44P, and L45R, relative to SEB, were compared in a competition ELISA with the anti-SEB sera. Increasing concentrations of SEB or any of the mutant proteins decreased the binding of SEB antibodies to toxin adsorbed onto microtiter plates (figure 3). Inclusion of SEA, which is serologically distinct from SEB, as a competitor demonstrated the binding specificity of the polyclonal serum for SEB and mutant proteins. Although Q43P, F44P, and L45R were obvious competitors in the assay, relative to SEA, they were statistically different from SEB at 0.16- to 10-μg/mL concentrations ($P < .02$). Results from the two lowest competitor concentrations (0.01 and 0.04 μg/mL) of any mutant protein versus SEB were not statistically different ($P > .05$). The data suggested that the mutant proteins were serologically similar, but not identical, to SEB and had retained epitopes found on the native toxin.

Anti-SEB titers of immunized mice. Relative to SEB, the mutant proteins had greatly reduced biologic activities and were serologically similar, thus warranting further studies for vac-
Figure 1. Comparison of interferon (IFN-γ) (A), interleukin (IL)-6 (B), and tumor necrosis factor (TNF; C) serum levels in mice injected with 5 μg of SEB, Q43P, F44P, or L45R at -4 h plus 75 μg of lipopolysaccharide (LPS) at 0 h. Controls were given SEB or LPS alone. Data are mean readings of duplicate samples ± 5% for A and ± SD for B and C.

cine potential. To test individual sera for anti-SEB titers in an ELISA, mice were immunized three times with antigen plus alum, or alum only, and bled after the second and third injections. All animals (n = 15/group) immunized with SEB, F44P, or L45R seroconverted (absorbance at 405 nm >0.1 at a 1:200 dilution) after the second immunization (data not shown), whereas 87% of the animals immunized with Q43P seroconverted after two injections. All mice given Q43P developed SEB-reactive antibodies after three injections. Pooled sera from mice immunized with any mutant protein or native toxin had statistically similar ELISA titers (P > .05) that were still evident at a 1:51,200 dilution (figure 4). There were no detectable SEB antibodies in a 1:200 dilution of pooled sera from preimmune or alum-injected mice.

Differences among immunoglobulin sub-isotype titers in the immune mouse sera after three injections of SEB or any mutant protein were determined in an ELISA (figure 5). SEB, Q43P, and F44P induced statistically similar levels of IgG1, IgG2a, and IgG2b (P > .05). However, the highest titers of IgG2a and IgG2b resulted from immunization with L45R; these titers were statistically different from those induced by SEB (P < .02). We also observed similar sub-isotype patterns in sera collected after the second injection (data not shown). Anti-SEB immunoglobulins were absent in the NMS control.

Protection against lethal SEB challenge. Mice received a lethal combination of SEB plus LPS 10 days after the final immunization to compare the protection afforded by the mutant proteins versus SEB. There was 77%–100% survival among mice immunized with SEB or any mutant protein, in marked contrast (P < .005) to 0% survival among naive mice or alum-only controls (table 2). Immunization with Q43P, F44P, or L45R resulted in active immunity and statistically the same protective effects as SEB against a lethal SEB challenge. Passive immunization with pooled sera (200 μL/mouse) from animals immunized with Q43P, F44P, or L45R completely protected against a 2.5-μg SEB challenge, versus no protection among animals given SEB plus sera from alum-injected controls, suggesting that antibodies neutralized SEB toxicity, and
(21% of alum control stimulation index; \( P \leq .002 \)), Q43P (23% of alum control; \( P \leq .001 \)), or F44P (51% of alum control; \( P \leq .01 \)). Similar patterns of unresponsiveness were also observed for these mononuclear cells incubated with TSST-1, which shares some V\(_b\) specificities with SEB [5]. The stimulation indices of lymphocytes from animals immunized with SEB, Q43P, or F44P incubated with TSST-1 in vitro were respectively 30% \((P \leq .02)\), 33% \((P \leq .006)\), and 71% \((P < .05)\) of the alum control value. Unlike the other immunogens, L45R had a markedly less pronounced effect on lymphocyte proliferation due to SEB (98% of alum control; \( P > .05 \)) or TSST-1 (79% of alum control; \( P > .05 \)) stimulation in vitro.

**Discussion**

In this study, we characterized three mutant proteins of SEB that differed from native toxin by single amino acid substitutions in a hydrophobic loop that forms part of the interface with the DR\(\alpha\) subunit of MHC class II molecules [6, 30]. Mutants Q43P, F44P, and L45R had significantly reduced biologic activities in vitro and in vivo compared with those of SEB, yet SEB or any of the mutant proteins afforded comparable protection in immunized mice. Immunization with any of the mutant proteins or native toxin stimulated equivalent anti-SEB titers, but this was not surprising, as Q43P, F44P, and L45R were virtually indistinguishable from SEB in a competitive protection following active immunization was not a consequence of nonresponsive T cells.

