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Determination by Western Blot (Immunoblot) of Seroconversions to Toxic Shock Syndrome (TSS) Toxin 1 and Enterotoxin A, B, or C during Infection with TSS- and Non-TSS-Associated *Staphylococcus aureus*

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Serum antibody responses to toxic shock syndrome (TSS) toxin 1 (TSST-1) and staphylococcal enterotoxins A, B, and C were determined by Western blot (immunoblot) analysis of acute- and convalescent-phase paired sera from 18 TSS- and 31 non-TSS-associated *Staphylococcus aureus* infections. Compared with non-TSS cases, seroconversion to TSST-1 was significantly more frequent among both menstrual (5 of 8 versus 1 of 31; $P < 0.001$) and nonmenstrual (3 of 10; $P < 0.05$) patients. Seroconversion to staphylococcal enterotoxin A was also more frequent among both menstrual (2 of 8 versus 0 of 31; $P < 0.05$) and nonmenstrual (2 of 9; $P < 0.05$) TSS patients. In general, patients with TSS associated with TSST-1-positive *S. aureus* were more likely to seroconvert exclusively to TSST-1 (4 of 12 versus 0 of 6; $P = 0.16$), whereas those associated with TSST-1-negative *S. aureus* were more likely to seroconvert exclusively to enterotoxins (3 of 6 versus 0 of 11; $P < 0.05$). Concurrent seroconversions to multiple exoproteins were more frequent among both menstrual (3 of 8; $P < 0.05$) and nonmenstrual (2 of 9; $P < 0.05$) TSS patients compared with persons without TSS (0 of 31). These data suggest but do not prove that enterotoxins (especially staphylococcal enterotoxin A) in addition to TSST-1 may be involved in both menstrual and nonmenstrual TSS. Furthermore, since exposure to multiple exoproteins is more likely to occur during TSS-associated than non-TSS-associated *S. aureus* infections, the possibility of additive or synergistic effects of these putative toxins in the pathogenesis of TSS should be further explored.

Toxic shock syndrome (TSS) associated with menstruation and tampon use (menstrual TSS) is believed to be mediated by a staphylococcal exoprotein, TSS toxin 1 (TSST-1) (1, 15). More recently, cases of TSS in men and in women not associated with menstruation are increasingly recognized (7, 12). Nonmenstrual TSS is usually associated with focal *Staphylococcus aureus* wound or soft tissue infections. Whereas vaginal *S. aureus* isolates associated with menstrual TSS usually produce TSST-1 when tested in vitro (85 to 100% of strains), nonmenstrual TSS isolates are less often positive (only 62 to 76% of strains) (7, 14). Nonmenstrual and menstrual TSS isolates also differ phenotypically in other characteristics (4), suggesting different origins or pathogenesis in these two variants of TSS. Recently, Crass and Bergdoll (5) reported that 60% of TSS-associated *S. aureus* also produced staphylococcal enterotoxin A, B, or C (SEA, SEB, or SEC, respectively) and that acute-phase sera from patients with TSS had lower levels of antibody to these enterotoxins than did healthy controls. Previous studies, however, had not compared TSS-associated with non-TSS-associated *S. aureus* infections, and paired sera (acute and convalescent phases) were examined infrequently. In the present study, we investigated the seroconversion rates to TSST-1, SEA, SEB, and SEC by Western blot (immunoblot) analysis of paired acute- and convalescent-phase sera prospectively collected from 18 menstrual or nonmenstrual TSS patients and from 31 unselected patients with culture-proven *S. aureus* infection but no clinical manifestations of TSS. These data suggest that enterotoxins, especially SEA, in addition to TSST-1 may be implicated in both menstrual and nonmenstrual TSS and demonstrate that exposure to multiple staphylococcal exotoxins was significantly more common after TSS-associated than non-TSS-associated *S. aureus* infection.

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MATERIALS AND METHODS

Study populations and sera. Acute- and convalescent-phase sera were obtained from 8 menstrual and 10 nonmenstrual TSS patients seen in Vancouver between 1980 and 1987. All fulfilled the case definition of TSS according to the Centers for Disease Control criteria (3). Acute-phase sera were obtained within 10 days of onset of illness (median of 3 days); convalescent-phase sera were obtained between 11 days and 10 months (median of 26 days). In addition, paired sera were obtained during 1986 and 87 from 31 unselected patients with culture-proven *S. aureus* infection but no clinical features suggestive of TSS. Acute-phase sera were obtained within 7 days (median of 4 days) of positive culture; convalescent-phase sera were obtained between 2 and 6 weeks after (median of 22 days). The median age, sex, clinical nature of infection, and association with TSST-1-positive or -negative *S. aureus* for these TSS and non-TSS cases are summarized in Table 1.

