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Inability of Toxin Inhibitors To Neutralize Enhanced Toxicity Caused by Bacteria Adherent to Tissue Culture Cells

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Toxicity to Y-1 adrenal mouse cells caused by heat-labile toxin secreted by an enterotoxigenic strain of *Escherichia coli* (H-10407-p) was 40-fold enhanced in mixtures containing organisms capable of adhering to the Y-1 cells compared with monolayers exposed to organisms whose adherence was inhibited by mannoside. Severalfold the concentrations of anti-heat-labile toxin antibodies required to neutralize the toxicity of nonadherent bacteria were unable to neutralize the toxicity caused by adherent bacteria. The cytolytic activity toward tissue culture cells and mouse peritoneal macrophages caused by streptolysin S carried by *Streptococcus pyogenes* was severalfold increased in mixtures containing organisms capable of adhering to the target cells compared with mixtures containing nonadherent bacteria. The ability of trypan blue and RNA core to inhibit the cell-bound streptolysin S was determined in tissue culture cells containing adherent streptococci and mixtures of streptococci randomly colliding with erythrocytes. Both inhibitors were markedly less effective in neutralizing cytolysis than in their ability to neutralize hemolysis. We conclude that compared with toxins produced by nonadherent bacteria, those produced by bacteria adherent to cells are targeted more efficiently and become relatively inaccessible to neutralization by toxin inhibitors.

It is widely accepted that bacterial adherence endows the pathogen with the ability to withstand cleansing mechanisms operating on mucosal surfaces and hence is important at an early stage in tissue colonization (6, 18). Recently we became interested in determining whether adherent bacteria have advantages over nonadherent bacteria in their survival, proliferation, and ability to cause tissue damage. In our previous study we showed both a growth advantage and an enhanced effect of heat-labile enterotoxin (LT) for a K-12 derivative of *Escherichia coli* adherent to tissue cells compared with nonadherent isogenic bacteria (26). The growth advantage and the enhanced toxicity were found to be due to the accumulation of products secreted by the tissue cells and the bacteria, respectively, in crypts formed by the ruffle structure of the tissue cells and "lids" formed by the adherent bacteria.

According to this proposed model, diffusion of molecules from the bulk medium into the unstirred layer formed between the bacteria and the animal cell surface is limited. It follows that toxin inhibitors (e.g., antitoxin antibodies) in the surrounding medium may not be able to neutralize the enhanced toxicity caused by bacteria adherent to tissue cells. In the present study we examined this hypothesis in two systems of bacterial adhesion to tissue culture cells. One system consisted of LT-secreting *E. coli* cells and anti-LT antibodies as toxin inhibitors. The other system consisted of streptolysin S (SLS)-producing *Streptococcus pyogenes* cells and trypan blue or RNA core as inhibitors of SLS. In both systems, the toxin inhibitors at relatively high concentrations, sufficient to neutralize toxic activity caused by nonadherent bacteria, were unable to neutralize the enhanced toxicity caused by bacteria of either species that were adherent to tissue cells.

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MATERIALS AND METHODS

Bacteria. *S. pyogenes* M type 5, described previously (19, 21), was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C for 6 and 18 h to obtain logarithmic- and stationary-phase bacteria, respectively. The microorganisms were harvested and washed twice with 0.02 M phosphate–0.15 M NaCl (pH 7.4) (PBS) and suspended in this buffer to obtain a density of 5×10^9 bacteria per ml. The K-12 isogenic strains of *E. coli*, VL645, a Fim^+ strain, and its Fim^- derivative, VL647, each harboring the pBR322-derived, LT^+ plasmid pΔ299, have been described in previous studies (11, 26). An enterotoxigenic strain of *E. coli*, H-10407-p, lacking colonization factor antigen I but expressing type 1 fimbriae, was obtained from D. J. Evans, Jr., and D. G. Evans (9). The bacteria were grown in Casamino Acid-yeast extract medium for 48 h at 37°C under static conditions to promote expression of the type 1 fimbrial adhesin (22). The organisms were harvested, washed with PBS, suspended to a density of 10^{10} bacteria per ml, and kept on ice to be used as stock suspensions for the various assays.

