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Recombinant *Staphylococcus aureus* Exfoliative Toxins Are Not Bacterial Superantigens

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Staphylococcal scalded-skin syndrome is an exfoliative dermatitis characterized by the separation of the epidermis at the stratum granulosum. This disruption is mediated by one of two *Staphylococcus aureus* exotoxins, exfoliative toxins A and B (ETA and ETB). Both ETA and ETB have been reported to be bacterial superantigens. A controversy exists, however, as other data indicate that these exotoxins are not superantigens. Here we demonstrate that recombinant exfoliative toxins produced in *Escherichia coli* do not act as T-cell mitogens and thus are not bacterial superantigens. These data fit the clinical profile of the disease, which is not associated with the classic symptoms of a superantigen-mediated syndrome.

Staphylococcal scalded-skin syndrome (SSSS) is an exfoliative dermatitis of infants and children that results from infection with exfoliative-toxin-producing *Staphylococcus aureus* (1, 15). SSSS is characterized by the formation of large bullae without inflammatory cell infiltrate and separation of extended areas of the epidermis at the stratum granulosum leaving the keratinocytes intact. Two biologically and serologically distinct *S. aureus* exotoxins are responsible for the skin manifestations of SSSS in humans, exfoliative toxin A (ETA) and exfoliative toxin B (ETB) (25). ETA (26.9 kDa) is encoded on the bacterial chromosome and shares 40% amino acid identity with the plasmid-borne ETB (27.3 kDa) (2, 13, 19). Despite extensive studies, the exact mechanism responsible for the skin disruption is not known.

X-ray crystallographic structures of ETA and ETB (5, 20, 23, 24) suggest that the toxins are members of the trypsin-like serine protease family. Protease activity has not been demonstrated for either toxin in vitro, but both ETA and ETB have intrinsic esterase activity, which is associated with serine proteases (3). Thus, it is likely that both toxins are proteases. In addition to having possible protease activity, both ETA and ETB are reported to be bacterial superantigens (14, 16, 17, 24). Bacterial superantigens are a family of proteins able to bind simultaneously to the major histocompatibility complex and to the T-cell receptor (TCR), resulting in stimulation of a large number of T cells expressing specific Vβ subsets of the TCR repertoire (14).

Previously, Fleischer and Bailey reported that recombinant ETA expressed in a superantigen-free *S. aureus* background does not have mitogenic activity (7). They conclude that the activity seen by others is due to contamination of the toxin preparations with other superantigens. However, since this report, additional literature has addressed the superantigenic activity of ETA and ETB. Here we demonstrate that recombinant exfoliative toxins produced in an *Escherichia coli* background do not act as T-cell mitogens and thus are not bacterial superantigens.

Isolation, expression, and purification of recombinant exfoliative toxins. DNA fragments encoding ETA and ETB were obtained by utilizing PCR and DNA from exfoliative-toxin-producing bacterial strains. Toxins were expressed and purified using the Novagen (Madison, Wis.) pET expression system. In brief, a 780-bp DNA fragment encoding mature ETA was obtained using the oligonucleotide primers ETA-3 (5′-GCGCCTCGAGGTTTCAGCAGAAGAAATAAAA-3′) and ETA-4 (5′-GCGCCTCGAGTATAAAACATCCACGGATTTT-3′) and chromosomal DNA of an ETA-producing *S. aureus* strain. A 775-bp DNA fragment encoding mature ETB was amplified from plasmid pIJ002 (generously provided by Peter McNamara) using oligonucleotide primers ETB-3 (5′-GCGCCATATGAAAGAATACAGCGCA-3′) and ETB-4 (5′-CGCGGGATCCATATTGAAATATTAA-3′). The amplifications were performed using *Taq* DNA polymerase (Bethesda Research Laboratories, Gaithersburg, Md.) or *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Calif.). Primers were designed to amplify the coding sequences for the mature proteins without the amino-terminal signal sequences. They were also designed to contain restriction endonuclease sites that facilitated insertion of the fragments into the *E. coli* expression vector pET-15b (Novagen). The DNA sequences of both the ETA- and ETB-encoding fragments were determined and shown to be identical to the GenBank database sequences (*eta*, accession numbers L25372 and M20371; *etb*, accession numbers M17348 and M13775). In order to generate an appropriate negative control, the ETA-encoding fragment was mutated to code for mature ETA with an alanine residue replacing the putative active-site serine residue at position 195 (21, 22), using overlap extension mutagenesis (10). The complete nucleotide sequence of both strands of the mutated fragment was determined to confirm the presence of the desired mutation.