Inhibition of lymphocyte proliferation in vitro. We examined the sera from immunized mice for effects on SEB-induced lymphocyte proliferation, which is commonly associated with superantigenic activity [5, 9, 10, 14, 33]. Sera from mice immunized with SEB or any of the mutant proteins, plus alum, effectively inhibited murine lymphocyte proliferation by SEB relative to sera from naive or alum-injected controls (figure 6; \( P \leq .01 \)). These results suggested that the protective effects from immunization with either SEB or mutant proteins may be linked to preventing lymphocyte proliferation.

Effect of mutant proteins and SEB immunizations on lymphocyte proliferation. In vivo stimulation of T cells with a superantigen causes proliferation of V\(_{\beta}\)-specific lymphocytes and is frequently followed by an immunologically unresponsive state due to anergy or deletion of the reactive cells (or both) [9]. Although the SEB antibodies were protective, as suggested by survival of actively or passively immunized mice against a lethal challenge and inhibition of SEB-induced lymphocyte proliferation in vitro, protection may have partly resulted from unresponsive lymphocytes bearing toxin-specific V\(_{\beta}\) elements.

To test this possibility, mononuclear cells from mice immunized with mutant protein or SEB were stimulated with SEB or TSST-1 in vitro. The lymphocytes from naive or alum-injected mice were quite responsive to SEB. However, relative to lymphocytes from alum controls that were incubated with SEB in vitro, there was a statistically significant decrease in lymphocyte proliferation from animals immunized with SEB (21% of alum control stimulation index; \( P \leq .002 \)), Q43P (23% of alum control; \( P \leq .001 \)), or F44P (51% of alum control; \( P \leq .01 \)).
Figure 4. ELISA anti-SEB titers of pooled sera from mice immunized 3 times with SEB or mutant protein. Results are mean absorbance at 405 nm ($A_{405}$) of triplicate wells ± SD. Readings for control wells prepared without SEB, yet treated with a 1:200 dilution of anti-SEB, were 0.008 ± 0.002.

ELISA with SEB antisera. Anti-SEB titers correlated well with protection against a lethal SEB challenge. Together, these observations suggest that epitopes necessary for active immunity against SEB were retained in the mutant proteins, although the amino acid substitutions that affected DRα binding substantially reduced toxicity.

The Q43P, F44S, and L45R mutant proteins, unlike SEB, were not lethal in our mouse model at the tested doses. Reduced toxicity of the mutant proteins paralleled lower serum cytokine levels induced by these mutant proteins in LPS-treated mice. Because the MHC class II molecules of mice do not have lysine at DRα residue 39, they have lower affinity than the human molecules for staphylococcal superantigens [5], and the end result is that mice are less responsive to these toxins [14, 35]. However, we successfully used LPS to potentiate the biologic activities of bacterial superantigens in mice [14] and investigated the vaccine potentials of various mutant proteins.

The cytokine patterns elicited by LPS and bacterial exotoxins are complex, and each cytokine has a range of biologic activities that may include stimulation of immunoglobulin production, activation of T and B cells, and induction of other cytokines (reviewed in [36]). Proinflammatory cytokines such as IFN-γ, IL-6, and TNF can be protective as well as potentially damaging to an infected host. Compared with animals injected with SEB plus LPS, there were conspicuously reduced serum concentrations of TNF and IFN-γ in mice given any of the mutant proteins plus LPS, or either SEB or LPS alone. Results from another murine model for SEB, which used β-galactosamine as a potentiating factor, revealed that a monoclonal antibody against TNF afforded protection against lethality, thus suggesting that TNF is critical in TSS due to SEB [37, 38]. SEB induces a large increase in serum concentrations of TNF within 2–4 h after injection into β-galactosamine–sensitized mice [38]. In our animal model, maximum serum concentrations of TNF were present at 6 h after the SEB injection and 2 h after LPS. These findings are consistent with other murine studies for LPS-potentiated SEB [33], SEA [14], or TSST-1 [39]. Elevated levels of TNF, persisting for >3 days, are also produced in human mononuclear cells cultured with SEB, SEC, streptococcal pyrogenic exotoxin A, or TSST-1 [40].

