Toxin Levels in Serum Correlate with the Development of Staphylococcal Scalded Skin Syndrome in a Murine Model

LISA R. W. PLANO,¹ BECKY ADKINS,² MARKUS WOISCHNIK,²
RUTH EWING,³ AND CARLEEN M. COLLINS^{2*}

*Departments of Pediatrics,¹ Microbiology and Immunology,² and Developmental Pathology,³
University of Miami School of Medicine, Miami, Florida 33101*

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Staphylococcal scalded skin syndrome (SSSS) is an exfoliative dermatitis that results from infection with exfoliative toxin-producing *Staphylococcus aureus*. SSSS is seen primarily in infants and children. Here we ask if there is a specific maturation process that protects healthy adults from this syndrome. For these studies, an active recombinant exfoliative toxin A (rETA) was used in a neonatal mouse model. A time course generated on the susceptibility to the toxin as a function of mouse age indicated that BALB/c mice developed the characteristic symptoms of SSSS until day 7 of life. Between day 7 and day 8 of life there was a dramatic decrease in susceptibility, such that mice at day 9 of life were resistant to the effects of the toxin. This time course corresponds approximately to the time needed for maturation of the adaptive immune response, and SSSS in adults is often identified with immunocompromised states. Therefore, mice deficient in this response were examined. Adult mice thymectomized at birth and adult SCID mice did not develop the symptoms of SSSS after injection with the toxin, indicating that the adaptive immune response is not responsible for the lack of susceptibility observed in the older mice. SSSS in adults is also associated with renal disorders, suggesting that levels of toxin in serum are important in the development of the disease. rETA was not cleared as efficiently from the serum of 1-day-old mice compared to clearance from 10-day-old mice. Ten-day-old mice were given repeated injections of toxin so that the maximal level of toxin was maintained for a sustained period of time, and exfoliation occurred in these mice. Thus, whereas the adaptive immune response is not needed for protection of adult mice from SSSS, efficient clearance of the toxin from the bloodstream is a critical factor.

Staphylococcal scalded skin syndrome (SSSS) is an exfoliative dermatitis characterized by the formation of large bullae and separation of extended areas of the epidermis (for a recent review see reference 10). SSSS results from infection with exfoliative toxin A (ETA) or exfoliative toxin B (ETB) producing *Staphylococcus aureus* (4, 12) and is primarily a disease of infants and children (7, 9). In children, the syndrome is associated with a trivial infective focus, 3% mortality with appropriate antibiotic therapy, and infrequent bacteremia (9). In contrast, the syndrome in adults is usually associated with significant bacteremia and a 50% mortality rate even with appropriate antibiotic therapy. SSSS in adults is associated with immunocompromised states, renal deficiencies, diabetes mellitus, and old age (7).

In 1970, Melish et al. (12) demonstrated that a mouse less than 5 days old exfoliated after injection with exfoliative toxin producing *S. aureus*, while mice more than 7 days old did not exfoliate. This information, along with the clinical observation that SSSS is usually seen in children and only rarely in adults, led us to ask if there is a specific maturation process protecting adults from the action of the exfoliative toxins. As initial studies to determine the target and activity of ETA, a more exact time course of natural protection in the neonatal mouse model was generated, and we asked if maturation of the adaptive immune response protected mice from the effects of the toxins.

Because of the link between renal-deficient adults and the development of SSSS, the toxin levels in serum of adult and neonatal mice were examined. We conclude that maturation of the adaptive immune response does not play a role in protecting mice, while efficient clearance of the toxin from the serum correlates with protection.

Susceptibility to recombinant ETA (rETA) as a function of mouse age. In the original work with the mouse model of SSSS, it was demonstrated that neonatal CD1 mice would exfoliate in response to injection with exfoliative toxin producing *S. aureus*, but that mice more than 7 days of age would not exfoliate. As a first step to determine the mode of resistance in adult mice, we wished to determine the time course of this apparent decrease in susceptibility to the toxin.