Tissue culture cell lines and mouse peritoneal macrophages. The following cell lines were used: Y-1 mouse adrenal tumor cells (ATCC 79) (26), intestinal epithelial cells (CCL-6, ATCC 407), pharyngeal epidermal carcinoma cells (HEp-2), and a primary culture of foreskin. Cultivation of the tissue culture cell lines was described previously (14, 24). Briefly, the cells were seeded in Falcon 24-well (1.5 cm in diameter) polystyrene plates (Costar, Cambridge, Mass.) and grown in minimal essential medium (MEM) containing 10% fetal calf serum supplemented with 100 μg of streptomycin and penicillin per ml. After growth over a period of 3 to 4 days at 37°C in a 5% CO_2 atmosphere to obtain confluent monolayers (5×10^5 cells per well), the cellular monolayers were washed three times with warm (37°C) PBS before use in the assay.

Mouse peritoneal leukocytes were harvested as described elsewhere (19). The cells were suspended in Hanks MEM to a density of 10^6 cells per ml, and 1 ml of the cell suspension was distributed into flat-bottom wells of Falcon 24-wells

plates. The cells were allowed to adhere to the bottom of the wells for 1 h at 37°C. The macrophage monolayers were washed three times with warm (37°C) PBS before use in the assay.

Determination of LT activity and adhesion of *E. coli*. Aliquots (1 ml) of various dilutions of an *E. coli* suspension in PBS supplemented with a 1:100 dilution of anti-LT sera (to block any LT activity) were added to two sets of Y-1 cell monolayers. Suspensions of *E. coli* H-10407-p were supplemented with methyl α-mannoside or methyl α-glucoside, both at a 2.0% final concentration. After incubation for 30 min at 4°C, the monolayers were washed free of bacteria and anti-LT.

One set of monolayers was fixed with glutaraldehyde (0.25% in PBS for 10 min at room temperature followed by a 30-min treatment with a solution containing 0.2% glycine and 0.1% bovine serum albumin) to determine the number of bacteria adherent per monolayer by an enzyme-linked immunosorbent assay (ELISA), as described previously (5, 20). Briefly, the detection system in this ELISA consisted of antisera to VL647 or H-10407-p (diluted 1:1,000), horseradish peroxidase-conjugated anti-rabbit immunoglobulin G, and ABTS (2,2'-azino-di-3-ethylbenzthiazoline sulfonate) substrate (Amersham), all of which were added sequentially in a 1-ml volume. Adhesion of bacteria was measured as ELISA units obtained by reading 0.1-ml aliquots transferred to 96-well microtiter plates. To correlate the number of bacteria with the optical density values, a standard curve was constructed as described previously (5). Briefly, increasing numbers of bacteria in distilled water were incubated in the wells at 40°C until dry, fixed with methanol, and blocked with 4% bovine serum albumin for 30 min at 37°C. ELISA units were obtained by using the procedure described above. In separate experiments it was determined that adhesion reached saturation in monolayers exposed to a range of 2×10^9 to 5×10^9 bacteria per monolayer and that anti-LT at a 1:100 dilution had no effect on the adhesion (data not shown). The method is sensitive for the detection of $>10^4$ bacteria per monolayer.

The other set of monolayers was used to determine LT activity by counting percent rounding up of cells, as described elsewhere (8, 26). The washed monolayers that were preincubated with the Fim^+ strain, VL645, or H-10407-p suspended in glucoside were further incubated at 37°C for 5 h in sterile Earle MEM that was either supplemented or not supplemented with various dilutions of anti-LT sera. The washed monolayers that were preincubated with the Fim^- strain, VL647, or H-10407-p suspended in mannoside were further incubated for the same period of time with medium to which various numbers of VL647 or H-10407-p cells suspended in mannoside were added, respectively, in order to determine the amount of nonadherent bacteria needed to reach the degree of toxicity observed in monolayers containing adherent bacteria (i.e., monolayers preincubated with VL645 or H-10407-p suspended in glucoside). In parallel, the medium containing nonadherent bacteria was supplemented with various dilutions of anti-LT sera.

