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# Teichoic Acids of *Streptococcus agalactiae*: Chemistry, Cytotoxicity, and Effect on Bacterial Adherence to Human Cells in Tissue Culture

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The ratio of teichoic acid to lipoteichoic acid (LTA) in a strain of *Streptococcus agalactiae* type III was found to be 8:1, with the total amount of LTA being 0.1% of the dry weight of the organism. Purified teichoic acid contained D-alanine and possibly a small amount of D-glucose and was approximately 22 glycerol phosphate units in length. The linkage between each of these units was 1-3. In addition, LTA contained a complex lipid, more glucose, and an unusually high content of a short-chain fatty acid, tridecanoic acid. This LTA was cytotoxic for a variety of human cell monolayers in tissue culture, including one derived from the human central nervous system. Established human cells were more sensitive than primary cell monolayers to this LTA, with as little as 12.5 μg of LTA per ml being cytotoxic for HeLa cells. Teichoic acid (250 μg/ml) was nontoxic under identical conditions. These cytotoxicity results suggest an LTA involvement in group B streptococcal pathogenesis. Also, the first model system for the study of group B streptococcal adherence to primary human embryonic amnion cells in tissue culture is detailed. This system was used to quantitate pronounced differences in tissue tropism between *S. agalactiae* and *Streptococcus pyogenes* and showed enhanced binding by this group A coccus over that of *S. agalactiae* for amnion cell monolayers. The adherence of both streptococcal species to only a portion (40%) of these amnion cells suggested that host cell receptor expression may vary for primary cells in vitro. Finally, this strain of *S. agalactiae* was shown to adhere to amnion cells by a non-LTA-mediated mechanism. The possibility of an LTA-mediated versus a protein-mediated adherence mechanism for host cells that is related to the virulence of *S. agalactiae* is discussed.

Group B streptococci are important pathogens of infants during the neonatal period. These organisms also produce disease in adults (37). Of all the serotypes known, type III strains account for the largest proportion of infections in humans (16). Studies of the physiology of this pathogen have dealt with cell wall composition (9), extracellular enzymes (31), and the release of soluble antigens (11). However, except for an earlier study by McCarty (22) which established the presence of a glycerol-type teichoic acid (TA) in a group B streptococcus, serotype unknown, the TA of these cocci has not been detailed. The important role of lipoteichoic acid (LTA) in bacterial adherence to host cells (3, 4) and its putative role in immunological and immunopathological (35) maladies is now well established. More recently, the ability of LTA from a group A streptococcus to destroy human cells in tissue culture (10) and to result in defective collagen formation (20, 34) was documented. These findings have made a more detailed study of this polymer in the group B streptococci mandatory. This paper details the cellular content, structure, and cytotoxic potential of TA and LTA from a human isolate of *Streptococcus agalactiae* type III. It also describes the development of the first model system for the study of the adherence of this organism to normal human cell monolayers in tissue culture.

(A preliminary account of this work was first presented at the Annual Meeting of the American Society for Microbiology, Miami Beach, Florida, 1980, abstr. no. D-72. Parts of this work have been presented by J. Goldschmidt, Jr., as a Ph.D. thesis at Thomas Jefferson University.)

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## MATERIALS AND METHODS

**Bacteria.** *S. agalactiae* type III, a human isolate designated DS2242-77, was obtained from Richard R. Facklam, Centers for Disease Control, Atlanta, Ga. The *Streptococcus pyogenes* type 12 used as a control has been described (29).

**Culture media.** Stock cultures were kept on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% (vol/vol) sheep blood at 5°C and transferred from agar to Todd-Hewitt broth (THB; BBL) as needed. Viable counts were done on Trypticase soy agar without and with 5% (vol/vol) sheep blood for *S. agalactiae* and *S. pyogenes*, respectively. Adherence studies utilized each species grown in THB (37°C, 5% [vol/vol] inoculum) and harvested during their logarithmic phases of growth (after 3 h). Each organism was harvested by centrifugation (6,130 × *g*, 20 min) and washed twice with phosphate-buffered saline (PBS) (23) before use. For biochemical studies, 10-liter batches of THB containing 80 g of glucose and 36 g of $Na_2HPO_4$ were inoculated (5% [vol/vol]) with *S. agalactiae* and incubated for a total of 6 h at 37°C, corresponding to a late-logarithmic to early-stationary phase of growth. Cells were harvested by centrifugation as described above, washed twice with 0.9% (wt/vol) saline and once with distilled water, and lyophilized.

**Extraction, estimation and purification of TA and LTA.** TA was extracted from 10 g (dry weight) of cells with 10% trichloroacetic acid in the cold (4°C) and purified by Sephadex gel column chromatography as detailed previously (29). LTA was obtained from dried cells (10 g) by the cold (30) and hot (27) phenol extraction methods and purified by Sepha-

rose 6B column chromatography (30). As before, yields of TA and LTA were calculated from column chromatographic elution profiles by determining the amount of phosphorus present, excluding that ascribed to RNA, and the results were expressed as milligrams of TA and LTA phosphorus per gram (dry weight) of cells (29, 30). Because LTA is hygroscopic, its dry weight was calculated from the known phosphorus content of the sample multiplied by a conversion factor (i.e., 1 mg of phosphorus = 5.81 mg of TA) calculated from the experimentally determined chain length and molar ratio of highly purified TA, which is less hygroscopic (29). A determination of this factor by gravimetric analyses with purified TA yielded a value of 1 mg of phosphorus = 5.64 mg of TA, the difference between the theoretical and actual value being 3%. Therefore, the weights given for LTA in Table 1 are minimal as they do not include the lipid portion of LTA. Chromatographic recoveries of material taken for analysis, in terms of phosphorus, were always in excess of 95%.