BALB/c mice, bred and housed under barrier conditions in a pathogen-free environment at the Division of Veterinary Resources at the University of Miami School of Medicine, were inoculated with rETA previously demonstrated to be active in a neonatal mouse model (14). Mice at 1 to 10 days of life were given a single subcutaneous injection at the nape of the neck with a dose of 5 µg of toxin per g of body weight and were examined at 16 h postinjection for signs of exfoliation. Gross scores were assigned to the response based on both the appearance of the skin and tactile examination as follows: 0, no obvious skin changes; 1, Nikolsky's sign (permanent wrinkling of skin after being rubbed with slight pressure); 2, bullous formation of <3 mm in diameter; 3, bullous formation of >3 mm in diameter or webbing of the skin at the hind limbs or frank exfoliation; E, expired. Mice either died during the course of the experiment or were sacrificed by hypothermia at

* Corresponding author. Mailing address: Department of Microbiology and Immunology, P.O. Box 016960 (R-138), Miami, FL 33101. Phone: (305) 243-6118. Fax: (305) 243-4623. E-mail: ccollins@med.miami.edu.

FIG. 1. Response of mice to rETA exposure as a function of age. Presented as a graph are the median gross scores at each day of life. The table presents the raw data for each point.

the end of the observation period. Starting at 4 to 5 days of life, mice develop hair, and these animals were examined at their forepaws and ears for exfoliation. Mice at day 7 of life or younger were susceptible to the effects of the toxin (Fig. 1), and all showed a gross score of 2 or 3. However, between 7 and 8 days of life there was a dramatic decrease in response to the toxin, and by day 9 of life there was no gross evidence of exfoliation.

To confirm the above findings, mice at 1 through 10 days of life were injected with toxin and prepared for histological analysis at 16 h postinjection. Minimal tactile manipulation was performed when scoring these mice prior to placement in fixative. The histopathology seen was as predicted by the gross scores. Mice 1 to 7 days old had significant clefting at the stratum granulosum when the ears and forepaws were examined. Microscopic examination of areas with hair (back and muzzle) indicated that while gross exfoliation was not apparent at these sites, significant separation at the stratum granulosum was still observed. Mice at 8, 9, and 10 days of life that did not exhibit any gross appearance of exfoliation did not have significant separation at the stratum granulosum, although minor clefts were seen on occasional views.

Mice at days 9 and 10 of life were injected with 50 µg of rETA per g of body weight in an attempt to elicit exfoliation. They were observed for gross signs and were prepared for histological evaluation as described above. There was no evidence of gross exfoliation, and only occasional small areas of separation at the stratum granulosum were detected on histological exam (data not shown).

Role of adaptive immune response in the development of SSSS. One hypothesis to explain the lack of response to rETA in mice greater than 9 days old is that these mice were being protected by the adaptive immune response. It is well established that the neonatal immune system in both humans and mice is immunologically immature (1), and therefore, there might be components of the mature immune system that protect the adult and are missing in the neonate. This hypothesis is supported by the clinical data on adult patients. When seen, adult cases of SSSS often occur in immunodeficient patients and healthy adults receiving immunosuppressive drugs. In order to determine if the adaptive arm of the immune response is responsible in some manner for the protection of adults against SSSS, we examined the effect of rETA in adult mice deficient in this response. Two types of immunocompromised adult mice were tested: (i) mice depleted of mature T cells and

(ii) SCID mice, which are lacking in both T and B cells due to an early block in maturation of bone marrow precursors.

