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Inhibition of Staphylococcal Enterotoxin B Formation by Cell Wall Blocking Agents and other Compounds

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Enterotoxin B formation by *Staphylococcus aureus* S6 was inhibited by Tween 80, oleic acid, sodium deoxycholate, penicillin, D-cycloserine, or bacitracin. Toxin formation by strain 243 was sensitive to oleic acid, sodium deoxycholate, sodium lauryl sulfate, D-cycloserine, or bacitracin. The effect of D-cycloserine was reversed by D-alanine with strain 243 but not with strain S6. Neither penicillin nor bacitracin inhibited α-hemolysin or coagulase activity of strain S6; however, 0.118 μmoles of D-cycloserine per ml increased the α-hemolysin titer more than eightfold. Pigmentation of strain 243 was reduced by oleic acid, sodium deoxycholate, or methicillin, and was completely inhibited by D-cycloserine or bacitracin. Glucose was required for the inhibition by spermine of ^{14}C-valine incorporation into cellular protein of strain S6. These data indicate that the cell surface may contain sites important to the synthesis of enterotoxin B.

The inhibition of the formation of staphylococcal enterotoxin B by various nutritional inhibitors was reported previously (3). Although the nature of the inhibitory compounds suggested possible mechanisms involved, none has been elucidated. However, fluorescent antibody studies by Friedman and White (5) and more recently by Genigeorgis and Sadler (6) strongly suggested that enterotoxin was cell surface-associated. This view was strengthened further by Hartman and Goodgal (7), who reported that enterotoxin had the characteristics of a cell surface constituent of bacteria.

The present paper reports experiments designed to test the ability of *Staphylococcus aureus* to produce enterotoxin B in the presence of compounds known to affect bacterial cell walls. The results strengthen the hypothesis that staphylococcal enterotoxin is cell surface-associated.

MATERIALS AND METHODS

Organisms and culture methods. S. aureus strains S6 (17) and 243 (2) were used. Stock cultures, maintained on Trypticase Soy Agar (BBL) supplemented with 0.001% thiamine and nicotinic acid, were stored at 4 C and transferred every 2 weeks. Broth cultures were grown in PHP medium consisting of 2% Protein Hydrolysate Powder (Mead Johnson & Co., Evansville, Ind.) and 0.001% thiamine and nicotinic acid (initial *p*H 7.7). Oleic acid, the penicillins, D-cycloserine, and bacitracin were prepared as sterile solutions and added aseptically to autoclaved medium. All other additions were autoclaved with the medium. Cells for the preparation of inocula were grown at 37 C for 6 to 7 hr on Trypticase Soy Agar slants, removed with sterile deionized water, and adjusted to an optical density of 0.26 (655 mμ). This represented approximately 1.1×10^9 cells/ml. This suspension (0.2 ml) was inoculated into 25 ml of medium in a 250-ml Erlenmeyer flask so that the initial cell count was approximately 9×10^6/ml. Incubation was for 16 hr at 37 C on a reciprocating shaker making 100 excursions/min. At the end of the growth period, when the cultures were in the phase of decline, the contents of duplicate flasks were pooled, and turbidity measurements at 655 mμ were made on samples diluted 10-fold in fresh medium, with fresh medium as a blank. The cell density values presented do not represent the maximal growth attained by the cultures, but are given merely to indicate that sufficient growth did occur for the cells to form enterotoxin were it not for the presence of the inhibitor. A supernatant solution collected by centrifugation of the culture at approximately $1,000 \times g$ for 15 min then was subjected to serological assay for enterotoxin. Merthiolate (final concentration, 100 μg/ml) was added to maintain sterility. The packed cells for strain 243 were observed for loss of pigmentation.

Enterotoxin assay. Enterotoxin B content of the culture supernatant fluid was assayed quantitatively by a modification (13) of the single-agar diffusion method of Oudin (11), sensitive to a minimal concentration of approximately 5 μg of toxin/ml. Semiquantitative assays were done by use of the Ouchterlony double diffusion technique (10). Precipitin bands representing the reaction of antibody with as

little as 1 μg/ml of antigen could be observed after overnight incubation at 30 C if the plate was washed with 0.85% NaCl repeatedly for a minimum of 4 hr and then flooded with a saturated solution of $HgCl_2$. Antitoxin was prepared as described by Silverman (15).