Exfoliative-toxin-encoding DNA fragments were inserted into the expression vector pET-15b, previously modified to code for kanamycin resistance. The recombinant toxins were expressed in *E. coli* as described previously (pET system manual, 4th ed., Novagen). Purified toxins were digested with thrombin to remove the amino-terminal histidine-rich leader peptide, and the histidine-rich leader peptide was separated from the toxin by dialysis against phosphate-buffered saline (PBS). Thrombin was removed from the toxin preparations by chromatography over ρ-aminobenzamidine-agarose (Sigma Chemicals, St. Louis, Mo.). The resultant recombinant ETA (rETA) and recombinant ETA-S195A (rETA-S195A) consist of the mature forms of native ETA and ETA-S195A with five

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TABLE 1. Activities of rETA, rETB, and rETA-S195A in the neonatal mouse

Injectant	Dose	Response at[a]:	
		2 h	24 h
rETA	5 μg/g	0	3, E
	25 μg/g	1	3, E
rETB	5 μg/g	ND	2.8, E
	25 μg/g	ND	3, E
rETA-S195A	5 μg/g	0	0
	50 μg/g	0	0
S. aureus	3.0×10^8 CFU	0	3
PBS	10 μl	0	0

[a] One-day-old BALB/c mice injected with increasing concentrations of rETA, rETB, rETA-S195A, PBS, or exotoxin-producing *S. aureus* and observed at the stated times for gross appearance. The grading system was as follows: 0, no obvious signs of skin change; 1, Nikolsky's sign (permanent wrinkling of skin after rubbing with slight pressure); 2, formation of bullae <3 mm in diameter; and 3, formation of bullae >3 mm diameter, webbing of the skin at the hind limbs, or frank exfoliation. The values are averages of the gross scores; a minimum of five mice were used for each toxin dose or control inoculation. E, expired; ND, not done.

additional N-terminal amino acids (GSHML). The resultant recombinant ETB (rETB) consists of the mature form of native ETB with four additional N-terminal amino acids (GSHM). The recombinant toxins ran as single bands of approximately 27 kDa on stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. rETA appeared as a single band by Western blot analysis. Protein concentrations were determined using a bicinchonic acid protein assay kit (Pierce, Rockford, Ill.).

Recombinant exfoliative toxins produce the symptoms of SSSS in the neonatal mouse assay. The recombinant exfoliative toxins were tested for activity in neonatal mice (15). One-day-old BALB/c mice were injected subcutaneously at the nape of the neck with increasing concentrations of rETA, rETB, rETA-S195A, PBS (negative control), or ETA-producing *S. aureus* (positive control) and observed at various times postinjection for symptoms of SSSS. All mice were returned to lactating mothers and were observed at hourly intervals for gross appearance. They were graded on appearance and tactile examination (Table 1). Mice died during the course of the experiment or were sacrificed at the end of the 24-h observation period. After only 2 h, the mice injected with the highest dose of rETA (25 μg/g of body weight) showed obvious signs of exfoliation (Table 1). rETB and rETA exhibited similar activities, while rETA-S195A did not cause any visible signs of exfoliation even at 10 times the dosage that elicited a positive response with either rETA or rETB.

To confirm that the recombinant toxins were causing the characteristic cleavage at the stratum granulosum, skin samples from representative animals were prepared for histological examination. At the time of death, animals were placed in 10% formalin for fixation and embedded in paraffin wax for preparation of skin sections. Sections were stained with hematoxylin and eosin and examined via light microscopy for separation at the stratum granulosum. Injection of both rETA (Fig. 1) and rETB (data not shown) resulted in the diagnostic skin cleavage, while rETA-S195A (data not shown) did not. The cleavage was identical to that observed in mice injected in a similar manner with an ETA-producing *S. aureus* strain (data not shown). Thus, rETA and rETB, but not rETA-S195A, were able to produce the characteristic and diagnostic cleavage of SSSS in this animal model.