To test the effects of a T-cell deficiency, adult BALB/c mice that had been thymectomized within the first 24 h of life as previously described (2) or CB-17 SCID mice (Taconic, Germantown, N.Y.) deficient in both mature T and B cells were examined for susceptibility to the toxin. At between 7 and 11 weeks of life these mice were shaved to expose the skin and were injected with either 5 or 50 µg of rETA or phosphate-buffered saline (PBS) (as a negative control)/g of body weight. Injection of a neonate with toxin at these concentrations would result in a gross grade 3 exfoliation by 4 to 6 h (14). Mice were evaluated and scored as described above at 16 h after injection. All mice had either no response or a minimal response (0 to 1) (Table 1). Mouse skin samples were fixed for histological examination as described above. Histological evaluation showed minimal cleavage at the stratum granulosum in both the thymectomized and the SCID mice. The same degree of clefting was noted upon evaluation of normal adult mice similarly shaved and injected with sterile PBS. These data suggest that the changes observed were an effect of shaving the skin and not a response to the toxin.

As an additional measure of the role of the adaptive arm of the immune response, we attempted to protect neonatal mice with spleen cells from adult mice. Intravenous injection of adult spleen cells into neonates results in substantial (less than 20% of total cells) colonization of the host spleen within 24 h (15) and has been shown to protect neonatal mice from lethal cytomegalovirus infection (5). BALB/c mice at day 1 of life were intravenously injected with approximately 2×10^7 adult

TABLE 1. Response of immune-deficient adult mice to rETA 16 h after toxin injection

Mouse type or description	Dose of toxin (in µg) per g of body wt	Median score ^b	No. of mice	Range of scores
SCID	5	0	4	None
SCID	50	0	4	None
Control adult BALB/c	PBS ^a	0	2	None
Thymectomized	5	1	9	0-1
Thymectomized	50	1	11	0-1
Sham thymectomized	5	0	9	None
Sham thymectomized	50	0.5	10	0-1

^a Control mice were injected with PBS instead of toxin.

^b For an explanation of the scoring system, see the text.

FIG. 2. Mean serum levels of rETA in BALB/c mice, which are 1 (shaded diamond) and 10 (■) days old, after a single toxin injection and in 10-day-old (△) mice after repeated toxin injections.

splenocytes either just prior to or 24 h before injection with various doses of rETA. Mice were observed at 2, 4, 6, 10, and 24 h for signs of exfoliation and were scored as described above. No protection against exfoliation was conferred upon neonatal mice by adult splenocytes when given either immediately before or 24 h prior to injection of rETA (data not shown).

Because adult immune-deficient mice did not respond to the toxin and replacement of mature immune components from adults did not confer protection to the neonatal mice, we conclude that the adaptive immune response is not responsible for protecting adult mice from the effects of toxin.

Role of serum clearance of rETA in the development of SSSS in 1- and 10-day-old mice. Adults with renal disorders have a higher probability of contracting SSSS than healthy adults. It is therefore possible that clearance of the toxin from the bloodstream is an important factor in the development of SSSS. To address this hypothesis, rETA levels in serum of 1- and 10-day-old mice were determined at various times postinjection by sandwich enzyme-linked immunosorbent assay (ELISA) using standard protocols and biotinylated anti-ETA immunoglobulin G raised in rabbits.

One- and 10-day-old BALB/c mice were injected in the subcutaneous tissue at the nape of the neck as described above with rETA at a dose of 10 µg per g of body weight. Sera from these mice were obtained at time points between 0 and 16 h. The sera from two mice were pooled at each time point, and the experiment was repeated once. Serum samples were diluted up to 80,000-fold in assay buffer to fall in the linear range of the standard curve for the ELISA. Measurements were made in triplicate and repeated once for each sample. Each experiment generated a similar curve for mean serum concentration, and a representative experiment is shown in Fig. 2.

In both 1- and 10-day-old mice, the toxin entered the bloodstream rapidly and was detected at the first time point taken (15 min postinjection) (Fig. 2). For the 1-day-old mice, levels of rETA in serum peaked at 120 min postinjection, with a serum area under the concentration-time curve (AUC) at 360 min of 10,645 µg · min/ml. The rETA persisted in the serum of the 1-day-old mice and could still be detected in appreciable amounts after 16 h (data not shown). In the 10-day-old mice the levels of rETA in serum peaked at 60 min postinjection, and then the levels decreased sharply. At 6 h postinjection (360

min) insignificant amounts of rETA were detected by the ELISA in the serum of the 10-day-old mice. At 360 min the AUC was 3,015 µg · min/ml. Nikolsky's sign was observed in 1-day-old mice starting at 2 to 4 h after injection of the toxin, while no signs of exfoliation were seen in the 10-day-old mice throughout the complete time course.