α-Hemolysin and coagulase assays. Culture supernatant solutions without added Merthiolate were used in these studies. Erythrocytes from heparinized normal rabbit blood were separated from the plasma by centrifugation at 1,000 × *g* for 20 min, washed three times, and resuspended to 5% (v/v) in a diluent consisting of 0.85% NaCl, 0.03% $MgSO_4 \cdot 7H_2O$, and 0.03% KCl. To 0.4 ml of serially diluted sample was added 0.1 ml of 5% erythrocytes; this mixture then was incubated at 37 C for 1 hr, followed by centrifugation at approximately 210 × *g* for 3 min. Complete hemolysis was designated as a 4+ reaction. For the coagulase test, 0.5 ml of undiluted sample plus 0.5 ml of heparinized plasma were incubated at 37 C and examined every 15 min for clotting.

Isotopic experiment. The incorporation of ^{14}C-L-valine into the protein of whole cells of strain S6 was examined as described by Friedman and Bachrach (4), except that 1% PHP broth was used and the inoculum was more dense. Radioactivity was measured in a Nuclear-Chicago windowless gas-flow counter.

Chemicals. Penicillin G, crystalline sodium (The Upjohn Co., Kalamazoo, Mich.), sodium methicillin (Staphcillin, Bristol Laboratories Inc., Syracuse, N.Y.), sodium ampicillin (Polycillin-N, Bristol Laboratories), bacitracin (Calbiochem, Los Angeles, Calif.), and D-cycloserine (Sigma Chemical Co., St. Louis, Mo.) were used in these studies. Uniformly labeled ^{14}C-L-valine (specific activity, 200 mc/mmole) was purchased from Schwarz Bio Research Inc., Orangeburg, N.Y.

RESULTS

Effect of detergent-like substances. Tween 80 effectively inhibited enterotoxin formation by strain S6 but increased toxin yield by strain 243 (Table 1). The supernatant solution obtained after centrifugation of an S6 culture containing Tween 80 appeared turbid, whereas that from strain 243 did not, indicating that strain S6 but not 243 cleaved insoluble oleic acid from the Tween 80. Subsequently, when oleic acid was included in the growth medium, toxin formation by both strains was inhibited. In general, strain 243 was more sensitive to inhibition by oleic acid, sodium deoxycholate, or sodium lauryl sulfate than was strain S6. In fact, toxin formation by strain S6 was increased in the presence of sodium lauryl sulfate.

Effect of penicillins. Penicillin inhibited toxin formation by strain S6 at the low concentration of 0.0056 to 0.0393 mμmole/ml (Table 2). Methicillin and ampicillin also were effective. In the case of strain 243 (penicillin- and ampicillin-resistant), 0.449 mμmole/ml of penicillin allowed full growth of the organism but inhibited toxin formation 12%. Growth of this strain was retarded by 0.0015 μmole/ml of methicillin (penicillinase-resistant); however, the toxin yield

TABLE 1. *Inhibition of staphylococcal enterotoxin B synthesis by detergent-like substances*

Inhibitor (μmoles/ml)	*Staphylococcus aureus* S6			*Staphylococcus aureus* 243		
	OD[a] (655 mμ)	Entero-toxin (μg/ml)	Inhi-bition (%)	OD[a] (655 mμ)	Entero-toxin (μg/ml)	Inhi-bition (%)
None	0.29	29		0.31	42	
Tween 80[b]						
8.0	0.36	9	69	0.24	112	0
16.0	0.40	10	66	0.24	132	0
24.0	0.38	9	69	0.21	110	0
Oleic acid						
0.354	0.28	30	0	0.40	9	79
0.885	0.30	33	0	0.43	0	100
1.42	0.24	11	62	0.34	0	100
Sodium deoxy-cholate						
0.048	0.33	13	55	0.29	15	64
0.145	0.21	18	38	0.31	7	83
0.386	0.26	15	48	0.24	3	93
Sodium lauryl sulfate						
0.014	0.28	46	0	0.32	36	14
0.028	0.34	49	0	0.29	28	33
0.042	0.36	43	0	0.33	18	57

[a] Optical density of a 10-fold diluted 16-hr culture.