Determination of superantigen activity. rETA, rETB, and rETA-S195A were assayed for mitogenic activity using human peripheral blood mononuclear cells (PBMCs). Mitogenicity assays were performed as described previously (12). Briefly, heparinized whole blood from human adults was fractionated on Ficoll-Paque (Pharmacia Biotech, Piscataway, N.J.) and the PBMCs were harvested. Cells (10^5) were added to 96-well U-bottom plates in RPMI 1640 supplemented with 10% fetal calf serum and were incubated for 72 h with various concentrations of one of the following: rETA, rETB, rETA-S195A, rSpeA1 (purified in this laboratory as described previously [12]), staphylococcal V8 protease (Promega, Madison, Wis.), and PBS. Staphylococcal V8 protease is a serine protease structurally similar to the exfoliative toxins and was used as a negative control; the streptococcal superantigen rSpeA1 was used as a positive control. One microcurie of [^{3}H]thymidine (ICN Biochemicals, Costa Mesa, Calif.) was added to each well, and cells were incubated for an additional 24 h and harvested; the [^{3}H]thymidine uptake was then quantified. For each toxin or control, three to five distinct donors were used. Assays were performed a minimum of three times per recombinant toxin with different donor cells each time. The human PBMCs responded as expected to both rSpeA1 (Fig. 2) and the anti-CD3 monoclonal antibody OKT3, which served as a nonspecific T-cell mitogen (data not shown). However, there was no detectable mitogenic activity when PBS, rETA, rETB, rETA-S195A, or V8 protease was added to the cells. To determine if the lack of activity was due to the age of the donor, freshly isolated human umbilical cord blood was prepared as described above, as a source of neonatal mononuclear cells. Neonatal mononuclear cells had the same mitogenic response to rETA, rSpeA1, OKT3, and PBS as adult PBMCs (data not shown).

At this time a controversy exists over whether these toxin are superantigens. The X-ray crystal structures of both toxins (5, 20, 23, 24) indicate that ETA and ETB are members of the trypsin-like serine protease family. Both toxins contain the signature His-Ser-Asp catalytic triad of this family, and substitution of an Ala residue for any one of these residues in ETA results in toxin unable to cause exfoliation in the neonatal mouse model (21, 22; L. R. W. Plano and C. M. Collins, unpublished data). Both ETA and ETB have esterolytic activity, which is associated with serine proteases (3). Therefore, it is commonly believed that ETA and ETB are serine proteases. Either they have a highly specific target (likely, given that the stratum granulosum is the only site of damage seen in an intoxicated animal), or the conditions required for their catalysis have not been identified.

It was suggested in the early 1980s by Morlock et al. that the exfoliative toxins were mitogens (17). In 1989 Kappler et al. reported stimulation of specific human TCR Vβ-chain-expressing T-cell populations in response to exfoliative toxin (11). In the following years exfoliative toxin was reported to stimulate additional human Vβ- and mouse Vβ-expressing T-cell populations (6, 8) and to induce cutaneous lymphocyte-associated antigen expression in peripheral T lymphocytes (26). However, during these early studies investigators were unable to demonstrate binding of ETA to major histocompatibility complex class II receptors, a requirement for superantigen activity (9).

In more recent work, Vath and coworkers report that wild-type ETA and an ETA active-site mutant are mitogens (24). In a follow-up study Monday et al. report expansion of specific Vβ-expressing human T-cell populations not previously cited for ETA or ETB but fail to show expansion of the populations which were originally described for the exfoliative toxins (16).

