

Investigation by Syringe Method of Effect of Tampons on Production In Vitro of Toxic Shock Syndrome Toxin 1 by *Staphylococcus aureus*

AMY C. LEE, BARBARA A. CRASS, AND MERLIN S. BERGDOLL*

Department of Food Microbiology and Toxicology, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 22 July 1986/Accepted 7 October 1986

A syringe method was designed to test the effect of tampons on the growth of three toxic shock syndrome-associated strains of *Staphylococcus aureus* and their in vitro production of toxic shock syndrome toxin 1 (TSST-1) under different conditions. Various amounts of TSST-1 were recovered from different tampons inoculated with these strains. Generally, the addition of 10% porcine blood to the growth medium, incubation in the presence of 5% CO₂, or the combination of these two factors resulted in the stimulation of TSST-1 production.

Toxic shock syndrome (TSS), an acute multisystem disease associated with infection by certain strains of staphylococci (7, 13, 15), most frequently affects young women during their menstrual periods but can be associated with staphylococcal infections of virtually any type (13, 15). The TSS toxin 1 (TSST-1), produced by the staphylococci isolated from TSS patients, has been accepted as the major cause of TSS (2). The toxin has been shown to produce in baboons many of the clinical and laboratory manifestations of TSS (6). An association between menses-associated cases and the use of tampons has been reported (3, 5, 10, 13). Two investigations have reported on the effect of tampons on the production of TSST-1 by *Staphylococcus aureus*. Schlievert et al. (12) studied the effect of tampons in vitro on the growth of and production of TSST-1 by two *S. aureus* strains isolated from TSS patients. These authors reported that all tampons inhibited the production of TSST-1 but had little effect on growth of the staphylococci. Tierno and Hanna (14) also studied the effect of tampons on the growth of staphylococci and TSST-1 production. They reported that in the majority of cases increased amounts of TSST-1 were produced by the *S. aureus* strain used in these studies when compared with a control.

In this study, we used a syringe method (A. C. Lee and B. A. Crass, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B 27, p. 28) for determining the effect of tampons under different conditions on the growth of and TSST-1 production by three *S. aureus* strains isolated from TSS patients.

MATERIALS AND METHODS

Staphylococcal strains. Three *S. aureus* strains, FRI-1169, FRI-1183, and FRI-1187, isolated from the vaginas of TSS patients, were used. The three strains were from the culture collection of the Food Research Institute, University of Wisconsin-Madison.

Culture media. The culture medium (3 + 1), with an initial pH of 6.5, contained 3% NZ-Amine NAK (Sheffield Products, Norwich, N.Y.) and 1% yeast extract (Difco Laboratories, Detroit, Mich.) (1). Petri dishes for enumerating CFU contained 3 + 1 medium and 1.5% Bacto-Agar (Difco). Porcine blood was collected in 0.13 M sodium citrate buffer

(pH 7.4; 9 parts blood, 1 part buffer) and added when needed at a rate of 10% to 3 + 1 medium. A ready supply of human blood was not available because of the high percentage of people who have antibodies to TSST-1 (16).

Materials tested. The tampons were supplied in 1981 by the tampon manufacturers and were the tampons that were on the market at that time, except for Rely, which had been taken off the market in 1980. The major part of the work was done before 1983; experiments repeated at later dates with the tampons from the same packages gave essentially the same results as the experiments done earlier.

Assay for bacterial growth and TSST-1 production. The appropriate *S. aureus* strain was inoculated into 10 ml of 3 + 1 medium in a screw-cap culture tube and incubated at 37°C for 18 h. Fresh medium (300 ml) was inoculated with 3 ml of the culture to give a count of about 10⁷ CFU/ml (determined by plate count). Each tampon tested was weighed, and 3 ml of inoculated medium was added per g of tampon. Half of the inoculated medium was added to a 30-ml disposable syringe (with plunger removed) (Becton Dickinson and Co., Rutherford, N.J.); this was followed by the addition of the tampon and the remainder of the inoculated medium. The syringes were covered with sterilized aluminum foil and incubated in a chamber with a constant exchange of air or an air-5% CO₂ mixture at 37°C for 22 h. Culture fluids were expressed by inserting the plunger and forcing it down on the inoculated tampons. To determine the bacterial population, culture fluids were serially diluted in 0.1% peptone and surface plated on 3 + 1 medium-agar plates. Colonies were counted after overnight incubation at 37°C (18 to 20 h). The expressed fluids were centrifuged (10,000 × *g*, 10 min) to remove bacterial cells. The concentration of TSST-1 in the supernatant fluid was determined serologically by a gel diffusion method with a detection limit of 0.5 µg/ml (4). Antiserum to TSST-1 used in the assay was prepared in rabbits (9). Purified TSST-1 was prepared by column chromatography (8). All experiments were done in duplicate with the duplicates done at different times. Data were analyzed by the one-tailed Wilcoxon matched-pairs signed-rank test.