Determination of SLS activity and adhesion of streptococci. The adherence of *S. pyogenes* to tissue culture cell lines or macrophage monolayers and the amount of cell-associated fibronectin were evaluated by ELISA in 24-well plates, as described elsewhere (20, 24). Briefly, 1-ml aliquots of streptococcal suspensions (5×10^9 cocci per ml) were added to the monolayers and incubated for 30 min at 37°C. After three washes in PBS, the number of bacteria bound was enumerated, as described above, using the ELISA and antistreptococcal sera (diluted 1:1,000). Similarly, the cell-associated fibronectin was determined with antifibronectin sera (1:500). ELISA values obtained with plates in which known amounts of fibronectin or bacteria were immobilized on the bottom of wells served to generate standard curves for estimating the number of bacteria bound or micrograms of cell-associated fibronectin, as described above (5). In separate experiments it was determined that adhesion reached saturation in monolayers exposed to 5×10^9 cocci per monolayer (data not shown).

Hemolytic activity was determined as described previously (13) with minor modification. A serial twofold dilution of a streptococcal suspension containing SLS-activating mixture (glucose, 10 mg/ml; $MgSO_4$, 10 mg/ml; and base-free cysteine, 1.3 mg/ml) was added to 50 ml of 2% human blood group O erythrocytes in 96-well microtiter plates. After incubation at 37°C for 30 min the plates were centrifuged at $1,000 \times g$ for 5 min and the supernatant was transferred to 96-well plates and read in an ELISA reader at 415 nm. One hemolytic unit represents the reciprocal of the highest dilution of the streptococcal suspension causing 50% hemolysis of the erythrocyte suspension. One hundred percent hemolysis represents the reading obtained by suspending the same amount of erythrocytes in wells with 100 ml of distilled water, and 0% hemolysis represents the reading obtained in wells to which a streptococcal suspension not supplemented with SLS-activating mixture was added.

Cytolytic activity was determined in 24-well plates by exposing the cell monolayers to 1 ml of serial twofold dilutions of the streptococcal suspensions in PBS supplemented with SLS-activating mixture. Cytolytic units represent the reciprocal of the dilution of streptococcal suspension causing death, as assayed by trypan blue exclusion, of 50% of the cell monolayer (20) after incubation for 30 min at 37°C. When different streptococcal suspensions were tested, the results were expressed as cytolytic units per one hemolytic unit to normalize the results.

In some experiments CCL-6 monolayers were treated with 100 μg of fibronectin per ml as described previously (24), washed, and exposed to a streptococcal suspension as described above to determine adhesion and cytolysis. In parallel experiments, 1 and 0.1 ml of streptococcal suspension (equivalent to eight hemolytic units) were added to cell monolayers in 24-well plates or erythrocyte suspensions in 96-well microtiter plates, respectively, as described above. The mixtures were incubated at 37°C for 30 min to allow binding of the bacteria to the cells. One hundred or 10 ml of 10× concentrated SLS-activating mixture containing various concentrations of trypan blue and RNA core (Sigma Chemical Co., St. Louis, Mo.) inhibitors were added to each of the cell monolayers or erythrocyte suspensions, respectively. The minimal concentration of the inhibitor needed to cause 50% inhibition of the cytolytic or hemolytic activity was determined.

Determination of hydrophobicity of streptococci. Hydrophobicity was measured by the streptococcus-hexadecane adherence assay as described previously (21). The test was performed on the same streptococcal suspensions that were used in the cytolytic and adhesion assays.

Preparation of antisera. Rabbits immunized with pure cholera toxin (Swiss Serum and Vaccine Institute, Bern, Switzerland), which shares common epitopes with LT (7), served as the source of LT antibodies. The toxin was dissolved in saline to a concentration of 400 μg/ml, and the solution was mixed 1:1 with complete Freund adjuvant. The mixture was injected intramuscularly as described previ-

TABLE 1. Influence of adhesion and hydrophobicity on cytolytic activity of *S. pyogenes* toward mouse peritoneal macrophages[a]

Growth phase of culture	Pretreatment	Adherence (cocci/cell)	Hydrophobicity (%)	Cytolysis (U/HU)
Logarithmic	PBS	2 ± 0	9 ± 4	0.3 ± 0.05
Logarithmic	Hyaluronidase	11 ± 2	95 ± 6	2.3 ± 0.3
Stationary	PBS	15 ± 3	95 ± 5	2.0 ± 0.2