Western blot procedure. Partially purified TSST-1, SEA, SEB, SEC1, SEC2, and SEC3 (Toxin Technology Inc., Madison, Wis.) were electrophoresed (5 μg per sample) in 14% polyacrylamide gels containing 0.1% sodium dodecyl sulfate under nonreducing conditions in Tris-glycine buffer (pH 8.6) by the method of Laemmli (9). After electrophoresis, proteins were transferred to nitrocellulose paper by

TABLE 1. Clinical features of study patients with TSS-associated and non-TSS-associated *S. aureus* infections

Patient group (no. of men/no. of women)	Median age (range), yr	No. of patients with the following type of infection[a]:				No. of patients with TSST-1-positive *S. aureus* infection
		Tampon associated	Postsurgical	Skin and wound	Miscellaneous	
Menstrual TSS (0/8)	29 (19–44)	8	0	0	0	8
Nonmenstrual TSS (3/7)	20 (1.5–63)	0	6	4 (1)	0	4
Non-TSS associated (17/14)	52 (9–104)	0	11 (3)	14 (2)	6 (2)	8

[a] Number with bacteremia is shown within parentheses.

using a semidry electroblotter (Dimensions Laboratories) at 1.5 mA/cm^2 for 1 h according to the manufacturer's instructions. The nitrocellulose paper was then treated overnight in skim milk buffer containing 2% test serum or an appropriate control. After a wash with Tris-buffered saline containing 0.05% Tween 20, biotinylated goat anti-human immunoglobulin G (BRL Life Technologies, Inc., Burlington, Ontario, Canada) diluted 1:500 in Tris-buffered saline with 0.5% bovine serum albumin was added. The nitrocellulose strips were incubated at room temperature for 2 h on a rotating platform, washed with Tris-buffered saline containing 0.5% Tween 20 and 0.5% bovine serum albumin, and incubated for 30 min with streptavidin-horseradish peroxidase (BRL Life Technologies). Immunoreactive bands were visualized with 4-chloronaphthol substrate (BRL Life Technologies). Rabbit antisera with monospecific activity against TSST-1, SEA, SEB, SEC1, SEC2, and SEC3 (Toxin Technology Inc.) and pooled normal human serum were used in similar immunoblots to positively identify the exoprotein antigens and to assess cross-contamination or cross-reactivity of the exoprotein preparations. Preliminary studies by Coomassie and silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by immunoblots with rabbit monospecific antiserum or pooled normal human serum demonstrated excellent purity of the exoprotein preparations and absence of cross-contamination or cross-reactivity, except among SEC1, SEC2, and SEC3 when immunoblotted with pooled normal human serum, as expected. In nonreducing gels (without mercaptoethanol), the apparent molecular size of TSST-1 was 24 kilodaltons, and those of SEA, SEB, SEC1, SEC2, and SEC3 were all approximately 30 kilodaltons.

Statistical methods. Statistical comparisons between different patient groups were performed by the Fisher exact test.

RESULTS

The presence of immunoglobulin G antibodies to TSST-1, SEA, SEB, and SEC in the acute-phase sera from 8 menstrual TSS, 10 nonmenstrual TSS, and 31 non-TSS patients was first examined (Table 2). TSST-1-specific immunoglobulin G in acute-phase sera was more frequently absent from menstrual TSS patients than from non-TSS controls (6 of 8 versus 3 of 31; $P < 0.001$). Nonmenstrual TSS patients (3 of 10) were not significantly different from non-TSS controls. Rates of antibodies to SEA, SEB, and SEC among these patient groups were not significantly different. Patients who had negative antibodies in acute-phase sera but subsequently developed positive antibodies in paired convalescent-phase sera were then separately analyzed (Table 2; Fig. 1 and 2). Compared with non-TSS cases, seroconversion to TSST-1 was significantly more frequent among both menstrual TSS (62 versus 3%; $P < 0.001$) and nonmenstrual TSS (30%; $P < 0.05$) patients. Interestingly, seroconversion to SEA was also more frequent for both menstrual TSS (25 versus 0%; $P < 0.05$) and nonmenstrual TSS (22%; $P < 0.05$) patients. Seroconversion to SEB occurred in only one instance in a menstrual TSS patient who also seroconverted to TSST-1 and SEA (Fig. 1). The seroconversion rates to SEC were

TABLE 2. Antibodies to TSST-1, SEA, SEB, and SEC in acute- and convalescent-phase sera from patients with TSS-associated and non-TSS-associated *S. aureus* infection