RESULTS

The amount of TSST-1 recovered per gram of tampon, the CFU per gram of tampon, and the amount of TSST-1 per 10⁹

* Corresponding author.

TABLE 1. Growth of *S. aureus* FRI-1169 and production of TSST-1 in tampons^a

Tampon	μg of TSST-1/g of tampon (log CFU/g of tampon-μg of TSST-1/10 ⁹ CFU) in 3 + 1 medium with:			
	Air	5% CO ₂	10% Blood, air	10% Blood, 5% CO ₂
Kotex security				
Regular	7.6 (10.93-0.09)	7.6 (10.23-0.44)	10.0 (8.74-18.43)	9.3 (9.10-6.0)
	1.5 (10.33-0.07)	6.3 (10.30-0.32)	1.7 (9.16-1.18)	24.0 (9.62-5.7)
Super	3.2 (10.44-0.12)	9.4 (10.09-0.75)	10.6 (8.89-13.52)	31.3 (9.22-19.29)
	1.3 (10.12-0.09)	15.3 (10.20-0.95)	4.6 (8.68-9.63)	58.6 (9.68-10.65)
Kotex stick				
Regular	37.2 (10.75-0.67)	94.0 (10.96-1.04)	55.8 (8.90-70.83)	121.2 (9.83-18.13)
	48.0 (10.65-1.07)	110.9 (10.74-2.00)	72.6 (8.75-130.56)	92.6 (10.25-5.23)
Super	9.7 (10.72-0.49)	121.4 (10.62-2.89)	50.6 (8.87-67.92)	210.1 (9.57-56.52)
	20.0 (10.56-0.54)	85.0 (10.74-1.54)	59.9 (8.56-165.52)	188.6 (10.01-18.38)
OB				
Regular	0.9 (10.88-0.01)	3.8 (10.70-0.08)	10.1 (8.18-62.7)	38.2 (9.11-29.58)
	3.1 (10.84-0.04)	2.9 (10.84-0.04)	6.6 (8.40-26.19)	39.2 (9.35-17.19)
Super	4.0 (11.03-0.04)	18.4 (10.63-0.43)	18.2 (8.15-130.0)	71.0 (9.39-28.57)
	2.5 (10.85-0.03)	15.6 (10.78-0.26)	13.5 (8.85-19.11)	77.1 (9.03-71.88)
Super-plus	11.8 (10.95-0.13)	38.7 (10.59-1.0)	37.8 (8.06-332.22)	28.7 (8.96-31.37)
	8.6 (11.01-0.08)	18.2 (10.56-0.5)	33.5 (8.88-43.70)	75.2 (9.26-44.55)
Playtex nondeodorant				
Regular	22.4 (9.43-8.3)	0.0 (7.28-0.00)	2.7 (8.15-19.77)	0.0 (7.58-0.00)
	3.2 (8.67-6.74)	30.5 (9.23-17.92)	6.7 (8.69-13.59)	5.7 (8.04-50.56)
Super	0.7 (8.12-3.88)	42.4 (9.29-12.88)	3.2 (8.10-25.73)	1.9 (7.22-115.38)
	0.8 (8.49-2.40)	10.2 (7.95-115.28)	20.1 (8.15-143.55)	10.8 (7.76-188.23)
Super-plus	53.4 (10.08-4.55)	155.0 (9.71-30.23)	76.4 (9.01-61.8)	73.0 (8.73-135.42)