[a] Streptococcal suspensions harvested at the indicated phase of growth and adjusted to a density of 5×10^9 bacteria per ml of PBS were used in all assays as described in the text. The results are given as the means of three separate experiments. Because the phase of growth and various treatments may influence the synthesis and surface expression of cell-bound SLS (7), the hemolytic activity of the streptococcal suspensions was determined in parallel, as described in the text, and the cytolytic activity is expressed as units per one hemolytic unit (HU) to normalize the results.

ously (22). Rabbit antifibronectin serum was prepared as described previously (29), using fibronectin purified from human plasma as described elsewhere (24). Anti-whole bacteria antibodies were prepared as described previously (19). Briefly, 0.5 ml of the bacterial suspension (2×10^9 bacteria per ml) was injected intravenously three times weekly for two consecutive weeks. Immune sera were collected at 4 weeks.

Chemicals and media. All the chemicals and media used in these experiments were of the highest quality and purity available.

RESULTS

Enhanced cytotoxicity caused by SLS expressed by adherent streptococci. Encapsulated streptococci harvested at the logarithmic phase of growth were less hydrophobic and adhered less well to the macrophage monolayer compared with hyaluronidase-treated bacteria or streptococci harvested at the stationary phase of growth (Table 1). Upon addition of activating mixture to induce the expression of SLS, the cytolytic activity per one hemolytic unit of the hydrophobic and adherent streptococci was about three times that of nonadherent streptococci (Table 1). All the cytolytic and hemolytic activities were inhibited by 20 μg of trypan blue, a specific inhibitor of SLS (12), per ml (data not shown). Furthermore, the supernatants of the reaction mixtures at the end of the experiment lacked any detectable hemolytic or cytolytic activity (data not shown).

To examine further whether streptococci adherent to tissue cells target their cell-bound cytolysin more efficiently than nonadherent bacteria, we employed various tissue cell monolayers that differ in their ability to bind streptococci. These monolayers contained different amounts of surface fibronectin, which is known to serve as a receptor that binds streptococci (23, 24). The different cell lines were exposed to streptococcal suspensions in the presence of an SLS-activating mixture to determine the streptococcal cytolytic activity or, in the absence of such a mixture, to determine the streptococcal adherence. We found that as the amount of fibronectin increased on cell surfaces, the more streptococci adhered and, concomitantly, the higher the cytolytic activity of the bacteria (Table 2). In particular, the results with the CCL-6 cells and CCL-6 cells treated with fibronectin showing a significant increase in cytotoxicity and adhesion can be attributed solely to the increase in the number of receptors that bind streptococci (24). Cytolysis was totally inhibited by 20 μg of trypan blue per ml, and the supernatants of the reaction mixtures at the end of the experiment lacked any detectable cytolytic activity (data not shown).

TABLE 2. Effect of trypan blue and RNA core on enhanced cytolytic activity of *S. pyogenes* adherent to tissue cells

Target cells	Amt of fibronectin expressed (pg/10^2 cells)[a]	Adherence (bacteria/cell)[b]	Cytolysis (U/ml)[b]	SLS inhibition (μg/ml) by[c]: TB	SLS inhibition (μg/ml) by[c]: RNA core
CCL-6	3.3 ± 0.1	15 ± 2	5 ± 1	1.4 ± 0.8	2,000
CCL-6 + Fn[d]	26 ± 2	30 ± 5[e]	10 ± 1[e]	ND[f]	ND
HEp-2	42 ± 4	40 ± 5[e]	13 ± 3[e]	2.4 ± 0.6	1,231
Foreskin	45 ± 5	50 ± 6[e]	16 ± 3[e]	2.8 ± 0.8	ND
Erythrocytes	0	Irrelevant	Irrelevant	0.06 ± 0	9.4 ± 1

[a] The amount of cell surface fibronectin was determined by ELISA techniques, as described in the text.

[b] The adherence and cytolysis tests were performed in triplicate using the same batch of streptococcal suspension (equivalent to 40 hemolytic units per ml [5×10^9 bacteria per ml]) in PBS supplemented or not supplemented with SLS-activating mixture to determine adhesion or cytolysis, respectively, as described in the text.

[c] Inhibition of cytolysis was performed with streptococcal suspensions (equivalent to eight hemolytic units per ml), supplemented with various amounts of the inhibitors (trypan blue [TB] or RNA core), to determine the minimal amount of the inhibitor needed to cause 50% inhibition of cytolysis.