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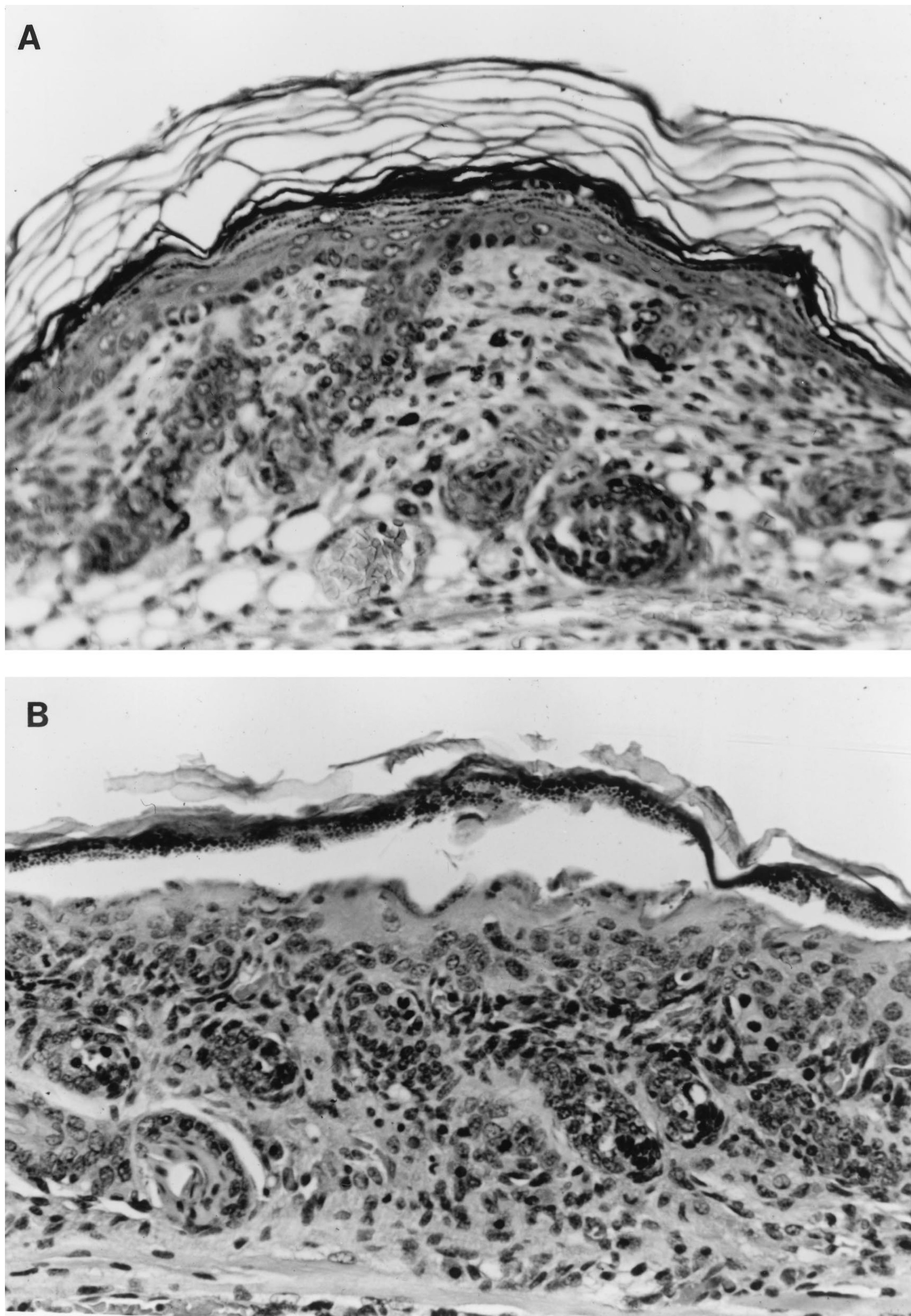

FIG. 1. Histological examination of neonatal mouse skin exposed to rETA and PBS. Sections are from the bases of the tails of 2-day-old mice sacrificed at 24 h postinjection and were stained with hematoxylin and eosin. Magnification, ×40. (A) Control mouse injected with PBS showing intact skin; (B) mouse injected with 5 μg of rETA/g of body weight showing the characteristic splitting at the stratum granulosum.

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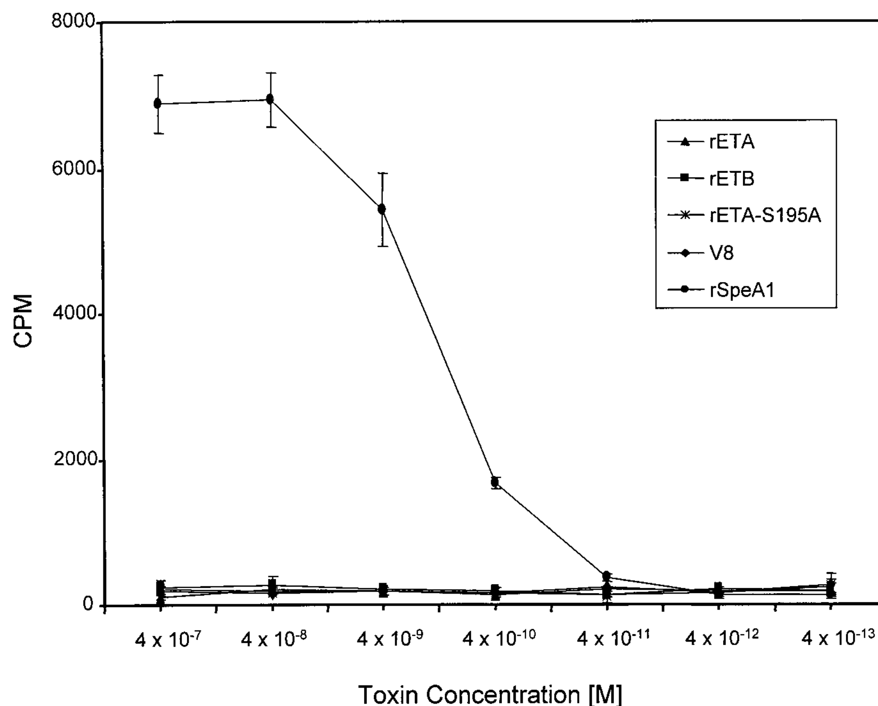