	35.0 (9.57-9.45)	1.1 (6.62-258.06)	75.6 (9.14-54.49)	39.8 (9.07-33.67)
Playtex deodorant				
Regular	0.0 (8.44-0.0)	0.0 (7.44-0.0)	1.6 (8.52-4.62)	1.9 (8.03-18.29)
	0.0 (9.32-0.00)	0.0 (7.16-0.00)	0.0 (8.23-0.00)	0.7 (7.81-10.2)
Super	0.6 (9.28-0.25)	5.9 (8.28-31.43)	7.6 (8.54-8.75)	2.4 (7.55-66.77)
	0.6 (9.18-0.29)	4.7 (8.02-44.71)	1.2 (7.94-14.28)	0.6 (7.6-16.13)
Super-plus	3.9 (9.39-1.62)	7.4 (8.75-13.04)	6.8 (9.10-70.35)	6.9 (7.04-636.36)
	0.7 (8.76-1.05)	0.7 (7.80-10.87)	0.0 (7.79-0.00)	13.7 (8.64-31.43)
Pursettes				
Regular	33.0 (10.80-0.53)	146.3 (10.70-2.92)	35.6 (8.71-70.35)	175.1 (9.71-34.09)
	35.5 (10.71-0.71)	151.0 (10.85-2.14)	39.8 (9.18-26.15)	187.2 (9.93-25.49)
Super-plus	13.0 (10.85-0.18)	51.1 (10.81-0.79)	20.2 (7.81-309.23)	66.2 (9.14-48.23)
	18.3 (10.69-0.37)	53.8 (10.94-0.61)	18.2 (8.59-46.30)	40.7 (8.87-55.10)
Rely				
Regular	86.3 (10.62-2.07)	138.7 (10.27-7.42)	78.5 (10.1-6.19)	278.2 (9.67-58.07)
	162.4 (10.81-2.51)	185.9 (10.33-8.78)	46.9 (9.71-9.18)	297.1 (10.21-18.5)
Super	89.8 (10.53-2.64)	144.8 (10.24-8.37)	82.9 (10.02-7.85)	293.4 (9.88-38.37)
	153.1 (10.81-2.36)	158.2 (10.44-5.75)	25.9 (9.64-4.75)	280.5 (10.11-21.56)
Tampax				
Regular	4.9 (10.93-0.06)	15.7 (10.9-0.2)	7.4 (7.61-177.78)	28.8 (8.65-146.67)
	5.9 (10.79-0.10)	16.8 (10.84-0.24)	5.8 (8.67-12.38)	27.5 (8.82-77.27)
Slender regular	2.7 (10.24-0.16)	10.3 (10.53-0.3)	1.6 (8.59-4.25)	40.6 (8.85-57.83)
	3.5 (10.18-0.25)	23.8 (10.39-0.96)	7.0 (8.53-20.47)	51.0 (8.89-68.18)
Super	7.4 (11.03-0.07)	20.9 (11.04-0.19)	26.7 (7.81-413.19)	45.0 (8.84-64.44)
	13.4 (10.86-0.17)	15.4 (10.59-0.4)	13.1 (8.67-27.5)	33.6 (8.67-72.41)
Super-plus	35.3 (9.82-5.39)	109.3 (9.27-58.93)	4.3 (7.91-54.39)	15.4 (7.86-212.96)
	58.7 (9.91-7.29)	124.7 (9.30-62.50)	5.8 (7.53-169.39)	23.0 (8.42-87.50)
Control^b	0.0 (9.21-0.00)	1.2 (9.80-0.75)	1.3 (8.02-15.89)	9.4 (8.95-10.56)
	0.0 (9.11-0.00)	0.0 (8.78-0.00)	0.5 (8.50-1.89)	2.6 (8.76-4.56)