### Table 2. Protection among immunized mice against lethal SEB challenge.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Challenge</th>
<th>No. live/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEB + alum</td>
<td>SEB + LPS</td>
<td>12/13</td>
</tr>
<tr>
<td>Q43P + alum</td>
<td>SEB + LPS</td>
<td>14/15</td>
</tr>
<tr>
<td>F44P + alum</td>
<td>SEB + LPS</td>
<td>10/13</td>
</tr>
<tr>
<td>L45R + alum</td>
<td>SEB + LPS</td>
<td>13/13</td>
</tr>
<tr>
<td>Alum only</td>
<td>SEB + LPS</td>
<td>0/13</td>
</tr>
<tr>
<td>None</td>
<td>SEB + LPS</td>
<td>0/14</td>
</tr>
<tr>
<td>None</td>
<td>LPS only</td>
<td>14/14</td>
</tr>
</tbody>
</table>

**Note.** Challenge dose per mouse was 10 μg of SEB plus 75 μg of lipopolysaccharide (LPS) or 75 μg of LPS only.
Antibody against IFN-γ reduces the circulating levels of IFN-γ and severity of SEB intoxication in d-galactosamine-treated mice but does not affect TNF or IL-6 levels, which suggests that IFN-γ also contributes to shock in this model [41]. This current study with SEB in the LPS-potentiated mouse model, as well as previous work with SEA or TSST-1 [14, 39], reveals that IFN-γ and TNF are good correlates of lethality. In contrast to findings in mice injected with a staphylococcal superantigen plus LPS or d-galactosamine, mice infected with lymphocytic choriomeningitis virus and subsequently injected with SEB plus a neutralizing monoclonal antibody against IFN-γ died, suggesting that IFN-γ is not intimately involved in the lethal effects of these toxins [42]. Obviously, there are fundamental differences among murine models for bacterial superantigens; such differences lead to different interpretations of the factors (i.e., cytokines) intimately involved in lethality. All of these assays use different potentiating compounds, which further complicates comparisons of data from various laboratories. The development of a reliable, inexpensive animal model for bacterial superantigens, minus any exogenous potentiation, would be of interest.

Of the three cytokines that we measured in mouse sera, the IL-6 concentrations induced by the mutant proteins and LPS were relatively high but comparable to those in LPS controls. IL-6 can evidently protect mice against the lethal effects of SEB in a d-galactosamine–induced toxic shock model [37], and antibodies against IL-6 enhance circulating levels of this cytokine in mice, presumably by the slow release of IL-6 from the antibody–IL-6 complex [41].

Besides a lack of lethal effects in mice and diminished cytokine levels relative to SEB, the mutant proteins that we tested also did not effectively stimulate human PBMC proliferation. It has been well established that many biologic properties of the staphylococcal superantigens depend on T cell activation [5, 7, 8, 14, 17–19, 29]. Specific populations of T cells are expanded in diseases of known or suspected superantigen etiology [5]. For example, T cells with Vb2 receptor elements are contrast to findings in mice injected with a staphylococcal superantigen plus LPS or d-galactosamine, mice infected with lymphocytic choriomeningitis virus and subsequently injected with SEB plus a neutralizing monoclonal antibody against IFN-γ died, suggesting that IFN-γ is not intimately involved in the lethal effects of these toxins [42]. Obviously, there are fundamental differences among murine models for bacterial superantigens; such differences lead to different interpretations of the factors (i.e., cytokines) intimately involved in lethality. All of these assays use different potentiating compounds, which further complicates comparisons of data from various laboratories. The development of a reliable, inexpensive animal model for bacterial superantigens, minus any exogenous potentiation, would be of interest.

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The mutant proteins we studied had single amino acid substitutions in a region of the SEB molecule important for binding to a cellular receptor, the MHC class II molecule. Superantigen activity involves binding to the MHC class II molecules of antigen-presenting cells and the Vβ domain of the T cell receptor [30, 44]. The primary MHC class II binding sites in SEB consist of a polar pocket, which contains a glutamate and two tyrosines that interact with K39 of DRα, and a hydrophobic loop [44]. Residues 44–47 of SEB form a hydrophobic interface with the α chain of DR1 [6, 30]. Residue F44 is favorably situated for MHC class II binding because its side chain is exposed to solvent, as demonstrated by radiographic crystallography [45]. Substituting arginine for leucine at residue 45 likely

Figure 5. Anti-SEB immunoglobulin isotypes from pooled immune mouse sera after 3 injections. SEB-coated wells (5 μg/mL) were incubated with pooled sera (diluted 1:100). Data are mean absorbance at 405 nm (A405) of duplicate wells ± SD.
Figure 6. Anti-proliferative effects of pooled sera from immunized mice. Naive mouse mononuclear cells were incubated with medium containing SEB and 1:128 dilution of pooled sera. Data are mean cpm of triplicate samples ± SD.

causes steric effects in this loop that markedly diminish binding to the MHC class II molecules [30]. It is noteworthy that this leucine is conserved in all SEs, streptococcal pyrogenic exotoxin A, and TSST-1 [30, 44]. By changing this specific leucine to glycine in SEA, which is analogous to L45 of SEB, there is >90% reduction in T cell proliferation compared with an equivalent concentration of SEA [46].

