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Production of Toxic Shock Syndrome Toxin 1 by *Staphylococcus aureus* as Determined by Tampon Disk-Membrane-Agar Method

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The influence of 17 commercially available tampons on production of toxic shock syndrome toxin 1 (TSST-1) by *Staphylococcus aureus* was investigated by using a tampon disk method. Filter membranes overlaying agar medium (with or without blood) in small petri dishes were spread inoculated with a TSST-1-producing strain of *S. aureus*. Disks cut from unrolled tampons were pressed and laid on the inoculated membranes; incubation was for 19 h at 37°C with 5% CO_2 in air. CFU on the membranes and in the disks were enumerated, and the presence of TSST-1 in the disks and in the agar layers was determined. Tampons made of different materials supported characteristic levels of cell growth and toxin production in the tampon. Colonization of the interface surface of the tampon disks was heavy. The number of CFU extracted from the tampon disks ranged from 5×10^{10} to 82×10^{10}. There was little variation in the CFU recovered from the membranes ($[1.9 \pm 0.4] \times 10^{11}$). Sixty to 170 μg of TSST-1 was recoverable from the agar, with an additional 10 to 90 μg recoverable from tampon disks, depending on the type of tampon disk. The amount of toxin in the agar layer from the various tampon disks was relatively constant and indicated an important contribution of toxin from vaginal *S. aureus* cells not growing in the tampon. The main role of tampons in toxic shock syndrome may be that of providing a fibrous surface for heavy colonization and sufficient air for TSST-1 production.

The use of tampons has been associated with menstrually occurring toxic shock syndrome (TSS) (2, 5, 8, 12), a severe and sometimes fatal multisystem disease associated with infection or colonization by *Staphylococcus aureus* (14). The disease has been associated with the production of a toxin by the staphylococci isolated from TSS patients (1, 11) which has been given the name TSS toxin 1 (TSST-1) (M. S. Bergdoll and P. M. Schlievert, Lancet ii:691, 1984). Although tampons have been associated with the occurrence of menstrual TSS, their role has yet to be completely elucidated. Two investigations of the in vitro influence of tampons on staphylococcal cell growth and production of TSST-1, in which culture and a tampon were introduced into an Erlenmeyer flask and either shaken (10) or held stationary (13), have resulted in conflicting conclusions. A third investigation (4) used a syringe method to mimic in vivo limitation of tampon swelling. In this method, each tampon, housed in the barrel of a syringe, contained subsaturation volumes of inoculated medium. Cell growth and toxin production which took place mainly within the tampon were affected dissimilarly by different tampons, with a broad range in toxin output.

In this communication, we report the effect of commercially available tampons on TSST-1 production in a disk-membrane-agar (DMA) method, with incubation at 37°C for 19 h under 5% CO_2 in air.

MATERIALS AND METHODS

TDs. This study included 17 tampons of different sizes and types from four major manufacturers; all were commercially available in the spring of 1984. The different sizes of tampons were included in this investigation even though the method was not designed to test this parameter. The tampons were divided into three groups according to composition: (i) cotton or rayon or both, (ii) polyacrylate, and (iii) polyacrylate with deodorant. The tampons were aseptically unrolled, held flat between sterile 47-mm Gelman cellulose absorbent pads, and cut to size. The residual folding patterns of the tampons were greatly reduced by pressing the tampon disks (TDs) under 3,348-lb/in^2 pressure in a modified Dennison 4.5-ton (4,081.9-kg) press. This was done primarily to duplicate the compactness of the original tampons as well as to obtain reasonable uniformity in the disks so that good contact with the inoculated membrane could be achieved. The uptake of moisture resulted in the expansion of the disks similar to the expansion of the intact tampons upon their coming in contact with moisture.

Staphylococcal strain. *S. aureus* strain FRI-1169, a vaginal TSS isolate that produces relatively large amounts of TSST-1 under laboratory conditions, was used. Additional strains were not tested because Lee et al. (4) showed in the syringe method that *S. aureus* strains that produced differing amounts of TSST-1 gave results proportional to the amount of TSST-1 produced.