^a Incubated at 37°C for 22 h. Two sets of data are shown for each tampon, corresponding to results from duplicate experiments.

^b 9 ml of inoculated medium in syringe [micrograms of TSST-1 per milliliter (log CFU per milliliter-micrograms of TSST-1 per 10⁹ CFU)].

CFU are given in Table 1. Incubation under 5% CO₂ resulted in essentially equivalent growth to that obtained with air, but in general more TSST-1 was produced with 5% CO₂ ($P < 0.00003$). The addition of blood to the medium resulted in a decrease in growth of as much as one to two logs. However, the amount of TSST-1 produced was higher in medium with blood ($P < 0.0089$), accompanied by a very pronounced increase in micrograms of TSST-1 per CFU. Incubation under 5% CO₂ in the blood medium also resulted in significant increases in TSST-1 production when compared with incubation in air alone ($P < 0.00003$). The amount produced

per CFU was much higher than that produced under 5% CO₂ without blood.

The amounts of TSST-1 obtained with *S. aureus* strains FRI-1183 and FRI-1187 paralleled those obtained with strain FRI-1169 and were proportional to the amounts produced by the three strains in aerated cultures (2 to 3 μg/ml versus 12 μg/ml) (results not shown).

DISCUSSION

Although the association of the use of tampons with the occurrence of TSS in menstruating women has been re-

ported (5, 10, 13), it has not been determined how tampons may be involved. The syringe method described here was an attempt to determine what some of the in vitro factors are that influence growth of staphylococci and toxin production in the presence of tampons. The syringe is similar in size to the syngyna used by the tampon manufacturers to measure the fluid capacity of tampons and prevents undue expansion of the tampons when fluid is added. This is considered important because in the in vivo situation the tampons are placed in a restricted area and are unable to expand to their full capacity of fluid uptake. In addition, the narrow diameter and small size of the syringe limit the amount of air available to the organisms. We added 3 ml of inoculated medium per g of tampon because it was less than the fluid capacity of the least absorbent tampon (4 ml/g for Kotex stick regular) in the 30-ml syringe. All of the fluid was taken up by the tampons, resulting in the growth of the staphylococci in close contact with the materials from which the tampons were made.

The control we used was the incubation of 9 ml of inoculated medium in a 30-ml syringe along with the syringes containing the tampons. Very little TSST-1 was produced because the staphylococci were growing in a static un-aerated culture medium, which is not conducive to good toxin production. Placement of a tampon in the syringe disperses the medium over a large surface area, thus exposing the organisms to the available air in the tampons.

S. aureus is facultative; however, it grows better and produces more TSST-1 under aerobic conditions than under anaerobic ones (11). The amount of oxygen available to the staphylococci in the syringe method is limited, yet a relatively large amount of TSST-1 was produced in many instances, particularly with the larger tampons. The fact that somewhat more air would be present in the larger tampons may account for this. Wagner et al. (17) showed that the O₂ tension in a woman's vagina rose to almost atmospheric levels with the introduction of a tampon. Even so, the amount of air that would be present in tampons is limited, which might indicate that only a relatively small amount is required.

The experiments that were conducted under CO₂ indicated that CO₂ also is important during incubation (Table 1). Incubation with the 3 + 1 medium under CO₂ resulted in at least a twofold increase in TSST-1 production without an accompanying increase in growth. Incubation under 5% CO₂ of the tampons to which the blood medium had been added resulted in increased growth and production of the largest amount of TSST-1 for most of the tampons.

In an attempt to make the medium more comparable to menstrual fluids, blood was added to the 3 + 1 medium for some of the experiments. This addition resulted in an increase in the production of TSST-1, with the exception of Rely and Tampax super-plus tampons (Table 1). The increase in toxin production was accompanied by a decrease of up to two logs in staphylococcal growth, with the result that the productivity (toxin per 10⁹ CFU) was greatly increased in most instances.

The chemical nature of the materials used in tampons may be important in the stimulation of the production of TSST-1. Tampons that are made from polyacrylate have a high fluid capacity and have been implicated in TSS (13); however, not as much TSST-1 was produced with these tampons as with some of the cotton-rayon tampons. They do have a marked buffering effect in that the pH of the fluid expelled from them after the incubations was 6.9 to 7.4, depending on the type of experiment, versus pH 8.0 to 8.8 of the fluids from the other tampons. The buffering effect by these tampons may have

been the reason for the slower growth of and lower TSST-1 production by the staphylococci; however, the effect in vivo may be very different because the major growth area is in all probability at the interface of the tampon and the vaginal wall.

Rely tampons were made from small pieces of polyester foam and carboxymethylcellulose chips which were placed in a bag. The results of our testing by the syringe method showed that Rely tampons were among the leaders in the quantities of TSST-1 recoverable. Experiments we did with the foam and the carboxymethylcellulose chips showed that the foam stimulated TSST-1 production and the carboxymethylcellulose chips did not. Although the polyester foam may be the major factor in the stimulation by Rely tampons, the tampon bag, the physical construction of the tampon, or any treatments the foam may have received may contribute also to the stimulation of TSST-1 production. In any event, the differences in TSST-1 production with the different brands appear to be related, at least in part, to the materials from which they are made. The only apparent difference between Playtex deodorant and nondeodorant tampons is the deodorant solution applied, yet the deodorant tampons did not stimulate the production of TSST-1 as did the nondeodorant ones. Apparently, the inhibition in toxin production was due to the presence of the deodorant solution. If the major area of growth of the staphylococci in vivo is at the interface of the tampon and the vaginal wall, it is likely that the deodorant would be removed from the growth area by the influx of the menstrual fluids into the tampon. Thus, one would expect in this case for the in vivo results to be different from those obtained with our method.

The syringe method is not a duplicate of the in vivo situation; however, the following conclusions can be drawn from its use in the in vitro study of tampons: (i) the presence of O₂ does result in an increase in growth and a large increase in the production of TSST-1; (ii) incubation under 5% CO₂ in a restricted system results in increased production of TSST-1; (iii) the presence of blood in the medium, particularly with incubation under 5% CO₂, results in an increase in the production of TSST-1 in most cases; and (iv) the composition of the tampons and the treatments they receive may have an effect on the production of TSST-1.