The biologic activities of Q43P, F44P, and L45R were similar in most respects; however, immunization with L45R stimulated higher titers of anti-SEB immunoglobulin sub-isotypes IgG2a and IgG2b and did not cause specific lymphocyte anergy and/or deletion. These results suggest that L45R interacts differently with the immune system than does SEB, Q43P, or F44P. The immunosuppressive properties of the SEs and TSST-1 are linked to unresponsive T lymphocytes [9, 10, 16–18], which suggests that a potential superantigen vaccine, versus native toxin, should induce minimal T cell activation and not subsequently anergize or delete specific T cell populations involved in humoral responses. In this sense, L45R may be a better vaccine candidate than the other two mutant proteins.

Protection in our studies was not due to an inability of lymphocytes to respond to SEB, as evidenced by the results from mice immunized with L45R and the passive immunization experiments. There were no signs of anergy or specific lymphocyte deletion in L45R-immunized mice relative to alum controls, plus they had an anti-SEB titer and were protected (100%) against a lethal SEB challenge, unlike the alum controls (0% survival). Although mice immunized with SEB, Q43P, or F44P had comparable anti-SEB titers and were afforded degrees of protection equivalent to that induced by L45R, it is conceivable that some protective effects seen in mice immunized with SEB, Q43P, or F44P could be attributed partly to an inability of SEB-reactive lymphocytes to respond to the toxin challenge.

The SEB mutants that we have studied will need further evaluation in other animal models to assess their utility as vaccines. Although our mouse model has proven useful for evaluating the biologic activities and immunogenicities of superantigen vaccine candidates, monkey feeding studies remain the only means of examining the emetic activities of SEs [2]. Emesis and superantigenicity are evidently separate properties of SEA and SEB [46, 47]. A mutant protein of SEB (F44S), which does not activate T cells [48], is emetic at 300 μg when given to rhesus monkeys by nasogastric intubation [46]. However, a glycine mutation at the analogous F47 site of SEA produces a molecule that has neither T cell proliferative nor emetic effects [46]. Another SEA mutant, L48G, is emetic at 500 μg/animal but does not induce proliferation of murine splenocytes. Obviously, residue location and type of substitution can have a profound effect on the biologic activities of the SEs.

Colonization with *S. aureus* strains that produce SEs or TSST-1 is widespread among humans, and much of the population has measurable antibody titers against one or more of these toxins [27]. Because TSS is a serious, potentially life-threatening disease caused by various staphylococcal toxins such as SEB [7, 8] and TSST-1 [9, 10], an effective vaccine...
is needed to protect those persons who are especially at risk. Patients with TSS not only have lower antibody titers to TSST-1 than do healthy persons with no history of the disease, but they tend not to produce significant anti–TSST-1 titers during the acute and convalescent stages [26, 27]. Although these persons have no general immunodeficiency, they are especially susceptible to TSS, and the immunomodulatory activities of SEs and TSST-1 play a role in the poor immune response to these toxins by some patients [26]. A recent report shows that SEB and the influenza virus can cause a lethal synergism in mice [49]. This finding may be particularly relevant, since strains of \textit{S. aureus} that produce SEB or TSST-1 have been recognized as a potentially lethal complication of influenza in humans [15].

As Q43P, F44P, and L45R were effective immunogens and had diminished biologic activities, compared with SEB, we believe that these three mutant proteins merit further study regarding their suitability as vaccines. In particular, L45R looks the most promising because it stimulated a unique antibody response and induced minimal immunologic unresponsiveness among lymphocytes. We have shown that Q43P, F44P, and especially L45R lost many of the typical superantigenic properties of the parent molecule. Future studies should hopefully determine how mutant proteins of a superantigen such as SEB are processed and presented to T cells.

Acknowledgments

The expert laboratory skills of Jay Brissey, Marilyn Buckley, Kathleen Callahan, Beverly Dyas, and Francis Sexton were invaluable in completing these studies. We thank Sara A. Grove for statistical analyses, Sina Bavari for instruction and help with lymphocyte cultures, and Lilee Cuff for invaluable discussions.

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