Growth media. The media consisted of 3.7% brain heart infusion (BHI) plus 1% yeast extract and 1.2% Bacto-Agar (Difco, Detroit, Mich.), with or without the addition of citrated whole rabbit blood (10%, by volume).

DMA method. A 10-ml portion of agar medium in the bottom of a glass petri dish (60 by 15 mm) was overlaid with a Gelman GN-6 0.45-μm filter membrane (Gelman Sciences, Inc., Ann Arbor, Mich.) and spread inoculated with 0.05 ml (approximately 10^9/ml) of an overnight still culture of *S. aureus* FRI-1169. The TD was laid on the membrane and gently pressed down for uniform contact with the inoculated membrane. The petri dish was covered with a Falcon plastic no. 1007 lid (Becton Dickinson Labware, Oxnard, Calif.), put into a humidified chamber (Billups Rothenberg modular incubator chamber; Vanguard International Inc., Neptune, N.J.), and flushed with 5% CO_2 in air for 0.5 h before being sealed and incubated at 37°C for 19 h. An expanded representation of the petri dish arrangement is shown in Fig. 1. After incubation, the TD and membrane were extracted by

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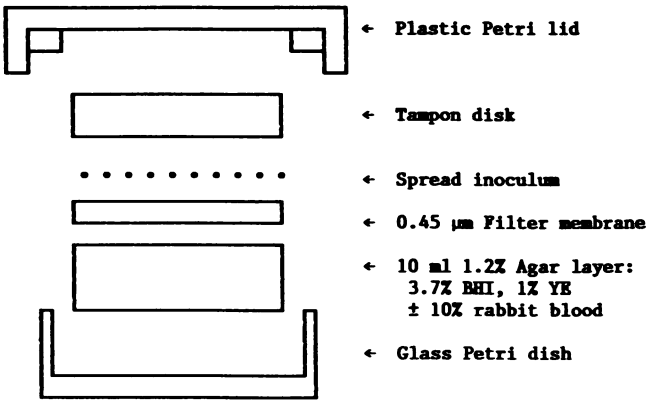

FIG. 1. Expanded view of a petri dish (60 by 15 mm) and contents as used in the DMA method. YE, Yeast extract.

massage in separate 18-oz. (510-g) Whirl-Pac (NASCO) bags containing 4 ml of 0.02 M phosphate-buffered saline, pH 7.4. A second wash of 3 ml of phosphate-buffered saline was added directly into the TD and expressed into the first extract.

CFU. Plate count agar (Difco) was used for enumeration of colonies in the TD and membrane extracts.

TSST-1. For determination of the toxin content of the agar layer, the volume of the agar was restored to 10 ml with phosphate-buffered saline, the agar plus phosphate-buffered saline was homogenized with a homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.), and the agar was removed by centrifugation. The TSST-1 concentrations of the centrifuged TD extract and agar homogenate were quantitatively determined by the single gel diffusion tube method (3), using anti-TSST-1 prepared in rabbits (7). The toxin recovered from the TDs was calculated by multiplying the TSST-1 concentration of the TD extract by the extract volume. For the total toxin in the agar layer, the TSST-1 concentration of the decanted centrifuged agar homogenate was multiplied by the total volume of the agar layer (10 ml). Replicate assays were done on different days with three replicates per sample.

Controls. An inoculated membrane without any disk served as a measure of the amount of toxin produced under favorable conditions of aeration and nutrition. A Gelman absorbent cellulose pad which provided relatively poor conditions for *S. aureus* colonization was used as a negative control.