[b] Milligrams per milliliter.

TABLE 2. *Inhibition of staphylococcal enterotoxin B by penicillin G*

Penicillin (mμmoles/ml)	*Staphylococcus aureus* S6			*Staphylococcus aureus* 243		
	OD[a] (655 mμ)	Entero-toxin (μg/ml)	Inhibi-tion (%)	OD[a] (655 mμ)	Entero-toxin (μg/ml)	Inhibi-tion (%)
0.0	0.30	39		0.31	49	
0.0056	0.26	34	13			
0.0112	0.25	21	46			
0.0168	0.21	14	64			
0.0224	0.19	7	82			
0.0281	0.19	4	90			
0.0335	0.15	3	92			
0.0393	0.24	0	100			
0.2245				0.32	43	12
0.4490				0.32	43	12

[a] Optical density of a 10-fold diluted 16-hr culture.

was almost twice that of the control culture containing no antibiotic.

Effect of D-cycloserine and reversal by alanine. D-Cycloserine, which is also active against bacterial cell wall synthesis, but at a different site than penicillin (16), was tested. The toxin-synthesizing systems of both strains were sensitive, although the growth of S6 was decreased by lower concentrations of D-cycloserine (Table 3). With 0.118 μmoles of inhibitor per ml, cell turbidity was equal to the control, but toxin was reduced 83%. However, it was consistently observed that, with a slight increase in concentration, growth decreased but toxin yield did not. When inhibitor content was increased further, both growth and toxin yield decreased. The activity toward strain 243 was reversed to a great extent by D-alanine but not by the L or DL isomers; however, none of these compounds antagonized the effect on strain S6 (Table 4). The reason for this became apparent when it was shown that D-alanine itself was inhibitory to enterotoxin formation by strain S6 but not by strain 243.

Effect of bacitracin. Bacitracin, also active against cell wall formation (1), inhibited the synthesis of enterotoxin B (Table 5). The toxin-forming system of strain S6 was more sensitive to bacitracin than that of 243; however, the growth of strain 243 was inhibited at a lower concentration of bacitracin than that of S6.

Inhibitory effects on other macromolecular substances. Penicillin and bacitracin, at concentrations that did not inhibit growth, had little or no effect on the α-hemolysin or coagulase activity of cultures of strain S6. However, α-hemolysin titers of the supernatant solutions of cultures grown in the presence of 0.118 to 0.157 μmole of D-cycloserine per ml were at least eightfold greater than those of control cultures. At this range of concentration of inhibitor, the yield of enterotoxin was consistently greater than that when the organism was grown in the presence of the next lower concentration of antibiotic. D-Cycloserine had no effect on coagulase activity. Bacitracin and D-cycloserine did not affect α-hemolysin or coagulase activity of strain 243. The control supernatant solution of strain 243 cultures contained more than eight times the α-hemolysin activity of the strain S6 control culture.

Pigmentation of strain 243 was not affected by growth in the presence of Tween 80, sodium lauryl

TABLE 3. *Inhibition of staphylococcal enterotoxin B synthesis by D-cycloserine*

D-Cycloserine (μmoles/ml)	*Staphylococcus aureus* S6			*Staphylococcus aureus* 243		
	OD[a] (655 mμ)	Enterotoxin (μg/ml)	Inhibition (%)	OD[a] (655 mμ)	Enterotoxin (μg/ml)	Inhibition (%)
0.0	0.29	29		0.27	49	
0.039	0.29	21	28	0.27	29	41
0.078	0.34	21	28	0.26	26	47
0.118	0.29	5	83	0.29	26	47
0.157	0.13	19	34	0.30	16	67
0.196	0.04	8	74	0.31	11	78
0.235	0.02	4	86	0.29	11	78
0.274	0.01	0	100	0.18	7	86

[a] Optical density of a 10-fold diluted 16-hr culture.

