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Characterization of *Staphylococcus aureus* Isolates from Patients with Toxic Shock Syndrome, Using Polyethylene Infection Chambers in Rabbits

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Isolates of *Staphylococcus aureus* from patients with toxic shock syndrome (TSS) were compared with non-TSS strains of *S. aureus* with respect to their virulence in rabbits. When the organisms were injected into subcutaneous chambers (perforated polyethylene golf balls) to assess virulence, a rapid mortality was observed with TSS but not with non-TSS strains. Of 16 TSS strains, 11 caused lethal infections in 33 rabbits tested, and none of the 5 control strains caused mortality in 10 rabbits. This evidence of enhanced virulence associated with TSS strains did not appear to be associated with the size of the inoculum. In addition, strains which produced lethal infections appeared to do so despite a reduction in the size of the original inoculum during the first 24 h. All of the TSS strains and none of the non-TSS strains elaborated extracellular protein(s) with a neutral pI when grown in a dialyzed beef heart medium. No other physiological difference was noted between the TSS and non-TSS strains.

Toxic shock syndrome (TSS) is a recently recognized clinical entity which is reported primarily in young women during menses (4, 8). The symptoms of TSS include acute onset of fever, hypotension, vomiting, diarrhea, myalgia, conjunctivitis, and the development of an erythematous rash with subsequent desquamation (3, 8, 11, 12). An etiological role for *Staphylococcus aureus* was recognized in the earliest cases, but the pathogenesis of TSS remains to be elucidated. Since TSS is multisystemic and results from localized *S. aureus* infections, most attention has been directed toward the extracellular products of *S. aureus* as potential effectors of the disease. Schlievert et al. (7) reported that 28 of 28 strains from TSS patients, but only 5 of 32 control isolates, produced a substance which they designated as pyrogenic exotoxin C. This exotoxin, when recovered after differential ethanol precipitation, exhibited an apparent isoelectric point of 7.2 when focused on thin-layer polyacrylamide gels.

Bergdoll et al. (2), in a similar study, found that 61 of 65 TSS strains and only 3 of 26 control strains produced an "enterotoxin-like" protein which they designated enterotoxin F. This extracellular product, which was recovered by chromatographic procedures, was reported to have an isoelectric point of 6.8.

Despite the impressive association of these two extracellular products with TSS strains relative to non-TSS isolates, their relationship to the actual disease remains obscure. Barbour, in fact, found that TSS culture filtrates were less toxic to both chicken embryos and rabbits than non-TSS filtrates when injected intravenously (1).

The present report summarizes our results comparing TSS and non-TSS strains by using artificial infection chambers in rabbits. This animal model was selected because it permitted a controlled, localized, staphylococcal infection similar to that observed in TSS cases.

MATERIALS AND METHODS

Staphylococcal strains. Most of the TSS strains were obtained from patients' attending physicians by one of us (J.J.K.). Others were provided by J. Todd (Children's Hospital of Denver), R. Martin (Baylor Medical School), and J. Picardi (Pensacola, Fla.). Other cultures, both TSS and non-TSS, were provided by R. Anderson (Centers for Disease Control, Atlanta, Ga.). A non-TSS control isolate from a wound infection was obtained from Talmadge Memorial Hospital, Augusta, Ga., and *S. aureus* Wood 46 was obtained from the American Type Culture Collection, Bethesda, Md.

Characterization of strains. All of the *S. aureus* strains used in this study were coagulase positive, hemolytic on 5% sheep blood agar plates, grew on mannitol salt agar, and gave characteristic colonies on Baird-Parker medium. Each culture was lyophilized upon receipt and maintained on beef heart infusion agar (Difco Laboratories, Detroit, Mich.) slants at 4°C.

