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Immunological Protection of Rabbits Infected with *Staphylococcus aureus* Isolates from Patients with Toxic Shock Syndrome†

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Toxic shock syndrome toxin-1 (TSST-1) isolated from the growth medium of *Staphylococcus aureus* 1169 and 555 was used to immunize male rabbits before infection with either a TSST-1$^+$ or a TSST-1$^-$ strain of *S. aureus* isolated from cases of TSS. None of the immunized rabbits died as a result of the infections, whereas 50% of the nonimmunized rabbits infected with the TSST-1$^-$ strain, D4508, and 75% of those infected with the TSST-1$^+$ strain, 555, died. Western blots of crude extracellular protein preparations probed with sera from immunized rabbits indicated that the TSST-1$^-$ strain produces a 30,000-molecular-weight protein that cross-reacts with antiserum to TSST-1. Because both organisms caused similar diseases in rabbits, we propose to designate the cross-reacting protein as TSST-2.

Staphylococcus toxic shock syndrome (TSS) is an acute multisystemic disease characterized by fever, vomiting, diarrhea, rash, and hypotension. Most *Staphylococcus aureus* isolates from patients with menstrual TSS produce an exoprotein that has a neutral pI and an M_r of 22,000 to 24,000 and has been designated as TSS toxin-1 (TSST-1) (2). However, when *S. aureus* isolates from well-characterized, nonvaginal cases of TSS were examined, a much greater percentage was found to be nonproducers of TSST-1 (12 of 32 compared with 3 of 41 [6]). Moreover, the TSST-1$^-$ isolates were more likely to be associated with a fatal outcome. These observations led Garbe and co-workers to propose that other staphylococcal products might play a role in the pathogenesis of TSS (6).

Earlier reports from this laboratory described the use of subcutaneous infection chambers in New Zealand White rabbits which permitted a controlled, localized infection with *S. aureus* strains (3, 14). This rabbit model for TSS also has been used by others to compare different *S. aureus* strains in vivo (1, 6, 11). These studies have shown that TSS isolates are generally more virulent than non-TSS isolates, that male rabbits are more susceptible to the lethal effects of infection, and that TSST-1 is a likely virulence factor. However, Garbe et al. (6) reported that TSST-1$^-$ strains produce clinical signs and histochemical changes similar to those produced by TSST-1$^+$ strains. Because we had found that rabbits immunized with a preparation containing TSST-1 are protected from the lethal effects of infection by a TSST-1$^+$ strain (3), we sought to determine whether immunizing rabbits with TSST-1 might afford protection from the lethal effects of infection by a TSST-1$^-$ strain. This report summarizes these studies, which indicated that a TSST-1$^-$ isolate from a TSS patient produces an exoprotein that cross-reacts with antiserum to TSST-1.

All of the *S. aureus* strains used in this study were isolated from patients with TSS. Strain 555 was isolated from a 14-year-old female and provided by J. Todd (Children's Hospital of Denver, Denver, Colo.). Strain 1169, which was isolated from a menstrual TSS case, was provided by J. J. Kirkland (Procter and Gamble Co., Cincinnati, Ohio). Strain D4508, which was isolated from an 18-year-old male, was provided by A. Reingold (Centers for Disease Control, Atlanta, Ga.). All cultures were maintained on beef heart infusion agar (Difco Laboratories, Detroit, Mich.) slants at 4°C. Crude extracellular proteins were concentrated from the supernatants of overnight cultures at 37°C in beef heart dialysate medium (16) by filtration through Centricon10 microconcentrators (Amicon Corp., Danvers, Mass.). The retentates were washed with deionized water, and their protein concentration was determined by the method of Lowry et al. (8).