TABLE 4. *Effect of alanine on inhibitory activity of D-cycloserine toward the formation of staphylococcal enterotoxin B*

Staphylococcus aureus strain	Additions to medium (μmoles/ml)	OD[a] (655 mμ)	Enterotoxin (μg/ml)	Inhibition (%)
S6	None	0.30	19	
	D-Cycloserine (0.118)	0.32	3	84
	+L-alanine (1.35)	0.30	4	79
	+L-alanine (2.02)	0.33	5	74
	+D-alanine (0.45)	0.29	5	74
	+D-alanine (0.90)	0.32	6	68
	+DL-alanine (0.45)	0.32	3	84
	+DL-alanine (0.90)	0.35	3	84
243	None	0.29	54	
	D-Cycloserine (0.235)	0.29	12	78
	+L-alanine (1.57)	0.29	12	78
	+L-alanine (2.54)	0.33	14	74
	+D-alanine (2.02)	0.38	38	30
	+D-alanine (2.54)	0.30	37	31
	+DL-alanine (1.57)	0.38	10	81
	+DL-alanine (2.54)	0.38	14	74

[a] Optical density of a 10-fold diluted 16-hr culture.

TABLE 5. *Inhibition of staphylococcal enterotoxin B synthesis by bacitracin*

Bacitracin (μmoles/ml)[a]	*Staphylococcus aureus* S6			*Staphylococcus aureus* 243		
	OD[b] (655 mμ)	Enterotoxin (μg ml)	Inhibition (%)	OD[b] (655 mμ)	Enterotoxin (μg/ml)	Inhibition (%)
0.0	0.29	32		0.28	54	
0.004	0.29	31	3	0.29	43	20
0.009	0.32	9	72	0.32	39	28
0.013	0.32	9	72	0.32	28	48
0.017	0.32	8	75	0.23	14	74
0.021	0.28	8	75	0.12	12	78
0.026	0.28	6	81	0.02	0	100
0.030	0.15	2	94	0.00	0	100

[a] Assuming a molecular weight of 1,411.

[b] Optical density of a 10-fold diluted 16-hr culture.

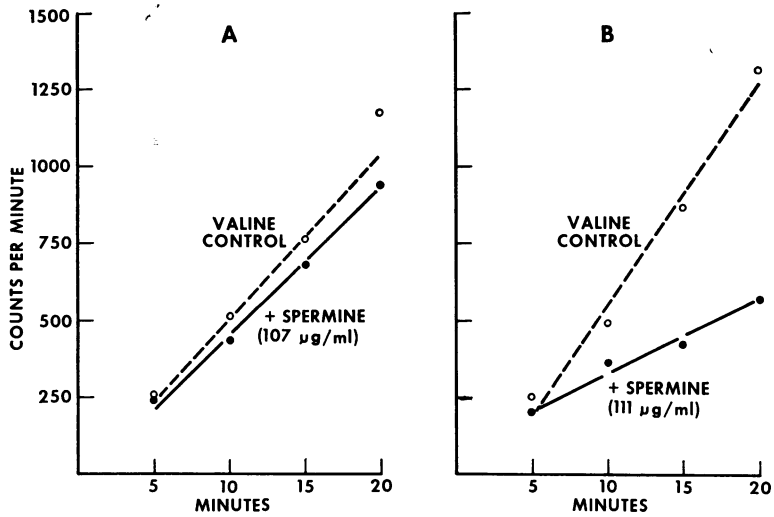

FIG. 1. *Effect of spermine on incorporation of* ^{14}*C-valine into growing log-phase cells of Staphylococcus aureus S6. (A) Medium containing no added glucose. (B) Medium prepared with 0.2% glucose. The pH of the cultures was adjusted to 7.8 with concomitant addition of spermine and* ^{14}*C-L-valine (0.09 µc or 0.05 µg/ml). Organisms were grown in 20-ml cultures in 250-ml Erlenmeyer flasks with shaking in a water bath (37 C).*

sulfate, or penicillin. Slight loss occurred in the presence of greater than 1.24 µmoles of oleic acid per ml, 0.145 µmole of sodium deoxycholate per ml, or 0.0015 µmole of methicillin per ml. Complete loss of pigmentation occurred in the presence of 0.021 µmole of bacitracin per ml and approximately 0.235 µmole of D-cycloserine per ml; however, the pigment was regained when the action of D-cycloserine was reversed by D-alanine. Subculture of nonpigmented cultures in the absence of inhibitor also resulted in the recovery of pigmentation.