[d] Fn, Fibronectin. Monolayers were pretreated with 100 μg of fibronectin per ml, as described in the text.

[e] These values are significantly different, statistically, compared with those obtained with CCL-6 cells ($P < 0.02$).

[f] ND, Not done.

Ability of SLS inhibitors to neutralize cytolysis of SLS-bearing streptococci. The ability of SLS inhibitors to neutralize the cytolytic effect of SLS-bearing streptococci was examined in organisms bound to tissue culture cells and, in parallel, in organisms randomly colliding with erythrocytes. Various concentrations of trypan blue and RNA core inhibitors in SLS-activating mixture were added to organisms preincubated with either tissue cell monolayers or erythrocyte suspensions. The amount of the inhibitors needed to inhibit 50% of the cytolysis caused by streptococci adherent to tissue culture cells was 100 to 200 times that needed to inhibit hemolysis caused by the same suspension of streptococci (Table 2).

Enhanced toxicity of LT secreted by adherent *E. coli*. The toxicity of LT secreted by enterotoxigenic *E. coli* H-10407-p was determined by exposing Y-1 monolayers to suspensions of bacteria supplemented with methyl α-mannoside to inhibit adherence (5, 6). As a control, monolayers were exposed to suspensions supplemented with methyl α-glucoside. In parallel we exposed the Y-1 cells to LT-producing, isogenic strains of *E. coli* that differ only in their ability to express type 1 fimbriae (26). The titer of LT secreted in the growth medium after 48 h of bacterial growth at 37°C was 1:40 for strain H-10407-p and 1:160 for both the fimbriate and afimbriate K-12 derivatives. The Y-1 cell monolayer exposed to the Fim^+ strain, VL647, bound 6.0×10^6 bacteria (31 bacteria per cell), and that exposed to the suspensions of the H-10407-p strain supplemented with glucoside bound 4.7×10^6 bacteria (24 bacteria per cell). No binding could be detected in monolayers exposed to the Fim^- VL647 bacteria or to the H-10407-p strain supplemented with mannoside (Table 3). Compared with that of nonadherent bacteria, the LT-mediated toxicity of adherent bacteria was several orders of magnitude greater for both the H-10407-p and VL645 strains (Table 3). For example, whereas 2×10^8 H-10407-p cells suspended in mannoside (nonadherent bacteria) were needed to cause 15% rounding up of Y-1 cells, only 3.6×10^6 adherent bacteria suspended in glucoside were needed to cause the same effect, representing a 40-fold enhancement of

TABLE 3. Effect of anti-LT on LT-mediated toxicity caused by *E. coli* cells nonadherent or adherent to Y-1 adrenal cells[a]

Strain	No. of bacteria in monolayers		% Rounding up of cells			
	Adherent	Nonadherent	−Anti-LT	+Anti-LT		
				1:800	1:200	1:100
VL 645 (Fim$^+$)	6.0×10^6	$<10^4$	27 ± 4	22 ± 1	25 ± 5	28 ± 5
VL 647 (Fim$^-$)	$<10^4$	1.7×10^{8b}	41 ± 6	18 ± 3	<5	<5
H-10407-p + 2% αMG[c]	4.7×10^6	$<10^4$	22 ± 3	ND	ND	19 ± 2
H-10407-p + 2% αMM[c]	$<10^4$	2.0×10^{8b}	14.6 ± 3	ND	<5	ND

[a] Y-1 tissue culture cells were exposed to 1 ml of the various *E. coli* strains at a density of 5×10^9 bacteria per ml in PBS supplemented with a 1:100 dilution of anti-LT sera for 30 min at 4°C. After being washed, one set of monolayers was fixed to estimate the number of adherent bacteria and the other set was further incubated for 5 h at 37°C in Earle MEM, not supplemented or supplemented with various dilutions of anti-LT sera, to determine the percent rounding up of cells.

[b] The indicated number of nonadherent bacteria were added to wells prior to the determination of the percent rounding up of cells. Wells to which nonadherent bacteria were not added showed <5% rounding up of cells after 5 h of incubation (data not shown).