Patient group	Toxin	No. of patients with acute- and convalescent-phase sera both positive/total	No. (%) of patients with acute-phase serum negative	No. (%) of patients who seroconverted
Menstrual TSS	TSST-1	2/8	6 (75)[a]	5 (62)[a]
	SEA	1/8	7 (87)	2 (25)[b]
	SEB	4/8	4 (50)	1 (12)
	SEC[c]	4/8	4 (50)	1 (12)
Nonmenstrual TSS	TSST-1	7/10	3 (30)	3 (30)[b]
	SEA	5/9	4 (44)	2 (22)[b]
	SEB	6/9	3 (33)	0
	SEC[c]	3/9	6 (67)	2 (22)
Non-TSS associated	TSST-1	28/31	3 (10)	1 (3)
	SEA	13/31	18 (58)	0
	SEB	21/31	10 (33)	0
	SEC[c]	10/31	21 (68)	5 (16)

[a] $P < 0.01$ compared with non-TSS patients (Fisher exact test).
[b] $P < 0.05$ compared with non-TSS patients (Fisher exact test).
[c] SEC1, SEC2, SEC3, or any combination.

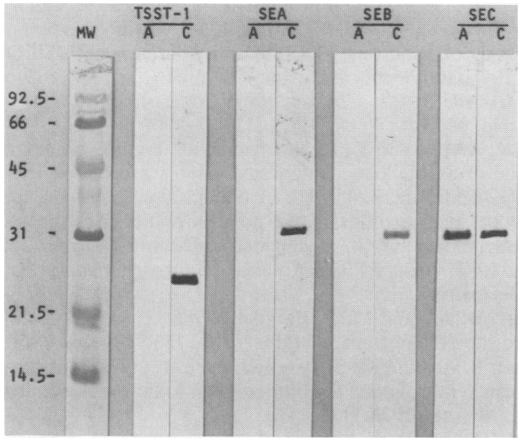

FIG. 1. Western blot analysis of acute- and convalescent-phase sera from a menstrual TSS patient. Partially purified TSST-1, SEA, SEB, and SEC were electrophoretically transferred to nitrocellulose and probed with paired acute-phase (A) or convalescent-phase (C) sera. MW, Molecular weight standards. This patient clearly demonstrated an absence of immunoglobulin G against TSST-1, SEA, and SEB in her acute-phase serum and subsequent multiple seroconversions to these exoproteins in her convalescent-phase serum.

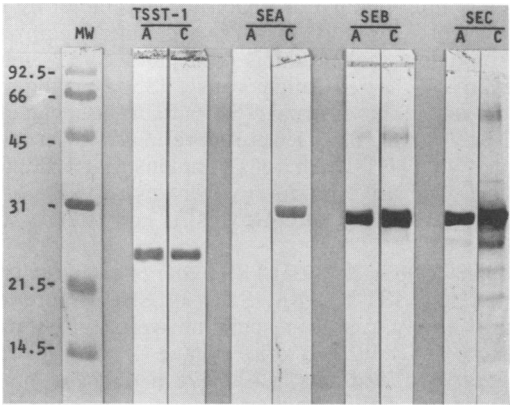

FIG. 2. Western blot analysis of acute- and convalescent-phase sera from a nonmenstrual TSS patient, demonstrating preexisting antibodies to TSST-1, SEB, and SEC in her acute-phase serum and seroconversion exclusively to SEA during convalescence.

similar in all three patient groups. The relationship of TSST-1-positive *S. aureus* and seroconversion to TSST-1 and enterotoxins was further examined (Table 3). Among the eight menstrual TSS cases, all of which were associated with TSST-1-positive *S. aureus*, five (62%) seroconverted to TSST-1; two (25%) seroconverted exclusively to TSST-1, whereas none seroconverted exclusively to enterotoxins. Among the 10 nonmenstrual TSS patients, only 4 were associated with TSST-1-positive *S. aureus*; of these, 2 (50%) seroconverted exclusively to TSST-1 and none seroconverted exclusively to enterotoxins. In contrast, among the six nonmenstrual TSS cases associated with TSST-1-negative *S. aureus*, three (50%) seroconverted exclusively to enterotoxins, whereas none seroconverted to TSST-1. Thus, TSS patients associated with TSST-1-positive *S. aureus* were more likely to seroconvert exclusively to TSST-1 (4 of 12 versus 0 of 6; $P = 0.16$), whereas TSS patients associated with TSST-1-negative *S. aureus* were more likely to seroconvert exclusively to enterotoxins (3 of 6 versus 0 of 9; $P < 0.05$). Concurrent seroconversion to multiple exoproteins (Fig. 1) was more frequent among both menstrual TSS (3 of 8; $P < 0.01$) and nonmenstrual TSS (2 of 9; $P < 0.05$) patients compared with non-TSS-associated controls (0 of 31).

