Defense Against Biologic Warfare with Superantigen Toxins

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Abstract

Background: Superantigens produced by *Staphylococcus aureus* and *Streptococcus pyogenes* are among the most lethal of toxins. Toxins in this family trigger an excessive cellular immune response leading to toxic shock.

Objectives: To design an antagonist that is effective *in vivo* against a broad spectrum of superantigen toxins.

Methods: Short peptide antagonists were selected for their ability to inhibit superantigen-induced expression of human genes for cytokines that mediate shock. The ability of these peptides to protect mice against lethal toxin challenge was examined.

Results: Antagonist peptide protected mice against lethal challenge with staphylococcal enterotoxin B and toxic shock syndrome toxin-1, superantigens that share only 6% overall amino acid homology. Moreover, it rescued mice undergoing toxic shock. Antagonist peptides show homology to a β-strand/hinge/α-helix domain that is structurally conserved among superantigens, yet remote from known binding sites for the major histocompatibility class II molecule and T cell receptor that function in toxic T cell hyperactivation.

Conclusions: The lethal effect of superantigens can be blocked with a peptide antagonist that inhibits their action at the top of the toxicity cascade before activation of T cells occurs. Superantigenic toxin antagonists may serve not only as countermeasures to biologic warfare but may be useful in the treatment of staphylococcal and streptococcal toxic shock, as well as in some cases of septic shock.

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Biologic toxins may soon replace traditional chemical weapons, given the ease and low cost of toxin production coupled with their high lethality. Thus, the development of protective countermeasures has become an urgent mission. It is also the best means to deter the use of toxin weapons, be it in the military arena or by terrorists.

The main microbial threats – anthrax, cholera, plague, tularemia and Q fever – can be combated to a varying degree with antibiotics; anthrax spores are sensitive to surfactant nanoemulsions [1]. Whereas antibodies against botulinum toxins are available and vaccines are under development, there is currently no effective defense, whether as antidote or vaccine, against the bacterial exotoxins of the superantigen family, which rate with botulinum toxin among the most lethal of toxins [2]. By comparison, other candidate toxins, such as ricin or the T-2 mycotoxins, require far greater amounts to achieve a similar degree of lethality, making them less suitable as weapons. Though deadly, tons of ricin would be needed for an effective open-air attack [2].

Superantigens are stable proteins that bind directly to most major histocompatibility class II molecules [3] and stimulate virtually all T cells bearing particular domains in the variable portion of the β chain of the T cell receptor, without need for processing by antigen-presenting cells [4–8]. The TCR interacts with superantigens via the outer face of its Vβ domain, a region not involved in ordinary antigen recognition [9]. Bypassing the restricted presentation of conventional antigens, superantigens can activate up to 50% of T cells to divide and produce cytokines. Thus, superantigens activate the cellular immune response at least 5,000-fold more strongly than do ordinary antigens. Toxic shock results from a sudden and massive induction of T helper 1 cell-derived cytokines that include interleukin-2, interferon-γ and tumor necrosis factor [10]. Death from capillary leak syndrome results within 24–48 hours, but even at concentrations several logs below lethal ones these toxins can elicit severe and prolonged incapacitation in humans, including nausea, vomiting and diarrhea [2,11]. Staphylococcal food poisoning is caused by low concentrations of superantigen toxins.

The family of about two dozen pyrogenic exotoxin superantigens secreted by *Staphylococcus aureus* and *Streptococcus pyogenes* includes at least 10 staphylococcal enterotoxins among which SEB is most prominent, as well as toxic shock syndrome toxin-1, and streptococcal pyrogenic exotoxins, among them SPEA. To compound the problem of protection, the amino acid sequences of superantigens are highly divergent. Thus, SEA and SEB are 27% homologous [12], whereas TSST-1 shares only 6% sequence homology with SEB [13]. SEB was weaponized by the United States before 1969. However, current scenarios of biologic warfare and bioterror are more likely to entail the use of superantigen toxins in natural mixtures, readily obtained by culturing the bacteria. All the staphylococcal enterotoxins are potent emetic agents and most of the bacterial superantigens induce toxic shock syndrome, though with individual characteristics [11]. This complexity demands the development of broad-spectrum countermeasures.