RESULTS

BHI agar with 10% blood. Results of growth and TSST-1 production by *S. aureus* FRI-1169 when 10% blood was added to BHI agar are given in Table 1. There was heavy colonization of *S. aureus* at the interface of the disk and the membrane as observed by dense orange colonies, with little

TABLE 1. Growth of *S. aureus* FRI-1169 and production of TSST-1 as determined by the DMA method[a]

Tampon group	Wt (g)	μg of TSST-1			% in agar	CFU, 10^{10}			% in disk	μg of TSST-1/ 10^9 CFU
		Agar	Disk	Total		Membrane	Disk	Total		
1[b]										
KScR	1.4	140	44	184	76	22	12	34	35	0.54
KScS	1.7	136	40	176	76	20	5	25	20	0.70
KStR	1.5	163	97	260	61	20	59	79	75	0.33
KStS	1.9	137	110	247	55	23	43	66	65	0.37
OBR	1.2	173	46	219	78	11	67	78	86	0.28
OBS	1.6	152	45	197	76	18	80	98	82	0.20
OBS+	2.4	92	29	118	78	26	52	78	67	0.15
TR	1.3	130	60	190	68	18	64	82	78	0.23
TSlR	1.2	120	68	188	63	20	82	102	80	0.18
TS	1.6	108	58	166	64	13	37	50	74	0.33
2[c]										
TS+	2.7	67	64	131	51	13	5	18	28	0.73
PR	1.3	160	75	235	68	21	10	31	32	0.76
PS	1.9	136	58	194	70	17	6	23	26	0.89
PS+	1.8	130	68	198	65	20	6	26	23	0.76
3[d]										
PDR	1.3	65	9	74	88	19	17	36	47	0.21
PDS	1.9	57	12	69	82	21	10	31	32	0.22
PDS+	1.9	71	13	84	86	16	20	36	56	0.23
Control[e]										
G Pd	0.5	6	0	6	100	6	8	14	57	0.04
0		280		280	100	59		59		0.47

[a] Blood was incorporated into the BHI-yeast extract agar layer.
[b] Group 1, Cotton or cotton-rayon tampons: KScR, Kotex Security Regular; KScS, Kotex Security Super; KStR, Kotex Stick Regular; KStS, Kotex Stick Super; OBR, OB Regular; OBS, OB Super, OBS+, OB Super Plus; TR, Tampax Regular; TSlR, Tampax Slender Regular; TS, Tampax Super.
[c] Group 2, Rayon polyacrylate: TS+, Tampax Super Plus; PR, Playtex Regular; PS, Playtex Super; PS+, Playtex Super Plus.
[d] Group 3, Rayon polyacrylate deodorant: PDR, Playtex Deodorant Regular; PDS, Playtex Deodorant Super; PDS+, Playtex Deodorant Super Plus.
[e] Control: G Pd, Gelman pad; 0, no disk or pad.

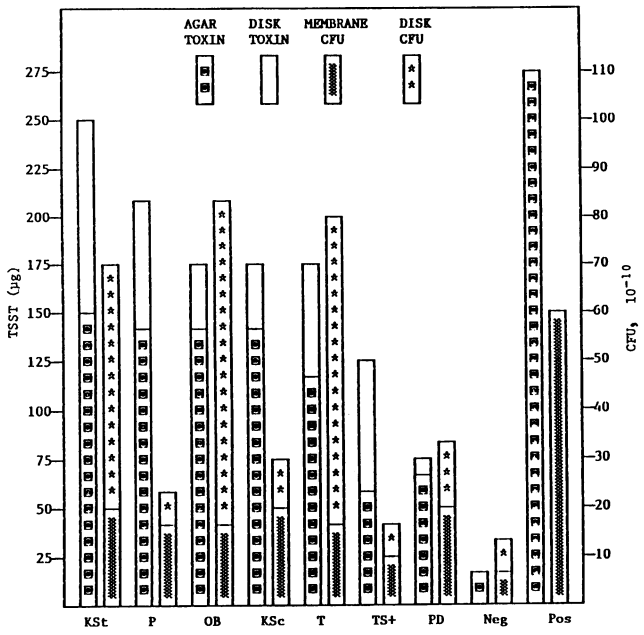

FIG. 2. Distribution of TSST-1 in the DMA method. Composite values are given for all tested sizes of tampons of the same composition and construction. KSt, Kotex Stick Regular and Super; P, Playtex Non-deodorant Regular, Super, and Super Plus; OB, OB Regular, Super, and Super Plus; KSc, Kotex Security Regular and Super; T, Tampax Regular, Slender Regular, and Super (these have similar composition and the same construction); TS+, Tampax Super Plus; PD, Playtex Deodorant Regular, Super, and Super Plus.