DISCUSSION

Our studies of seroconversion to TSST-1 by Western blot analysis presented here confirm and extend our earlier serologic data by enzyme-linked immunosorbent assay (13) and those of Stolz et al. (17) and others (2, 3). These data lend further support to the etiologic role of TSST-1 in the pathogenesis of both menstrual and nonmenstrual TSS. Furthermore, since seroconversion rates to SEA were also significantly higher among both menstrual and nonmenstrual TSS patients compared with non-TSS-associated controls, this staphylococcal enterotoxin may also be implicated in some cases of TSS. Crass and Bergdoll (6) observed that although TSST-1 was the major exoprotein produced in TSS-associated *S. aureus* (over 90% of strains), enterotoxins (SEA and SEC) were also produced in over 60% of these isolates. Among 55 *S. aureus* isolates associated with nonmenstrual TSS, only 12 (22%) produced TSST-1 alone, whereas 34 (62%) produced SEA or SEC in addition to TSST-1, 8 (14%) produced SEB alone, and 1 produced neither TSST-1 nor enterotoxins. Schlievert (14) reported that SEB was significantly correlated with TSST-1-negative TSS-associated *S. aureus* compared with non-TSS-associated control isolates. Garbe et al. (7) and Scott et al. (16) provided evidence that TSST-1-negative strains of *S. aureus* associated with nonmenstrual TSS produced illness and death in the rabbit similarly to TSST-1-positive strains. Parsonnet et al. (10) found that TSST-1-negative TSS-associated *S. aureus* frequently produced enterotoxins in vitro, primarily SEA and SEB, and that such strains were found to be significantly more potent inducers of interleukin-1 in human monocytes, compared with TSST-1-negative non-TSS-associated control strains. These investigators further reported that purified SEA was more potent than TSST-1 in inducing interleukin-1 and causing lethality in the rabbit by constant subcutaneous infusion (8, 11). They postulated that

TABLE 3. Relation of TSST-1-positive *S. aureus* and seroconversion to TSST-1, SEA, SEB, and SEC

TSST-1 production	Patient group	No. of sera positive/total for:						
		TSST-1	TSST-1 only	Enterotoxins	Enterotoxins only	Both TSST-1 and enterotoxins	Neither TSST-1 nor enterotoxins	Multiple exoproteins
Positive	Menstrual TSS	5/8	2	3/8	0	3/8	3/8	3/8
	Nonmenstrual TSS	3/4	2	1/3	0	1/3	1/3	1/3
	Non-TSS associated	1/8	1	1/8	1	0/8	6/8	0/8
Negative	Nonmenstrual TSS	0/6	0	3/6	3	0/6	3/6	1/6
	Non-TSS associated	0/23	0	5/23	5	0/23	19/23	0/23

induction of interleukin-1 or tumor necrosis factor by TSST-1 and enterotoxins may provide a common pathway by which staphylococcal products from both TSST-1-positive and -negative *S. aureus* might cause TSS. Our finding that menstrual and nonmenstrual TSS patients associated with TSST-1-positive *S. aureus* were more likely to seroconvert exclusively to TSST-1, whereas nonmenstrual TSS patients with TSST-1-negative *S. aureus* were more likely to seroconvert exclusively to enterotoxins, is consistent with this hypothesis.

Although 7 of our 18 (39%) TSS patients failed to seroconvert either to TSST-1, SEA, SEB, or SEC, this does not necessarily imply that these patients were not exposed to these exotoxins. We and others have previously demonstrated that TSS patients may have a deficient antibody response and that this postulated deficiency could explain the high risk for recurrence in some TSS patients (2, 5, 13, 17). However, such occurrence must be rare, since only one of our nine patients who lacked TSST-1 antibody in their acute-phase sera and were infected with TSST-1-producing *S. aureus* failed to seroconvert during convalescence. Conversely, demonstration of specific antibody by the very sensitive Western blot technique does not necessarily indicate the existence of protective titers to the exoproteins in question. The seroconversions confirmed by Western blot in our patients, however, should suggest the minimum rate of exposure to these exoproteins during either TSS-associated or non-TSS-associated *S. aureus* infection. It is also of interest that concurrent seroconversions to multiple exoproteins were significantly more frequent during TSS-associated than non-TSS-associated illness. This raises the possibility that these toxins could interact additively or synergistically in vivo in producing the clinical manifestations of TSS. Crass and Bergdoll (5) noted that TSS-associated isolates producing both SEC and TSST-1 were associated with a higher case fatality rate than was any other toxin combination. Interactions of different staphylococcal toxins in vivo may also explain the variable spectrum of clinical illness in human disease and in animal models of TSS. The role of these exoproteins and their possible interactions in the pathogenesis of TSS should be further explored.

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