[c] αMG (methyl α-glucoside) and αMM (methyl α-mannoside) were added to the streptococcal suspensions during the adhesion phase of the assay and in the Earle MEM during the cytotoxicity phase of the assay, as described in the text.

toxicity. The number of cells of the afimbriate K-12 derivative needed to cause 41% rounding up of Y-1 cells was 30 times the number of cells of the adherent, Fim$^+$ strain needed to cause 27% rounding up of cells. As in a previous study (26), the medium after 5 h of incubation with monolayers containing adherent bacteria lacked any detectable LT activity.

Effect of anti-LT toxin on adherence-dependent toxicity of *E. coli*. The effect of the LT toxin secreted by the afimbriate bacteria was readily neutralized by as high as a 1:800 dilution of the anti-LT toxin, whereas that secreted by the fimbriate bacteria was uninhibited even by >8× concentrated anti-LT (Table 2). Likewise, a 1:200 dilution of the anti-LT toxin neutralized the effect of toxin secreted by the nonadherent strain H-10407-p suspended in mannoside, whereas >2× concentrated anti-LT did not inhibit LT toxin secreted by the same bacteria that were suspended in glucoside and that were adherent to the cells.

DISCUSSION

In our previous study we employed isogenic derivatives of *E. coli* that differ only in their adherence abilities and showed that LT secreted by adherent *E. coli* accumulates at high concentrations in the tissue culture cell vicinity and thus is targeted more efficiently toward the Y-1 cells (26). The purpose of the present study was to examine the phenomenon of enhanced toxicity of adherent bacteria in other strains of *E. coli* as well as in other bacterial species. More importantly, it was of interest to determine whether such enhanced toxicity escapes neutralization by toxin inhibitors.

The results obtained with the enterotoxigenic strain H-10407-p suggest that there was 40-fold enhancement of toxicity caused by LT in Y-1 monolayers exposed to organisms capable of adhering to the Y-1 cells compared with monolayers exposed to organisms whose adherence was inhibited by mannoside. Although the Fim$^+$ strain, VL645, secreted four times the amount of LT secreted by strain H-10407-p, both strains were equally toxic when adherent to the target Y-1 cells. These results confirm our previous data and show that adhesion-dependent toxicity is not restricted to the genetically manipulated strains employed in the previous study (26). Moreover, at bacterial densities that were equally toxic to the adrenal cells, neutralization by anti-LT was in excess of 1,000 times more efficient when the neutralization target was LT secreted by the nonadherent bacteria than when the target was LT secreted by the adherent bacteria (Table 2).

The phenomenon of adhesion-dependent toxicity was also examined in *S. pyogenes*. These pyogenic cocci produce a highly potent cytolytic toxin known as SLS, a peptide with a molecular weight of about 1,800 (4, 17), which may exist in two forms. One form is released from the bacterial cells in a complex with a suitable carrier molecule. The other form is bound to the cell surface and is known as cell-bound hemolysin or streptolysin (12). The surface expression of SLS requires an activating mixture consisting of glucose and cysteine. Upon contact with animal cells, this surface-expressed SLS is transferred to the cell membrane of the animal cell where it causes lysis. It has been postulated that the SLS peptide is transferred directly from the streptococcal surface to the cell membrane of the target cell, because the culture supernatant lacks any detectable hemolytic or cytolytic activity (12). It is not known whether streptococci randomly colliding with the target cells are as efficient as adherent bacteria in the ability to cause cytolysis. This tissue was examined by comparing the abilities of bacteria to cause hemolysis or cytolysis. Erythrocytes served as prototypes of cells that are believed to undergo lysis due to random collision with the streptococci (12, 13). Importantly, erythrocytes do not hemagglutinate with streptococci and are devoid of surface fibronectin (3).

We assayed cytolysis in two different systems: one varied the bacterial adherence phenotype, and the other varied the ability of the cell monolayer to bind bacteria. In the first we compared the ability of adherent and nonadherent streptococci to cause cytolysis in monolayers containing mouse peritoneal macrophages. In contrast to *E. coli*, it is impossible to obtain a viable streptococcal mutant that is deficient in the adhesin that binds the streptococci to host cells, in this case lipoteichoic acid (6). Instead, we took advantage of two important properties of streptococci to alter their binding and toxin-expressing phenotypes. With regard to adherence, the ability of streptococci to associate with monolayers of mouse peritoneal macrophages is dependent on the phase of growth and presence of a surface hyaluronate capsule (25), which apparently masks the lipoteichoic acid-adhesin function (21). With regard to toxin expression, resting streptococcal suspensions preincubated with an activating mixture to induce expression of SLS are capable of lysing monolayers of mouse peritoneal macrophages (17).