Previous efforts to develop antidotes against toxic shock concentrated on blocking downstream phenomena in the toxicity cascade, mainly by inhibiting the action of tumor necrosis factor with monoclonal antibodies or soluble receptors. However, the extremely high levels of cytokines produced in response to superantigens render this approach difficult and ineffective.

We have explored the possibility of blocking superantigen action at the top of the toxicity cascade, before activation of T cells occurs. We describe antagonist peptides that inhibit the induction of human Th1 cytokine gene expression by a variety of superantigens.

TCR = T cell receptor
Vβ = variable portion of the β chain of the T cell receptor
SEB = staphylococcal enterotoxin B
SEA = staphylococcal enterotoxin A
TSST-1 = toxic shock syndrome toxin-1

These antagonist peptides are capable of protecting mice from the lethal effects of superantigen toxins as widely different as SEB and TSST-1 and can rescue animals already deep into toxic shock.

Methods

Toxin antagonist peptides

Peptides were synthesized using fluoronyl-methoxycarbonyl chemistry. They were cleaved and the side chain deprotected with triflouroacetic acid. Triflouroacetic acid-peptide salts were soluble in culture medium. *D*-Ala was linked to *N*- and *C*-termini using the same procedure. High pressure liquid chromatography showed that peptides were > 95% pure.

Protection of mice against toxic shock

Groups of 10 female BALB/c mice (Harlan, Jerusalem, Israel), aged 10–12 weeks, were challenged by intraperitoneal injection of 10 μg of SEB (Sigma, USA) mixed with 20 mg of *D*-galactosamine, or of 5 μg TSST-1 (Sigma) mixed with 40 mg of *D*-galactosamine. Unless otherwise shown, an amount of 25 μg of antagonist peptide was injected intraperitoneally 30 minutes before the toxin. Survival was monitored. SPSS for Windows 9.0 software was used for analysis of variance between groups. All experiments involving the use of mice were in accordance with protocols approved by the Animal Care and Use Committee of the Hebrew University-Hadassah Medical School.

Structures of superantigens

Molecular modeling using RasMol 2.6 software was based on atomic coordinates derived by X-ray diffraction for SEB (Protein Data Bank code 1SEB) [14–18] and TSST-1 (1TSS) [19]. Domains with partial homology to antagonist peptide p*12* are $SEB_{150-161}$ (Thr-Asn-Lys-Lys-Lys-Val-Thr-Ala-Gln-Glu-Leu-Asp) and $TSST\text{-}1_{119-130}$ (Phe-Asp-Lys-Lys-Gln-Leu-Ala-Ile-Ser-Thr-Leu-Asp).

Results

Superantigen toxin antagonist

In an attempt to obtain antagonists of SEB, short peptides were synthesized that consist of amino acid sequences from SEB domains known to be essential for binding to the TCR, to the MHC class II molecule, or both [20]. In addition, peptides were synthesized with partial homology to SEB residues 150-161, a domain that is conserved among superantigens not yet known to be involved in binding to either TCR or MHC class II molecule [14–18]. When present in up to 200-fold higher molar amounts than SEB, none of these peptides had significant SEB agonist activity, shown by the lack of ability to induce expression of mRNA encoding the Th1 cytokines, IL-2 and IFNγ, in human peripheral blood mononuclear cells [20].