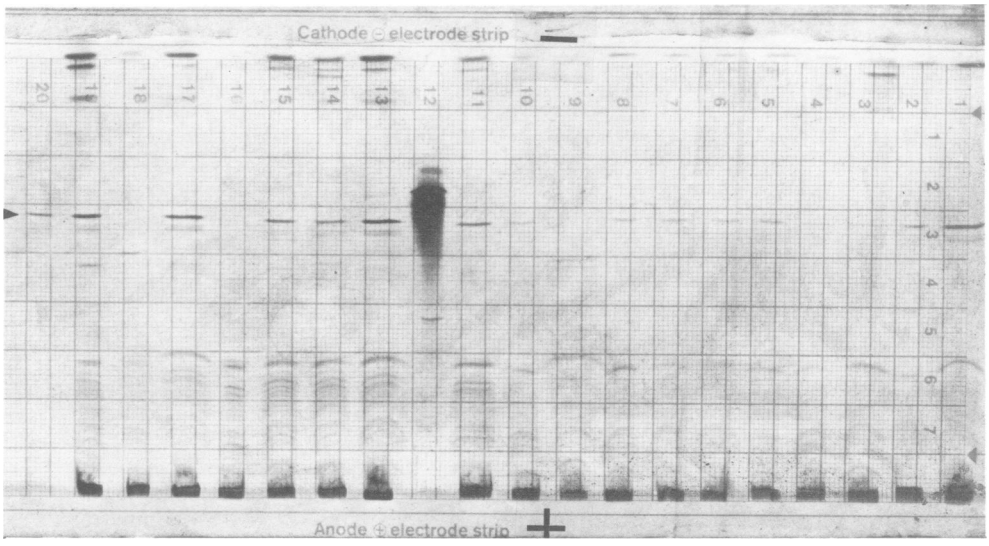

FIG. 1. Isoelectric focusing profiles of crude extracellular, alcohol precipitable proteins. Samples, 80 to 100 μg of protein, were applied near the anode, and focusing was carried out as described in the text. From left to right, the samples were from strain 755, 521, 551, 552, 555, 564, 602, 636, 638, 656, 807, crude bovine hemoglobin, 822, 828, 830, 837, 852, 1020, and 925. The rightmost channel contained 5 μg of neutral protein obtained from strain 587. Strains 638, 837, and 1020 are non-TSS strains. Arrow, proteins with a pI of 6.8 to 7.2.

Rabbits. New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with ketamine (75 mg/kg) and xylazine (7.5 mg/kg) intramuscularly. Two autoclaved polyethylene chambers (Sportsotron, Inc., Smithtown, N.Y.) were implanted subcutaneously (one on each side) in the lumbar region through a single midline dorsal incision. The rabbits were used 4 to 8 weeks after the implantation of the chambers, when the straw-colored transudate (usually about 25 ml/chamber) contained a stable leukocyte population (10).

Growth of *S. aureus* in rabbit infection chambers. A 0.5-ml amount of transudate was removed aseptically, and its leukocyte concentration was determined by using an automatic cell counter (Coulter Electronics, Inc., Hialeah, Fla.; model D2N). Log-phase cells grown in a beef heart dialysate medium (13) were washed and suspended in phosphate-buffered saline such that their concentration in the inoculum would yield the desired ratio of *S. aureus* to leukocytes (usually from 1:1 to 30:1). After inoculation, the contents of the chambers were mixed by gently drawing and expelling fluid with a syringe and a 20-gauge needle. Samples of the fluid were taken immediately after mixing and daily thereafter for plating and leukocyte enumeration.

Preparation of extracellular proteins. Each strain was grown overnight in 100 ml of a beef heart dialysate medium prepared according to the method of Watson (13). The cells were removed by centrifugation at 15,000 × *g* for 15 min, and the proteins in the supernatant solutions were precipitated with 4 volumes of absolute ethanol at −20°C. The resulting mixtures were placed at 4°C overnight, and the precipitates were collected by centrifugation and then resuspended in 3 ml of distilled water. After the preparations were dialyzed against distilled water, water-insoluble material was pelleted by centrifugation, and the supernatant solutions were lyophilized (6, 7).