FIG. 2. Mitogenic activities of rETA, rETB, rETA-S195A, staphylococcal V8 protease (V8), and rSpeA1. Shown are the results obtained from one representative experiment. All experiments had similar results.

They conclude that the exfoliative toxins are less potent in inducing T-cell proliferation and less toxic in a rabbit model than the conventional staphylococcal superantigens.

In contrast to the results from the above-mentioned studies, others have not seen superantigenic activity with these toxins. In their 1992 report Fleischer and Bailey could not demonstrate a mitogenic response from rETA expressed in *S. aureus* (7). They conclude that the activity seen by the other groups was caused by contamination of the commercial preparations used. Cavarelli et al. reported that the purified toxin they used to generate an X-ray structure was not able to stimulate cutaneous lymphocyte-associated antigen expression in T cells (5).

Here we demonstrate that rETA and rETB expressed and purified from *E. coli* do not act as superantigens. These recombinant toxins are able to produce all the characteristic signs of SSSS in the neonatal mouse model, and the histopathology of skin samples from mice injected subcutaneously with either purified rETA or rETB is identical to that of mice injected with ETA-producing *S. aureus*. Therefore, while these toxins have slight modification at the amino-terminal end compared to wild-type ETA and ETB, they are fully active as exfoliatins. However, in our standard T-cell proliferation assay neither recombinant toxin was mitogenic. No activity was observed above the background level generated by buffer alone or by the control protein staphylococcal V8 protease. The rSpeA1 protein used as a positive control was expressed in the same *E. coli* expression system as used for the rETs and also has a slightly modified N-terminal sequence.

The X-ray structures of the exfoliative toxins do not resemble the structures of the other superantigens from gram-positive bacteria (5, 20); therefore, the exfoliative toxins do not belong to that family. From this finding, and the lack of mitogenic activity seen on our assays, we conclude that ETA and ETB are not bacterial superantigens. Admittedly, it can be argued that the fact that our recombinant toxins are modified at the amino terminus explains why they are not mitogens. Also, regarding the structural data, there are superantigens with a second activity (4, 18), and possibly the exfoliative toxins are proteases with a second activity. However, our main reason for arguing that ETA and ETB are not superantigens is that SSSS does not resemble a superantigen-mediated disease. Classic superantigen-mediated diseases, such as the toxic shock syndromes, are associated with erythematous rash, hypotension, multiorgan failure, and high mortality rates. In contrast, among young SSSS patients, mortality is low with appropriate antibiotic therapy, and systemic manifestations of the infection generally are not present, with the exception of the exfoliative rash. This rash is due to the direct effect of the toxin on the skin and does not resemble the erythematous rash of the toxic shock syndromes. Hypotension and possible organ failure can be found in SSSS only in severe cases where there are extensive areas of denuded skin with significant fluid loss or with the onset of sepsis with either *S. aureus* or a secondary infecting organism.

Mortality is associated with SSSS in the adult even with appropriate antibiotic therapy, but it is likely secondary to bacterial sepsis associated with the underlying clinical state of these patients, who are often immunocompromised, aged, or afflicted with other clinical problems, such as renal compromise.

Viewing the data as a whole, we conclude that SSSS is not a superantigen-mediated disease and that the exfoliative toxins are not superantigens. The data and the clinical picture of SSSS support our thesis that these toxins are most likely unique serine proteases which act on an unknown target in the upper epidermis.

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