or no colonization visible elsewhere. The distribution of CFU between the membrane and the disks is given in Table 1 and Fig. 2. The overall average CFU on the membrane for each of the three groups was $(1.9 \pm 0.4) \times 10^{11}$; however, there was considerable variation in CFU associated with the disks for the three groups, with an average of $(6.1 \pm 1.6) \times 10^{11}$ for the cotton or rayon tampons, $(7.5 \pm 3) \times 10^{10}$ for the polyacrylates, and $(1.5 \pm 0.8) \times 10^{11}$ for the polyacrylates with deodorant.

TSST-1 production. The amount of toxin produced increased with all tampons when blood was added to the BHI agar medium, with an average of 42% over that without the addition of blood (data not shown). Decreasing amounts of TSST-1 were recovered from the agar layer as the tampon size increased. The average value for all tampons in one group is an adequate representation of the effect of the composition of the tampons in that group. The average amount of TSST-1 in the agar layer for the cotton or rayon tampons (135 ± 24 μg) was only slightly different from that for the polyacrylate tampons (144 ± 30 μg), but the amount for the polyacrylate tampons with deodorant was greatly reduced (65 ± 10 μg). The amounts present in the agar layer for the three groups represented 69, 68, and 87% of the total TSST-1 (agar layer plus disk), respectively. In all but two cases, the variation in total toxin was due to differences in the toxin associated with the disks.

Tampon additives. The effects of Aqualon (Hercules, Inc., Wilmington, Del.), one surfactant, and one deodorant used in tampon manufacture are given in Table 2. The presence of Aqualon in the tampons appeared to have little or no effect on growth and TSST-1 production by the *S. aureus* strain used. The surfactant resulted in a decrease in CFU recovered from the disk with a corresponding decrease in TSST-1 production associated with the disk. The deodorant or surfactant associated with the deodorant had little effect on the growth of *S. aureus* but did reduce the amount of TSST-1 produced.

DISCUSSION

Tampons are made of a core consisting of either a single fiber or a blend of fibers and may contain deodorants or fragrances, absorbents, and surfactants. Some tampons include a wrap, which may have a binder as well as a surfactant. The tampon composition, both fiber and additives, does affect cell growth and toxin production associated with the TD as demonstrated by the data presented. This is in agreement with the results reported by Lee et al. (4). However, cell growth on the membrane and the TSST-1 in the agar layer were unaffected for the most part by tampon composition in all but two cases. Agar layer toxin represents that portion of the total toxin which may be available for absorption by the tampon user in contrast to that which is absorbed by the tampon and, hence, is relatively unavailable. The two types of tampons (Tampax Super Plus and Playtex Deodorant) with which there were reduced amounts of TSST-1 in the agar layer have been associated with TSS (5). Consequently, all tampons have potential risks for TSS development in susceptible women infected with a TSST-1-producing strain of *S. aureus* at the time of tampon use.

The results presented here do not delineate the effect of tampon size on TSST-1 production. The heavier TDs overtaxed the limits of the small test system (10-ml volume), resulting in drier disks on a per-gram basis when compared with the lighter disks. The main purposes of the method were to determine (i) the major area of staphylococcal growth and production of TSST-1 in an in vitro system that was reasonably similar to the in vivo system and (ii) the effect of different materials on TSST-1 production.