Characterization of extracellular proteins by analytical isoelectric focusing. Lyophilized ethanol precipitates were dissolved in distilled water, and the protein concentrations of the solutions were determined by the method of Lowry et al. (5). Filter paper wicks containing 80 to 100 μg of protein were applied to the anode end of PAGplate gels (LKB Instruments Inc., Rockville, Md.; pH 3.5 to 9.5), and focusing was carried out at 7°C and 8 W constant power for 30 min. The wicks were removed, and focusing was continued for 75 min at 15 W constant power. Channels of the gel were divided into 5-mm sections and placed in 1.5 ml of water to determine the pH gradient. The remainder of the gel was fixed in 3.5% sulfosalicylic acid–11.5% trichloroacetic acid and stained overnight with a 0.5% Coomassie brilliant blue R-250 solution containing 0.1% cupric acetate, 10% acetic acid, and 25% isopropanol. The gels were destained with 25% ethanol–8% acetic acid. The positions of protein bands were compared with the pH gradient.

RESULTS

All of the strains used in this study were screened for the ability to produce neutral proteins during growth in dialyzed beef heart broth. Figure 1 shows the protein profiles obtained with analytical isoelectric focusing. Each of the 16 TSS strains and 3 of the 5 non-TSS strains were included on the gel shown. All of the strains produce an array of proteins, but only the TSS strains produced proteins with a pI of 6.8 to 7.2 (arrow in Fig. 1).

Aside from the fact that the polyethylene golf

TABLE 1. Staphylococcal infection of artificial chambers in rabbits with TSS and non-TSS strains[a]

Group	Strain designation	Leukocyte count (cells/ml)	Staphylococcal challenge level		Survival time
			CFU/animal	CFU/leukocyte	
Non-TSS strains, neutral protein negative (n = 5)	Wood 46	1×10^7	2.5×10^8	1.0	7 days
	1020	4×10^6	2.5×10^8	2.5	7 days
	638	2×10^6	5.0×10^8	10.0	7 days
	Wood 46	1×10^6	5.0×10^8	22.0	7 days
	638	1×10^7	7.5×10^8	3.0	7 days
	1025	2×10^6	7.5×10^8	15.0	7 days
	638	1×10^6	7.5×10^8	30.0	7 days
	1020	8×10^6	1.5×10^9	7.5	7 days
	837	8×10^6	2.5×10^9	12.5	7 days
	1020	ND	4.8×10^9		7 days
TSS strains, neutral proteins produced (n = 16)	755	6×10^5	1.5×10^7	1.0	7 days
	760	6×10^5	1.8×10^7	1.2	<48 h
	755	9×10^5	2.5×10^7	1.1	7 days
	555	5×10^5	5.0×10^7	4.0	24 h
	740	2×10^6	1.5×10^8	3.0	<48 h
	730	5×10^6	1.8×10^8	1.4	7 days
	602	3×10^6	1.5×10^8	3.0	7 days
	555	5×10^5	2.5×10^8	20.0	24 h
	945	3×10^6	2.3×10^8	3.0	7 days
	730	1×10^7	2.5×10^8	1.0	7 days
	828	2×10^6	2.5×10^8	5.0	<48 h
	557	8×10^6	5.0×10^8	2.5	7 days
	720	3×10^6	5.0×10^8	6.6	7 days
	555	1×10^6	5.0×10^8	20.0	<24 h
	720	8×10^5	5.0×10^8	28.0	<24 h
	555	8×10^5	5.0×10^8	28.0	24 h
	830	3×10^6	7.5×10^8	10.0	48 h
	852	2×10^6	7.5×10^8	15.0	7 days
	852	2×10^6	7.5×10^8	15.0	48 h
	555	1×10^6	7.5×10^8	30.0	<24 h
	555	8×10^6	7.5×10^8	38.0	72 h
	587	4×10^6	8.0×10^8	12.0	<24 h
	945	4×10^6	1.0×10^9	10.0	7 days
	564	3×10^6	1.0×10^9	13.0	7 days
	564	3×10^6	1.0×10^9	13.0	7 days
	602	3×10^6	1.25×10^9	17.0	48 h
	602	3×10^6	1.25×10^9	17.0	7 days
	852	5×10^6	1.75×10^9	14.0	7 days
	852	4×10^6	2.0×10^9	20.0	7 days
	720	ND	4.8×10^9		<24 h
	955	ND	4.8×10^9		<24 h
	950	ND	4.8×10^9		24 h
	730	ND	4.8×10^9		7 days