The powerful ability of superantigens to activate T cells involves their tight binding to the TCR and MHC class II molecule, stabilized by interactions at multiple sites [14–18]. A short peptide would not be expected to exhibit that property, yet we conjectured that should it compete with SEB for an essential site, it might prevent cooperative interactions. We thus assayed each peptide for its ability to inhibit SEB-mediated induction of human IL-2, IFNγ and tumor necrosis factor-β mRNA expression [20]. No peptide derived from SEB domains that interact with the TCR and/or MHC class II molecule was inhibitory, but strong antagonist activity was exhibited by dodecapeptide p*12* whose sequence, Tyr-Asn-Lys-Lys-Lys-Ala-Thr-Val-Gln-Glu-Leu-Asp, is a variant of SEB domain 150-161. p*12* inhibited not only the action of SEB but also that of the staphylococcal and streptococcal superantigens SEA, TSST-1, and SPEA [20]. p*14*, a derivative of p*12*, carries two extra amino acids from $SEB_{148-149}$ (Val-Gln) preceding the p12 sequence [21]. p*12A* and p*14A* carry *D*-Ala residues abutted to the *N*- and *C*-termini to render them more protease-resistant.

Protection and rescue of mice from lethal shock [Figure 1]

The *D*-galactosamine-sensitized mouse, an accepted animal model for studying lethality of the superantigens, was used to investigate the protective activity of p*12A* [20] and p*14A* [21]. Mice exposed to antagonist peptide alone remained viable and showed no detectable side effects. Of the mice challenged with SEB, only 30% were still alive 24 hours after toxin exposure and 20% at later times. However, all of the SEB-challenged mice were protected by p*14A* given 30 min before toxin ($P = 0.0003$) [Figure 1A]. Surviving animals showed no signs of distress and remained indistinguishable from normal

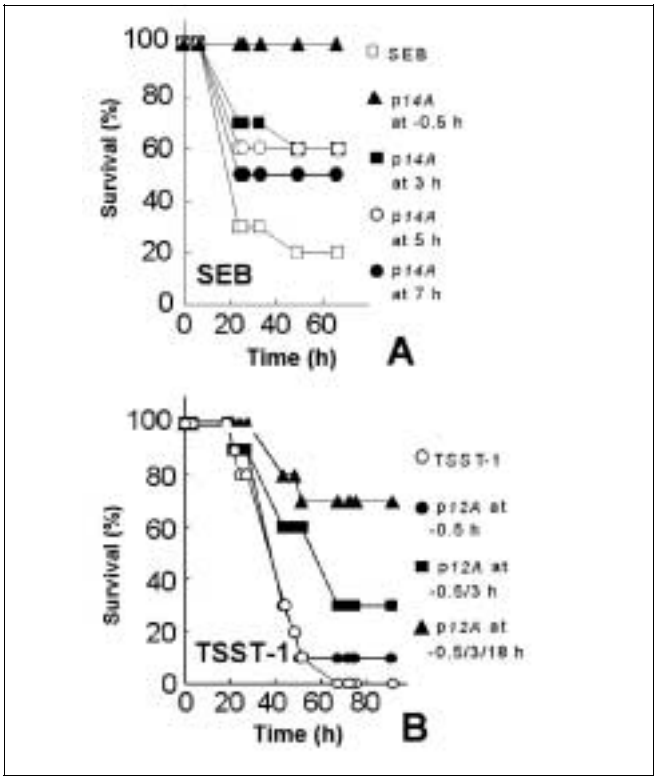

Figure 1. Antagonist peptide protects and rescues mice from SEB-induced lethal shock and protects mice from TSST-1-induced lethal shock. Groups of 10 mice were challenged with SEB **[A]** or TSST-1 **[B]**, alone or in the presence of antagonist peptide.

Th1 = T helper 1
MHC = major histocompatibility complex
IL = interleukin
IFN = interferon

controls in behavior; they survived for as long as they were monitored, 2 weeks. Partial protection was obtained when administration of the antagonist peptide was delayed to 3 hr ($P = 0.05$), 5 hr ($P = 0.08$) or even 7 hr after lethal challenge. A progressively decreasing protective effect of p*14A* was seen between 20 and 40 hr, yielding 70%, 60% and 50% survival, respectively. Thus, p*14A* is not only fully protective when given before SEB challenge but is also able to rescue mice after they were exposed to toxin. Protection or rescue was observed when the antagonist peptide was in only a 20-fold molar excess over SEB, showing that the peptide is a potent superantigen antagonist *in vivo*.