ACKNOWLEDGMENTS

We acknowledge the help of the other members of the toxic shock syndrome research team, Raoul F. Reiser and Ruth N. Robbins.

The investigation was supported by the Procter & Gamble Co., Personal Products of Johnson and Johnson, Kimberly-Clark Corp., Tambrands, and the College of Agricultural and Life Sciences of the University of Wisconsin-Madison.

LITERATURE CITED

1. Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis. 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock syndrome *Staphylococcus aureus* isolates. *Lancet* i:1017-1021.
2. Bergdoll, M. S., and P. M. Schlievert. 1984. Toxic shock syndrome toxin. *Lancet* ii:691.
3. Davis, J. P., P. J. Chesney, P. J. Wand, and M. LaVenture. 1980. Toxic shock syndrome: epidemiological features, recurrence, risk factors, and prevention. *N. Engl. J. Med.* 303:1429-1435.
4. Kato, E., M. Khan, L. Kujovich, and M. S. Bergdoll. 1966. Production of enterotoxin A. *Appl. Microbiol.* 14:966-972.
5. Osterholm, M. T., J. P. Davis, R. W. Gibson, J. S. Mandel, L. A. Wintermeyer, C. M. Helms, J. C. Forfang, J. Rondeau, J.

- Vergeront, and the Investigation Team. 1982. Tri-state toxic shock syndrome study. I. Epidemiologic findings. *J. Infect. Dis.* **145**:431-440.
6. Quimby, F. W., and H. T. Nguyen. 1985. Animal studies of toxic shock syndrome. *Crit. Rev. Microbiol.* **12**:1-44.
 7. Reingold, A. L., K. N. Shands, B. B. Dan, and C. V. Broome. 1982. Toxic-shock syndrome not associated with menstruation: a review of 54 cases. *Lancet* **i**:1-4.
 8. Reiser, R. F., R. N. Robbins, G. P. Khoe, and M. S. Bergdoll. 1983. Purification and some physicochemical properties of toxic shock toxin. *Biochemistry* **22**:3907-3912.
 9. Robbins, R. N., and M. S. Bergdoll. 1984. Production of rabbit antisera to the staphylococcal enterotoxins. *J. Food Prot.* **47**:172-176.
 10. Schlech, W. F., III, K. N. Shands, A. L. Reingold, B. B. Dan, G. P. Schmid, N. T. Hargrett, A. Hightower, L. A. Herwaldt, M. A. Neill, J. D. Band, and J. V. Bennett. 1982. Risk factors for development of toxic shock syndrome: association with a tampon brand. *J. Am. Med. Assoc.* **248**:835-839.
 11. Schlievert, P. M., and D. A. Blomster. 1983. Production of staphylococcal pyrogenic exotoxin type C: influence of physical and chemical factors. *J. Infect. Dis.* **147**:236-242.
 12. Schlievert, P. M., D. A. Blomster, and J. A. Kelly. 1985. Toxic shock syndrome *Staphylococcus aureus*: effect of tampons on toxic shock syndrome toxin 1 production. *Obstet. Gynecol.* **64**:666-670.
 13. Shands, K. N., G. P. Schmid, B. B. Dan, D. Blum, R. J. Guidotti, N. T. Hargrett, R. L. Anderson, D. L. Hill, C. V. Broome, J. D. Band, and D. W. Fraser. 1980. Toxic-shock syndrome in menstruating women: association with tampon use and *Staphylococcus aureus* and clinical features in 52 cases. *N. Engl. J. Med.* **303**:1436-1442.
 14. Tierno, P. M., Jr., and B. A. Hanna. 1985. *In vitro* amplification of toxic shock syndrome toxin-1 by intravaginal devices. *Contraception* **31**:185-194.
 15. Todd, J., M. Fishaut, F. Kapral, and T. Welch. 1978. Toxic-shock syndrome associated with phage-group-I staphylococci. *Lancet* **ii**:1116-1118.
 16. Vergeront, M., S. J. Stotz, B. A. Crass, D. B. Nelson, J. P. Davis, and M. S. Bergdoll. 1983. Prevalence of serum antibody to staphylococcal enterotoxin F among Wisconsin residents: implications for toxic-shock syndrome. *J. Infect. Dis.* **148**:692-698.
 17. Wagner, G., L. Bohr, P. Wagner, and L. N. Peterson. 1984. Tampon-induced changes in vaginal oxygen and carbon dioxide tensions. *Am. J. Obstet. Gynecol.* **148**:147-150.