These data suggest that one key difference between 1- and 10-day-old mice is the ability to clear the toxin from the bloodstream. To determine if the older mice would exfoliate if levels of toxin in serum remained high, 10-day-old mice were given repeated injections of rETA and toxin levels in the blood were monitored. A dose of 10 µg of rETA per g of body weight was administered at hourly intervals starting at time zero for 5 h. Mice were observed at the forepaws, footpads, and ears for gross exfoliation, and serum samples were obtained at hourly intervals. Levels of toxin in serum at all time points were comparable to the maximum toxin levels achieved with the single injection into the 1- and 10-day-old mice (Fig. 2), and the AUC at 360 min was 15,116 µg · min/ml. Nikolsky's sign became evident by gross examination at hour 5, which corresponds to a total of 50 µg of toxin administered per g of body weight. Exfoliation did not occur in 10-day-old mice after a single injection of 50 µg of toxin per g of body weight (data not shown). Therefore, 10-day-old mice will exfoliate if toxin levels in serum are maintained.

Toxin levels in serum of 21-day-old mice showed a profile similar to that observed for the 10-day-old mice. In addition, similar clearance profiles were seen when 1- and 10-day-old mice were injected with an active site mutant form of the toxin (data not shown). These data indicate that the clearance of toxin from the serum is independent of toxin activity.

This work reopened questions initially addressed in the 1970s on the differences between neonates and adults that result in the neonatal susceptibility to SSSS. Our results indicate that mice aged 7 days or less developed the symptoms of SSSS after a single injection with purified rETA, while mice 8 days of age and older did not. There was not a subtle decrease in susceptibility with age; rather, a sharp drop-off was seen between 7 and 8 days of life. While it is established that mice less than 5 days old exfoliate in response to exfoliative toxin producing *S. aureus* and mice more than 7 days old do not, this is the first study in which a day-of-life response curve to purified toxin was generated. One hypothesis to explain the change in susceptibility to rETA is that the adaptive arm of the immune response is protecting the adult mice. The mouse adaptive immune system matures within the first 2 weeks of life, which correlates with the decrease in susceptibility. This hypothesis corresponds to the clinical observation that immunodeficient adults are significantly more likely to develop SSSS than are healthy adults. As demonstrated, however, the adaptive immune response does not play a role in protecting adult mice from the toxin.

These results address the question of the role of protective anti-ETA antibodies in human adults. Over time, healthy humans produce antibodies that recognize the exfoliative toxins. In one study, more than 50% of persons over age 10 possessed antibodies that recognize ETA (11). A conclusion drawn from this observation is that these antibodies are a factor in the protection of adults from the exfoliative toxins. It is not known if these antibodies were

raised against ETA or against a cross-reacting material. The prevalence of ETA-producing strains is low, representing only 5% of clinical isolates in some settings, and this argues against these being specific antibodies.

Despite this fact, however, anti-ETA antibodies clearly were not protecting the adult mice in the mouse model used here. The immunodeficient mice are not capable of producing antibodies, and adult immunodeficient mice were not affected by the toxin at the largest dose given (50 µg of toxin per g of body weight). The normal adult mice did not exfoliate after a single injection of this high dose of toxin. Although the normal mice would be able to generate antibodies, it is doubtful they came into contact with either *S. aureus* or ETA before they were inoculated with the toxin, as all mice used were maintained in pathogen-free conditions before inoculation. Finally, even if the normal mice had been exposed to toxin-producing *S. aureus*, there would not be sufficient time by day 9 of life to generate the antibody levels needed to provide protection.