Even though TSST-1 exhibits a mere 6% overall sequence homology with SEB, p*12A*, which protects mice from SEB [20], was also protective against this toxin. Relative to SEB, TSST-1 killed mice far more slowly, with half-maximal mortality occurring by 40 hr [Figure 1B]. In the control group, all mice died upon challenge with TSST-1. p*12A* did not protect when administered just before TSST-1 but afforded significant protection (up to 70%) when also injected after toxin challenge. The protective effect became progressively more pronounced with repeated administration at 3 and 18 hr. Survival of mice from TSST-1-mediated toxic shock thus depends on sustained presence of the peptide, suggesting that although its half-life is limited, it is long enough to protect mice against death. p*12A* is a broad-spectrum superantigen antagonist *in vivo*.

Mice protected from lethal toxin challenge by antagonist peptide rapidly became resistant to subsequent toxin challenges. Within 2 weeks, protective antibodies against superantigens were found in the serum of mice that were shielded from lethal shock by the antagonist peptide. This immunity is cross-protective against different superantigen toxins [20,21]. Apparently, by blocking the ability of the toxin to induce a cellular immune response leading to toxic shock, the peptide antagonist allows the superantigen to induce a vigorous humoral immune response directed against itself. Yet, antibodies against antagonist peptide could not be detected. Indeed, the small size and relatively rapid clearance of a short peptide (12–14 amino acids) constitute therapeutic advantages.

The antagonists target a structurally conserved domain [Figure 2]

Peptides deriving from the $SEB_{150-161}$ domain exhibit superantigen antagonist, whereas peptides from regions known to be essential for the interaction of SEB with the TCR and/or class II MHC molecule lack such activity [20]. Indeed, the 12-amino acid 150-161 domain in SEB is well removed from regions that participate in binding of TCR and/or class II MHC. Moreover, this SEB domain lies outside the region sufficient for mitogenic activity, the *N*-terminal 138 amino acids [22,23]. Thus, the ability of p*14A* and p*12A* [20] to act as SEB antagonists is surprising. The $SEB_{150-161}$ domain forms a central turn starting within a β-strand and connecting it, via another short β-strand, to an α-helix. This domain is conserved among pyrogenic toxins, with 10/12 amino acid identities for SEA, SEC1, SEC2 and SPEA, 9/12 for SEE, and 4/12 for TSST-1, the most remotely related member of the staphylococcal superantigen family [24]. Notwithstanding the great diversity in overall sequences of SEB and TSST-1, reflected also by distinct binding sites for the TCR Vβ chain in these toxins [11,16], their structures show spatial conservation. In particular, the β-strand/hinge/α-helix domain corresponding to residues 150-161 in SEB is found in TSST-1 residues 119-130. This domain in TSST-1 shares only 33% sequence similarity with the corresponding SEB domain, yet the domain folding is conserved. An antagonist peptide that shares a higher homology with SEB was nevertheless capable of protecting against lethal shock induced by TSST-1.