[a] ND, Not determined.

balls permitted the establishment of a localized staphylococcal infection in an experimental animal, we were also attracted to the use of these chambers by the previous report of Tight et al. (10), which established that the transudate in these chambers contains a stable population of both polymorphonuclear leukocytes (10%) and mononuclear leukocytes (90%) after about 30 days. Thus, if TSS strains were more virulent by virtue of an antiphagocytic mechanism, a possibility for any pyogenic coccus, the presence of granulocytes in the chamber fluids was an asset with respect to making the desired virulence comparisons between strains.

Preliminary measurements of the fluid in the subcutaneous chambers indicated that about 25 ml of transudate was present after 30 days, and this volume was subsequently assumed in calculating inoculum levels. Before actual infection of the chambers, however, a specific leukocyte count was made, and the actual inoculum was usually based on this value. Therefore, the challenge level of each infection could be expressed as colony-forming units (CFU) of *S. aureus* per leukocyte at time zero as well as CFU per animal or chamber infected.

TABLE 2. Cell populations after inoculation of infection chambers with staphylococcal strains

Group (no. of chambers)	Leukocyte count[a] (log [no./ml])			Staphylococcal count[b] (log [no./ml])		
	Time zero	24 h	48 h	Time zero	24 h	48 h
Non-TSS strains (13)	6.57 ± 0.11	7.31 ± 0.04	7.58 ± 0.10	7.37 ± 0.11	6.90 ± 0.41	6.27 ± 0.50
TSS strains, nonlethal infections (11)	6.63 ± 0.08	7.54 ± 0.13	7.70 ± 0.11	7.23 ± 0.12	5.96 ± 0.37	5.55 ± 0.52
TSS strains, lethal infections (9)	6.39 ± 0.12	7.17 ± 0.09	—[c]	7.42 ± 0.05	6.61 ± 0.31	—

[a] Immediately before the addition of *S. aureus* to the chambers (time zero) and at the other times indicated, a 0.5-ml sample of fluid was removed, and the leukocyte count was determined as described in the text.

[b] Suspensions of the desired *S. aureus* strains were injected into the chambers as described in the text. Samples (1 ml each) of the transudate were removed from the chambers at the time of initial injection (time zero) and at 24-h intervals subsequently. These were diluted and plated in Trypticase soy agar (BBL Microbiology Systems) to enumerate viable organisms.

[c] —, Too few of the rabbits with lethal infections survived for 48 h to permit sampling on day 3.

Table 1 summarizes the results of 43 infections with both TSS and non-TSS strains at a wide range of challenge levels. The infections in each group are arranged from low to high inoculum sizes at time zero. None of 10 rabbits infected with five non-TSS strains (neutral proteins not produced in vitro) at challenge levels between 1 and 30 CFU/leukocyte (2.5×10^8 to 48×10^8 CFU/animal) died within the 7 days the animals were followed. Significant mortality ($P = 0.012$ by the exact test of Fisher) was observed in 33 rabbits infected with the 16 TSS strains (isolated from TSS patients; produce neutral proteins) at a wide range of challenge levels. Over half of these rabbits died (17 of 33), and virtually all mortality occurred within the first 2 days after infection.

This evidence of enhanced virulence with TSS strains led us to sequentially monitor the populations of both bacterial cells and leukocytes in the chambers to determine whether the different strains elicited a different host response or had the ability to persist in the chamber fluids. Table 2 shows the population changes of bacteria and leukocytes from three types of infections: (i) infections with non-TSS strains, (ii) nonlethal infections with TSS strains, and (iii) lethal infections with TSS strains. As indicated, the population dynamics of the bacteria-leukocyte interaction offered no obvious indication of whether or not the infection was lethal. In each group of rabbits, there was an increase in the leukocyte count in the first 72 h, whereas there was a decrease in the staphylococcal count. Thus, the rabbits that died did so despite a reduction in the inoculum which is not statistically distinguishable from that observed in surviving rabbits.