TSST-1$^+$ strains 555 and 1169 were grown aerobically in beef heart dialysate medium (16) with shaking for 18 h at 37°C. The extracellular proteins were harvested from the culture supernatants by precipitation with four volumes of cold absolute ethanol. A fraction enriched in TSST-1 was obtained by flatbed isoelectrofocusing as previously described (3). The neutral proteins were subjected to flatbed isoelectrofocusing for 18 h with Pharmalyte (pH 6.5 to 9.0; Pharmacia Diagnostics, Piscataway, N.J.) to produce the pH gradient. The gel was fractionated with a grid, and the proteins were eluted with water. After numerous dialyses against water, the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel fractions that displayed only a single band that comigrated with TSST-1 were pooled, lyophilized, and stored at −20°C. This preparation from strain 1169 was used to immunize rabbits in experiment 1 as described below. For experiment 2, the pooled samples from strain 555 were dissolved in 0.05 M potassium phosphate buffer (pH 6.0) containing 0.1 M NaCl, chromatographed on a TSK 2000 SW molecular sieve column (Beckman Instruments, Inc., Norcross, Ga.), and eluted with the same buffer. The major peaks were pooled, dialyzed against deionized water, lyophilized, and stored at −20°C. The TSST-1 was dissolved in phosphate-buffered saline at a concentration of 32 μg/ml, and 20 mg of Ultradex (LKB Instruments, Inc., Rockville, Md.) was added per ml of solution. The resulting suspension was divided into eight fractions and stored at −20°C until used to immunize the rabbits.

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Artificial infection chambers were implanted subcutaneously in New Zealand White rabbits as previously described (3, 14). Starting 2 weeks after surgery, the rabbits were immunized by eight weekly subcutaneous injections in two separate sites of 0.25 ml of the TSST-1 suspension.

Actively growing (log-phase) cells of *S. aureus* 555 and D4508 were harvested from dialyzed beef heart medium, washed twice by centrifugation, and suspended in 0.1 M phosphate-buffered saline (pH 7.2) to give a cell suspension of 10^9 CFU/ml. The rabbits were infected within 30 min of the time the suspensions were prepared by palpating the chamber to locate a perforation, inserting a 21-gauge needle into the chamber, and injecting 1 ml of bacterial suspension. The rabbits were observed periodically after infection, and deaths were recorded at 24-h intervals.

The Iodogen procedure of Fraker and Speck (5) was used to iodinate protein A. After the bottom of a culture tube (75 by 100 mm) was coated with 50 μg of Iodogen (Pierce Chemical Co., Rockford, Ill.), 500 μCi of carrier-free $Na^{125}I$ and 10 μg of protein A (Sigma Chemical Co., St. Louis, Mo.) were added to the tube in a total volume of 100 μl of 0.02 M sodium borate-buffered saline (pH 8.2). The reaction was allowed to proceed in an ice bath for 10 min before the contents of the reaction mixture was transferred to a Sephadex P-10 column which had been equilibrated with phosphate-buffered saline and to which 200 μg of bovine serum albumin had been added. The iodinated protein A was eluted with 10 mM phosphate-buffered saline (pH 7.2).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by using a 3% stacking gel and a 3.3 to 20% resolving gel and a discontinuous buffer as described by Tsang, Peralta, and Simons (15). The protein samples were stacked into the 0.75-mm-thick gel at 6 mA per slab. When all of the protein had entered the gel, the current was increased to 13 mA, and electrophoresis was continued until the bromophenol blue dye reached the bottom of the gel. The gel was incubated for 30 min with two changes of 200 ml of transfer buffer–0.01 M sodium borate (pH 9.2) before the proteins were transferred to diazophenylthioether paper prepared as described by Reiser and Wardale (12). The transfer was accomplished in a Transphor apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) by using a current of 0.5 A for 1 h. After transfer, the paper was incubated for 30 min at room temperature in a buffer containing 0.1 M Tris hydrochloride, 10% ethanolamine, and 0.25% gelatin (pH 9.0) to inactivate any remaining diazonium groups. The paper was rinsed with deionized water, incubated serially with antiserum and ^{125}I-labeled protein A as described by Renart et al. (13), and autoradiographed.

Double-diffusion analysis in agar was performed by the method of Ouchterlony (10). Gels were cast on glass slides (50 by 75 mm) and consisted of 0.8% agarose in 0.02 M phosphate buffer (pH 7.4). Samples (5 μl) were added to 3-mm wells, and the plates were incubated in a moist container for 24 h at room temperature. Precipitation lines were visualized by staining with 0.1% Coomassie blue in 10% acetic acid–45% absolute ethanol–45% water.