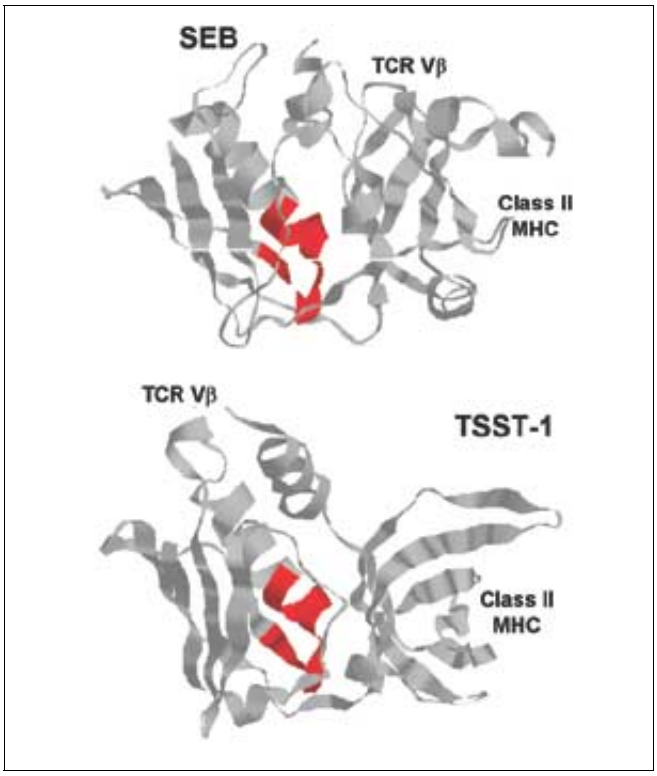

Figure 2. Structures of superantigen toxins SEB and TSST-1. The SEB domain having homology to antagonist peptide p*12* and the corresponding domain in TSST-1 are colored red. Binding sites of TCR Vβ chain and MHC class II molecule are indicated.

Discussion

Delivery of live bacteria is difficult, which limits their weapons potential. In contrast, certain microbial toxins are exceptionally stable, facilitating their dissemination. The superantigen toxins remain fully active even after prolonged boiling. This heat resistance is one reason for their potent ability to induce food poisoning; indeed, most cases of food poisoning with high and prolonged morbidity are caused by SEB.

Unlike chemical weapons, biologic toxins are not volatile and tend to be more toxic per weight than many chemical agents. Because they are produced by culturing microorganisms commonly found in nature, which in the case of staphylococcal superantigen toxins is readily accomplished by personnel with ordinary skills and equipment, they are also more easily available, enhancing their threat potential. For this reason, toxins may replace chemical weapons in the future.

The probability that a particular toxin may be selected for biologic warfare increases when an antidote or vaccine is lacking.

Once countermeasures are developed, its attractiveness as a potential biologic weapon is diminished. The antagonist peptides described here protect or rescue mice from lethal shock at a molar excess of as low as 20-fold over the toxin, implying that they bind tightly to a cellular target that is critical for superantigen action. These antagonist peptides show homology to a superantigen domain consisting of a β-strand/hinge/β-strand/α-helix motif that is conserved spatially and in sequence among all superantigens studied. This provides a plausible explanation for the broad-spectrum antagonist activity. Apparently, superantigens use the domain homologous to the antagonist to bind to a novel receptor on T cell or antigen-presenting cell critical for their action, and the nature of this receptor is under active investigation. By competing with superantigens in binding to this receptor, the antagonist peptide is able to block Th1 cell activation and the resulting toxicity. Superantigenic toxin antagonists may serve not only as countermeasures to biologic warfare but may be useful in the treatment of staphylococcal and streptococcal toxic shock, as well as in gram-positive septic shock. The latter is made relevant by the rising incidence of methicillin-resistant *S. aureus* infections.

The antagonist protects mice against lethal doses of superantigen toxin, and this protection is broad spectrum in nature. Moreover, the mice surviving a toxin attack are rapidly rendered resistant to recurrent toxin challenges, even in the absence of more antagonist and even with different toxins. This is because protective antibodies develop in these mice as soon as the antagonist blocks the toxicity cascade that leads to death. That is a significant bonus to the protective action of the antagonist.

During their convergent evolution, the superantigen toxins from *S. aureus* and *S. pyogenes* have acquired molecular structures designed to recognize the receptors of the human immune system critical for their function, among them TCR and MHC class II molecule. Therefore, these toxins are not likely to be amenable to facile improvement or to engineering of resistance against an antidote that disturbs the highly specific molecular interaction between toxin and target. Although these biologic toxins pose a serious threat, countermeasures designed against them will also be more difficult to overcome.

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