As opposed to antibody generation or any component of the adaptive immune response, levels of toxin in serum play a clear role in the protection of adult mice. One- and 10-day-old mice were given a single injection of rETA at the same micrograms per gram of body weight. In the 10-day-old mice the maximal concentration of toxin in serum was observed at 60 min postinjection, the toxin was cleared rapidly, and exfoliation did not occur. In the 1-day-old mice, the maximal concentration of toxin in serum was seen at 120 min postinjection, the toxin was not cleared even at 16 h postinjection, and exfoliation occurred. Ten-day-old mice might not exfoliate after a single injection of toxin because they are able to clear rETA more efficiently than 1-day-old mice. The hypothesis that exfoliation is a function of toxin levels in serum was tested by giving 10-day-old mice repeated injections of toxin. The concentration of toxin in serum of these animals reached the maximal levels observed with the single injection and remained at these maximal levels. Exfoliation occurred in these animals. The correlation of exfoliation with levels of toxin in serum might reflect that a specific epidermal concentration of toxin is needed for exfoliation to occur and that, in healthy adults (represented here by 10-day-old mice), the toxin is cleared from the serum before this critical concentration of toxin can accumulate. This corresponds to the observations that adult patients are often compromised and/or renal deficient. The levels of toxin in serum for these patients would be high, and the toxin would not be cleared efficiently.

Why is the toxin cleared more rapidly in adult mice than in neonatal mice? We did not address the mechanism of the differential decrease in levels of toxin in serum. However, in 1976 Fritsch et al. (8) demonstrated that iodinated ETA appears in the urine of adult mice at high levels within 2 h postinjection. This rapid and high level of clearance was not observed with neonatal mice. Therefore, we conclude that, at least in part, the protection of adult mice is due to a more rapid renal clearance of the toxin in the adult compared to that in the neonatal mouse. The work of Fritsch et al. did not demonstrate any specific tissue accumulation of this water-soluble toxin, and the role of the liver in toxin clearance is not known.

Our present hypothesis regarding the susceptibility of

adults compared to that of neonates is the following. The epidermal target of the toxin is present in both adult and neonatal skin. A specific concentration of toxin is needed in the skin for exfoliation to occur. At toxin levels below this concentration some small patches of cleavage might occur, but these are not enough to produce the exfoliation associated with SSSS. In the neonate, toxin is produced at a small focus of infection, it gains access to the bloodstream, and it disseminates to the skin. The kidneys and possibly the liver of the neonates are not able to clear and inactivate the toxin rapidly enough to prevent the needed accumulation in the epidermis. This is not the situation with a healthy adult. The toxin concentrations in serum of a healthy adult are not maintained at the levels needed for significant amounts of the toxin to reach the skin. However, when adults are immunocompromised, septic, or renal deficient, it is possible for levels of toxin in serum to remain high and allow toxin to collect in the skin. While anti-ETA antibodies might play a role in protecting human adults, in the mouse model they clearly are not necessary to prevent exfoliation.

SSSS is characterized by a specific separation of the epidermis at the stratum granulosum. This cleavage is associated with a disruption of the desmosomes, with the surrounding cells remaining intact. Recently, ETA was demonstrated to cleave the desmosomal protein desmoglein 1 (Dsg1), a member of the cadherin family of cell adhesion molecules (3). Both ETA and ETB share amino acid identity with staphylococcal V8 protease, and significantly, this identity includes residues of the V8 protease serine-histidine-aspartate catalytic triad, a signature sequence common to serine proteases. Mutation of any one of the three amino acids in this proposed catalytic site results in an inactive toxin. Structural studies indicate that both ETA and ETB are related to the trypsin family of serine proteases (6, 13, 16, 17). Thus, after much investigation, it can be concluded that SSSS results from the activity of skin-specific serine proteases that target the desmosomal protein Dsg1.

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