The TSST-1 preparations from both *S. aureus* 555 and 1169 were toxic to young male rabbits. Two of 10 rabbits died after their first subcutaneous injections of 8 μg of TSST-1 from strain 1169, and 4 of 16 died after their first injections and 2 of 12 died after their second injections of 8 μg of TSST-1 from strain 555. Somewhat greater toxicity was reported by Notermans and Dufrenne (9), who calculated the 50% lethal dose for TSST-1 to be 6 μg when given subcutaneously to 3.0- to 3.5-kg (body weight) rabbits.

TABLE 1. Effect of immunization with TSST-1 on young male rabbits[a]

Group (no. of rabbits)	Infecting strain	No. of deaths	
		Day 1	Day 2
Expt 1			
Immunized (4)	555	0	0
Immunized (4)	D4508	0	0
Nonimmunized (4)	555	2	1
Nonimmunized (4)	D4508	2	0
Expt 2			
Immunized (4)	555	0	0
Immunized (5)	D4508	0	0
Nonimmunized (4)	555	3	0
Nonimmunized (4)	D4508	1	1

[a] Immunized rabbits received eight weekly subcutaneous injections of 8 μg of TSST-1 prepared from culture supernatants of strains 1169 and 555 in experiments 1 and 2, respectively. All rabbits were infected with 10^9 CFU of the designated *S. aureus* strain. Deaths were recorded daily; rabbits that died in <24 h are given under day 1, and those that died in 24 to 48 h are given under day 2. No rabbits died on day 3.

However, their preparation was administered in the absence of anything that could be expected to retard the absorption of the toxin, such as the gel that we used.

Immunization with either preparation of TSST-1 was effective in affording protection from the lethal effects of infection with either *S. aureus* 555 or D4508 (Table 1). None of nine immunized rabbits died, whereas four of eight nonimmunized rabbits died after being infected with strain D4508 ($P = 0.029$, Fisher's exact test). Similarly, none of eight immunized rabbits died, whereas six of eight nonimmunized rabbits died after being infected with strain 555 ($P = 0.003$, Fisher's exact test). As was reported previously (3), the immunized rabbits appeared to be more alert and lacked the obvious signs of illness, such as nasal discharge, diarrhea, conjunctival hyperemia, and general listlessness, that were observed in the unprotected animals.

These results led us to examine preparations of extracellular proteins from the three strains by immunoblots with sera from the immunized rabbits and ^{125}I-labeled protein A as a probe. These analyses revealed a protein band in the strain D4508 channel that had an apparent M_r of 30,000 (Fig. 1). This cross-reacting band was not observed in the strain 555 channel but was seen in the strain 1169 channel. The relationship of this immunological cross-reactivity was obtained by double-diffusion analysis. A reaction of partial identity between the TSST-1 of 555 and the 30,000-molecular-weight protein of D4508 is shown in Fig. 2. This result makes it unlikely that the 30,000-molecular-weight band was the result of antibodies from a contaminant in our TSST-1 preparation. Rather, the D4508 protein most likely is related to TSST-1 and shares immunological determinants with it.

We examined other TSST-1$^-$ strains of *S. aureus* and observed similar higher-molecular-weight bands on immunoblots of their extracellular proteins (data not shown). To our knowledge, we are the fourth laboratory to report on a 30,000-molecular-weight protein associated with TSS strains. Cohen and Falkow (4) reported that *S. aureus* isolates from patients with TSS had two antigenic proteins that distinguish them from strains isolated from patients who do not have TSS. The proteins were detected on Western blots of total cellular proteins with TSS convalescent-phase serum as a probe and found to have M_rs of 30,000 and 33,000. Nakashima and Wuepper have described an