DISCUSSION

Since our intention in this study was to determine whether TSS strains exhibited enhanced virulence for rabbits compared with non-TSS strains, we included a variety of TSS strains from several sources without regard to the severity of the clinical case from which the culture was obtained. It is apparent from Table 1, however, that some TSS strains are more virulent than others. Four of the strains (755, 730, 564, and 945) were used to infect at least two rabbits each (a total of nine) and did not cause mortality. Another strain, 555, killed each of the six rabbits in which it was used at a wide range of challenge levels. Efforts to account for these differences in virulence have not been successful. As reported by Schlievert et al. (7) and Bergdoll et al. (2), each of our TSS strains produced proteins with neutral pIs on focusing gels. We observed apparent differences in the amounts of this material produced by strains in cultures processed simultaneously, but strains producing small amounts of neutral proteins (for example, 555) were often more virulent than other strains which produced larger quantities of neutral protein (for example, 755). This apparent variability in neutral protein production in dialyzed beef heart medium has not yet been confirmed by quantitative assays, but it is of interest to note that Schlievert et al. did not observe C-toxin production by the Harrisburg strain of *S. aureus* in a 1979 report (6) but stated that the strain was C-toxin positive in a subsequent report (7). Therefore, the neutral bands we observed in our gels could be enterotoxin F, pyrogenic exotoxin C, or both, and actual production by a given strain could be highly variable and dependent on culture conditions. This fact alone could complicate a determination of the relationship of these toxins to TSS pathogenesis. However, we did find the proteins a reliable marker, and none of our control strains produced them.

The results of our infections of subcutaneous chambers with different *S. aureus* strains re-

vealed several unusual and potentially significant aspects of TSS pathogenicity. First, the size of the infecting population does not appear to be determinative with respect to virulence. We compared inoculum differences of from 1.5×10^7 to 4.8×10^9 CFU/animal without observing evidence of a dose response. This observation might be related to a recent report of a TSS case in a postoperative male patient with a staphylococcal stitch abscess which was almost clinically inapparent (9). Thus, the pathogenesis of TSS could result from relatively few organisms and a critical threshold of toxin(s).

A second interesting aspect of our strain comparisons is the indication in Table 2 that rabbit mortality occurs despite a normal leukocyte infiltration into the chambers and a decrease in the numbers of the staphylococci used to produce the infection (original inoculum). Thus, the rapid mortality observed in our rabbits is apparently not the consequence of excessive proliferation by TSS strains of *S. aureus* in the presence of polymorphonuclear and mononuclear leukocytes. We did not sample the chambers at intervals shorter than the 24 h reported in Table 2, so it can not be stated whether there are earlier growth differences between TSS and non-TSS strains after injection into the chambers. However, in vitro cultivation of the isolates in the chamber fluids did not reveal differences in growth rates between the TSS and control strains (data not shown).

Even though the reported infections in subcutaneous chambers in rabbits showed differences in virulence between TSS and non-TSS strains, an obvious limitation of these results is that mortality was the only virulence parameter actually used to distinguish the strains. All rabbits developed fevers in the first 24 h after infection with either a TSS or non-TSS strain. Some, but not all, of the rabbits that died developed diarrhea, and all rabbits observed immediately before death had labored breathing. However, some rabbits infected with TSS strains developed labored breathing during the first 48 h, appeared extremely lethargic, and did not succumb to the infection. Thus, TSS strains which may produce less drastic effects in individual rabbits would not be evident from the results we present, and this could account for the observation that 5 of the 16 TSS isolates did not produce a lethal infection in rabbits and one strain, 852, killed only one of four rabbits infected.

For this reason, studies are in progress which will attempt to compare physiological responses of rabbits infected with TSS and non-TSS isolates and will necessarily include histopathology. Protection studies are also being done to determine which of several possible extracellular products of *S. aureus* could be responsible for the noted toxicity of TSS strains.

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