In the second cytolytic system, the streptococcal suspension was kept the same and various tissue cell monolayers that differ in their ability to bind streptococci were employed as target cells. As with the results seen in the first system of interaction, the hemolytic and the cytolytic activities were

inhibited by trypan blue, a specific inhibitor of SLS (12), and no cytolytic or hemolytic activities were detected in the culture supernatants. We conclude that in both systems the hemolytic and cytolytic activities were mediated by the cell-bound form of SLS (12, 13). Moreover, compared with data for nonadherent streptococci, the data obtained for cytolysis and adhesion in both systems tested suggest that the cytolytic activity is enhanced greatly in monolayers exposed to streptococci capable of adhering to the target cells.

To examine the ability of SLS inhibitors to neutralize the adherence-dependent cytotoxicity caused by SLS-bearing streptococci, we employed trypan blue and RNA core as inhibitors of the toxin. These inhibitors act by interacting with the SLS-bearing streptococci to form relatively stable, soluble complexes with SLS and hence act as carrier or "inducer" molecules for the SLS peptide (12). SLS in the cell-bound form is loosely bound to the streptococcal surface, whereas the carrier-SLS complexes are more stable, especially with trypan blue, which possesses a relatively high affinity for the SLS peptide (12). The carrier molecules act as inhibitors of the activity of the cell-bound form of SLS by preventing the transfer of the peptide from the bacteria to the target cells within 30 min of incubation (12, 19). The inhibitory activity of a carrier molecule, therefore, is dependent on the affinity of the carrier for SLS and the accessibility of the toxin on the streptococcal surface to the inhibitor. Both SLS inhibitors were 100 to 200 times less effective in neutralizing cytolysis than hemolysis, suggesting that the cell-bound form of SLS carried by streptococci adherent to tissue culture cells is less accessible to the inhibitors than SLS expressed by streptococci randomly colliding with the erythrocytes. These results support and extend those found with *E. coli*.

Previous studies have indicated that bacteria adherent to tissue cells gain several survival advantages over nonadherent bacteria. In addition to the classical mechanism of withstanding the host's cleansing mechanisms at mucosal surfaces, adherent organisms grow better because they are in a position to utilize the higher concentrations of nutrients trapped in the unstirred layer between the bacteria and the target cell (26). In addition, it now appears that adherent bacteria are significantly more toxic toward host cells, as has been shown in separate systems with different organisms, including LT-secreting *E. coli* (18; this study), lipopolysaccharide-mediated inflammation caused by P-fimbriated *E. coli* (11), and SLS-mediated cytolysis in streptococci (this study). The enhanced toxicity of adherent bacteria has been explained by postulating that toxin secreted by adherent *E. coli* accumulates at high concentrations in the tissue culture cell vicinity (26). The cell-bound form of SLS, however, may be aimed more efficiently at the target cell. Finlay et al. (10) showed that adhesion of *Salmonella* spp. to tissue culture cells induces de novo synthesis of bacterial proteins. The possibility, therefore, that adhesion induces the production and/or secretion of toxin in the cell vicinity cannot be excluded.

More importantly, we now show that another advantage gained by adherent bacteria is that the already enhanced toxicity can escape neutralization by a number of inhibitors, including antibodies. It appears that the larger the size of the inhibitor molecule, the less efficient it is in neutralizing the toxic activity of adherent bacteria. Antibodies, which are large molecules, are particularly inefficient, a finding relevant to the development of new vaccines against bacterial infections of mucosal surfaces. Thus, the ineffectiveness of an anti-cholera toxin vaccine against cholera (1, 2, 15) might now be interpreted as the inability of the antitoxin antibodies to neutralize the toxin secreted by adherent vibrios. Cholera toxin, however, is more readily secreted in the medium than LT, so that the comparison is not an exact one. Obviously, further studies are needed to determine the general relevance of adhesion-enhanced toxicity and the inaccessibility of the toxin for neutralization by inhibitors in infections caused by pathogens adherent to mucosal tissues and expressing and/or secreting tissue-damaging toxins.

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