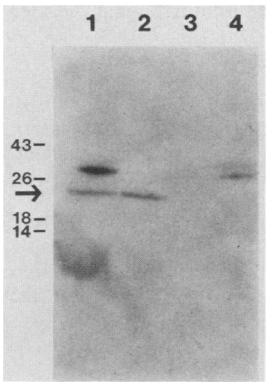

FIG. 1. Western blot analyses of *S. aureus* proteins. After being boiled in the presence of 1.7% mercaptoethanol, proteins were separated on a 3.3 to 20% polyacrylamide gel and transferred to diazophenylthioether paper. After transfer, the paper was incubated with a 1:500 dilution of anti-TSST-1 serum from experiment 2 and with ^{125}I-labeled protein A. Lanes 1, 2, and 4, Autoradiograms, from gel loaded with 66 μg of the extracellular proteins from cultures of strains 1169, 555, an D4508, respectively, per lane. Lane 3 was loaded with molecular weight standards, which included ovalbumin, alpha-chymotrypsinogen, beta-lactoglobulin, and lysozyme. The arrow indicates the TSST-1 band. Molecular weight markers (10^3) appear to the left.

exoprotein designated as TSS-1 which has a neutral pI and mitogenic properties similar to those reported for TSST-1 (H. Nakashima and K. D. Wuepper, Clin. Res. **31**:149A, 1983). Although antibodies directed against TSS-1 did not cross-react with TSST-1 isolated from *S. aureus* 1169, those researchers did not state whether they attempted to neutralize TSS-1 with antiserum to TSST-1. TSS-1 was reported first to have an M_r of 25,000 (Nakashima and Wuepper, Clin. Res. **31**:149A, 1983) and subsequently M_rs of 30,000 (Nakashima and Wuepper, Clin. Res. **31**:591A, 1983; Nakashima and Wuepper, J. Invest. Dermatol. **80**:313, 1983)

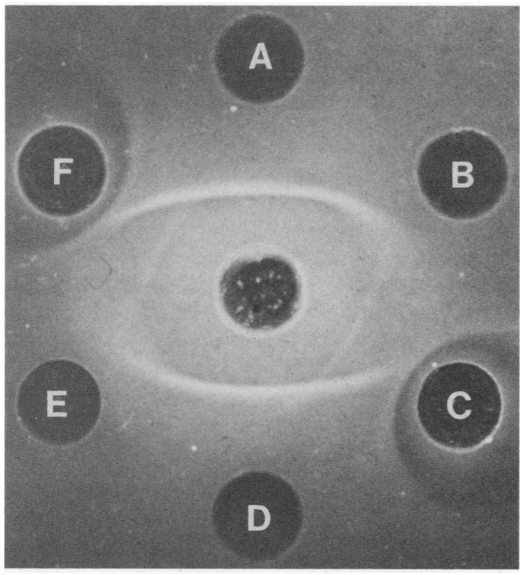

FIG. 2. Immunodiffusion comparison of TSST-1 and TSST-2. The center well contains rabbit serum (3 μl) directed against purified TSST-1. Wells A and D contain 5 μl of crude supernatant proteins (7 mg/ml) from *S. aureus* 555. Wells C and F contain 5 μl of crude supernatant protein (8 mg/ml) from *S. aureus* D4508.

and 20,000 (17). Ikejima and co-workers reported that culture filtrates of TSS-associated but not control strains contain a substance that induces interleukin-1 production by human blood monocytes (7). Moreover, the inducing activity was isolated from strain 1169 filtrates and found to reside in a protein designated as TSS toxin with an M_r of 30,000. Whether our 30,000-molecular-weight band that cross-reacted with anti-TSST-1 serum corresponds to any or all of these 30,000-molecular-weight proteins remains to be determined.

Attempts are under way to isolate the 30,000-molecular-weight protein from the extracellular proteins of strain D4508 so that we may characterize what could well be TSST-2. That rabbits immunized with TSST-1 were protected from the lethal effects of infection with a TSST-1$^-$ strain of *S. aureus* whose extracellular proteins include one that is recognized by the serum of the protected rabbit indicates that the recognized protein was responsible, at least in part, for the lethality caused by the TSST-1$^-$ strain. It is difficult to compare the relative toxicities of TSST-1 and the putative TSST-2, because *S. aureus* 555 makes only TSST-1 and has somewhat greater virulence for male rabbits than strain D4508, which makes only TSST-2 (28 of 34 compared with 17 of 25 lethal infections; $P = 0.16$, Fisher's exact test). However, TSST-1$^-$ strains are more likely to be associated with lethal infection in humans (6).

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