^{14}C-valine incorporation and the effect of spermine. Spermine, an inhibitor of enterotoxin formation (3), decreased the incorporation of ^{14}C-valine by log-phase cells of *S. aureus* S6, but only if glucose was contained in the growth medium (Fig. 1). The inhibition was not complete, because uptake of radioactivity proceeded at a rate which, though slower than that of the control, was steady.

DISCUSSION

The formation of staphylococcal enterotoxin B can be inhibited nutritionally by a number of compounds without affecting the growth of the organism (3). The mechanisms of inhibition most likely vary; none has been elucidated nor has the site of synthesis been discovered through these studies. However, there is strong indication that the cell surface is very important to these phenomena (5). The studies reported here, in which detergent-like compounds and agents blocking cell wall synthesis displayed the ability to inhibit enterotoxin formation, serve to strengthen the hypothesis that enterotoxin is associated with the cell surface.

Because penicillin, D-cycloserine, and bacitracin affect cell wall synthesis at different sites, it is most likely that their activities toward enterotoxin were not specific but affected the cell surface in such a way as to preclude the formation of enterotoxin. Growth of *S. aureus* in the presence of penicillin was shown to result in structural changes of the cell wall characterized by thinning and disturbance of the deposition of new wall material (9). It is possible that such "lesions" would occur at sites important to enterotoxin elaboration, resulting in complete loss of formation or premature sloughing off of toxic material or precursors, or both. The presence of enterotoxin in cultures of low density and the increase in enterotoxin and α-hemolysin concentrations in cultures containing 0.118 to 0.157 µmole/ml of D-cycloserine over that observed in the presence of lower amounts of the inhibitor suggest that structural changes may have progressed to a point where "leaking" of antigenic material occurred. If this is so, then observations made here and elsewhere (8), that enterotoxin is elaborated as a metabolic product that apparently does not accumulate in the whole cell, point further to the cell surface as the site of formation.

Further support for the hypothesis that the cell surface is the major site of enterotoxin synthesis is derived from the results of the following experi-

ments. It was demonstrated that a drop in the *p*H of a culture from 7.7 to 5.4, caused by the metabolism of glucose in the medium, resulted in the reversal of the ability of spermine to inhibit enterotoxin formation (3). Cells of *S. aureus* S6 grown in the presence of 0.2% glucose incorporated 21% of the ^{14}C-spermine measured in the whole culture, but cells grown in the absence of glucose incorporated 12% of the label (M. E. Friedman, *unpublished data*). Furthermore, experiments reported here demonstrated that glucose was necessary for the inhibition by spermine of the incorporation of ^{14}C-valine into cellular protein. The role of glucose in the activity of spermine was confirmed by the work of Razin and Rozansky (12). They showed that glucose was necessary in a buffer solution for the maintenance of *S. aureus* in an active metabolic state so that the bactericidal activity of spermine toward the cells could be demonstrated. Also, Schlenk and Dainko (14) found that the inhibitory effect of spermine toward ribonuclease activity on the cell surface of yeast did not occur in the presence of glucose, which was necessary for the assimilation of the polyamine into the cells. Thus, if spermine inhibits enterotoxin formation only in the absence of glucose, and little spermine enters the cells in the absence of glucose, we suggest that spermine acts at the cell surface to inhibit enterotoxin formation. In addition, the failure of penicillin, D-cycloserine, or bacitracin to block α-hemolysin or coagulase indicates that their activities were not toward bacterial protein in general, but more specifically toward those cellular products whose origin was at or near the cell wall; therefore, this outer surface must contain the site(s) of enterotoxin synthesis.

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