The role of the tampon appears to be generic. Important functions of tampons may be to support the vaginal infection by supplying a fibrous surface for heavy colonization and to provide a sufficiently aerobic environment for toxin production. Wagner et al. (15) demonstrated that at the time of insertion of a tampon into the vagina the level of oxygen is raised to that of the atmosphere followed by a slow decrease with time. Pickrum et al. (H. M. Pickrum, D. L. Lucas, and R. L. Stone, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982,

TABLE 2. Effect of additives present in one of a pair of similar tampons

Additive	Tampons[a]	μg of TSST-1		CFU, 10^{10}	
		Agar	Disk	Membrane	Disk
Surfactant					
Present	KScR,S	140 ± 30	40 ± 10	22 ± 4	9 ± 9
Absent	KStR,S	150 ± 55	105 ± 20	22 ± 6	51 ± 6
Aqualon					
Present	TSlR	120 ± 30	70 ± 10	20 ± 3	82 ± 29
Absent	TR	130 ± 30	60 ± 20	18 ± 3	64 ± 31
Deodorant					
Present	PDR,S,S+	65 ± 10	10 ± 5	19 ± 4	15 ± 8
Absent	PR,S,S+	144 ± 30	67 ± 3	20 ± 3	8 ± 3

[a] Tampons: KScR,S, Kotex Security Regular and Super; KStR,S, Kotex Stick Regular and Super; TSlR, Tampax Slender Regular; TR, Tampax Regular; PDR,S,S+, Playtex Deodorant Regular, Super and Super+; PR,S,S+, Playtex Regular, Super and Super+.

B57, p. 27) found that TSST-1 production increased during incubation with vigorous agitation or oxygenation when compared with incubation without agitation. Schlievert and Blomster (9) observed a 32-fold increase in TSST-1 production under aerobic conditions over that obtained under anaerobic conditions, with only a 2-fold increase in cell growth. In the syringe method of Lee et al. (4), cell growth under anaerobic conditions (replacement of the air in the tampons with nitrogen and incubation under anaerobic conditions) was observed in only two tampons with very low levels of TSST-1 production (unpublished data). Reiser et al. (6), using a sac method, showed that the presence of any tampon, or even a plug of glass wool, in an inoculated dialysis sac doubled the total number of CFU and greatly increased production of TSST-1 over that obtained with an inoculated empty sac, when each was incubated in a tube of agar medium solidified around the sac. Thus, it can be concluded that tampons do supply adequate air for stimulation of TSST-1 production.

The location of the cells producing TSST-1 that subsequently is absorbed into the body to cause TSS has not been established. In our experimental model, in the absence of inhibition, uniform amounts of toxin would be expected to originate from cells colonized on the membranes in each of the TD experiments because the cells grew to approximately the same number in each of the experiments. The remarkably constant amount of TSST-1 in the agar layer with the different experiments indicates a relatively uniform contribution from cells colonized at the interface of the disk and the membrane. It appears that production of TSST-1 by *S. aureus* colonized at the interface was influenced more by the nutritional environment than by the tampon composition. That approximately 70% of the TSST-1 produced in the DMA method appeared in the agar layer is a good indication that a substantial portion of TSST-1 produced in vivo would be available for absorption into the body.

The presence of the deodorant/surfactant in the Playtex polyacrylate tampons resulted in no change in growth of the *S. aureus* strain on the membrane over that obtained with the cotton or rayon tampons and the polyacrylate tampons without deodorant. However, there was a >50% decrease in the amount of TSST-1 recovered from both the agar layer and the TD with the deodorant/surfactant-containing tampons. Although similar amounts of TSST-1 were recovered from the agar layer with the Tampax Super Plus disks, there was an accompanying reduction in the number of CFU associated with both the membrane and the disk. Presumably the reduction of CFU was at the interface because the amount of toxin associated with the disk was unaffected. Thus, there is evidence of inhibition of TSST-1 production by additives such as the deodorant/surfactant used in Playtex deodorant tampons and by a decrease in TSST-1 production by inhibiting growth of *S. aureus*, as was observed in the case of the Tampax Super Plus tampons. Both may prove to be important in reducing the risk of TSS.

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