**Analytical methods.** Almost all of the analytical methods used previously for the structural characterization of TA and LTA from *S. pyogenes* and its L form were used again (26, 29, 30, 32). Protein as a contaminant of LTA and TA preparations was determined by the method of Lowry et al. (21), with purified LTA and TA containing 4.6 ± 0.4% ($n$ = 4) and 0.4 ± 0.2% ($n$ = 4) protein, respectively. However, reproducible protein values were only possible when large amounts of LTA (≥0.5 mg) were analyzed. Earlier, LTA from *S. pyogenes* with a similar protein content (as determined by the same method) was found to contain an amino acid content of only 0.45% when examined by amino acid analyses with ophthalaldehyde and fluorescence detection (34). Therefore, since the actual protein content of these LTA preparations was questionable, the method of Lowry et. al. was used instead to monitor only the relative amounts of protein in LTA and TA preparations during purification by column chromatography. Purification was terminated when the protein content of each preparation could not be lowered further. Others have also commented on the variability of protein values for LTA as determined by colorimetric procedures (36).

**Anti-LTA serum.** Anti-LTA serum was prepared in rabbits as described by Fiedel et al. (12) by using purified LTA extracted from *S. agalactiae* by the cold phenol method. This antiserum reacted with LTA from *S. agalactiae* and *S. pyogenes*; the titers obtained with both antigens were identical, 1:256 as determined by passive hemagglutination with rabbit erythrocytes.

**Tissue culture studies.** A variety of established primary cell lines were employed to determine the cytotoxic potentials of TA and LTA from this group B streptococcus. Established cell lines included HeLa (Flow Laboratories, McLean, Va.) and Girardi heart cells (CCL-27; American Type Culture Collection Repository, Rockville, Md.) and primary cell lines such as human embryonic kidney (GIBCO Laboratories, Grand Island, N.Y.), human embryonic brain (Flow 3000), and human embryonic amnion cells (GM472; Institute for Medical Research, Camden, N.J.) were used. HeLa, heart, and embryonic brain and kidney cells were grown in Eagle basal medium (MEM) with 10% (vol/vol) inactivated fetal bovine serum (FBS), 1 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml, pH 7.5. Amnion cells were propagated in RPMI 1640 medium with 20% (vol/vol) FBS, 2 mM L-glutamine, and the same antibiotics and pH described above. All media and reagents were obtained from GIBCO. Tissue culture cells were grown in flat 75-cm$^2$, 250-ml plastic bottles (Corning Glass Works, Corning, N.Y.) at 37°C in an atmosphere of $CO_2$ (5%) and air (95%). Viable cell counts were determined with Erythrocin B and a hemacytometer (10).

**Destruction of human primary and established cell monolayers by LTA.** Initial experiments employed confluent monolayers of cells on glass cover slips in round 16-mm wells. Purified streptococcal LTA, in a concentration range of 12.5 to 250 μg/ml, was dissolved in MEM (pH 7.5) with 1% FBS, 1 mM L-glutamine, and antibiotics as described above plus 1 U of mycostatin per ml. No appreciable change in pH was observed. One milliliter of the appropriate concentration of LTA was then added to each well as before (10). Where appropriate, TA served in lieu of LTA. All monolayers were then incubated for 48 h without refeeding, washed twice with PBS, fixed in absolute methanol, and stained with Giemsa blood stain (10). Morphological changes were observed with an inverted light microscope (×100) and photographed with a Nikon AFM camera equipped with an automatic exposure meter. HeLa cells were chosen for more detailed studies because of their ease in propagation and greater sensitivity to LTA. However, because of rapid proliferation, uniform monolayers were difficult to obtain and to quantitate accurately. For reproducible results, cell numbers had to remain relatively constant during exposure to LTA. This was achieved by seeding a sufficient number ($1.3 \times 10^5$) of viable HeLa cells into each tissue culture well to assure subconfluent monolayer production after overnight incubation. The extent of monolayer destruction after 48 h of exposure to LTA was estimated by counting under a microscope (×1,000) 10 representative fields per slide and expressing the data as the total number of cells attached per 10 fields after LTA treatment and, also, as a percentage of the untreated (control) cells (Table 2). Multinucleated cells were counted as one.

**Bacterial adherence assay.** Primary embryonic amnion and established HeLa cells were used for these comparative studies; the former because they are thought to be more representative of the natural host receptors for group B streptococci and the latter, with their atypical membrane as a result of transformation, because they are not. HeLa or embryonic amnion cells ($1.8 \times 10^5$) in 1 ml of appropriate medium were seeded into glass Leighton tubes (16 by 93 mm, containing 9 by 50 mm no. 1 glass cover slips; Bellco Glass Co., Vineland, N.J.) or plastic disposable tubes of the same size (with 9 by 55 mm cover slips; Costar, Cambridge, Mass.) and incubated for 24 and 48 h, respectively. Each resulting subconfluent monolayer was washed three times with PBS. Suspensions of *S. pyogenes* and *S. agalactiae* were prepared by using logarithmic-phase cells (after 3 h at 37°C) grown in THB that had been washed twice in PBS and suspended in RPMI 1640 medium (without antibiotics but with serum and glutamine as before) plus 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. All suspensions were made to an optical density of 0.4 at 860 nm, yielding viable counts of $2.5 \times 10^8$ and $1.39 \times 10^9$ CFU/ml for *S. agalactiae* and *S. pyogenes*, respectively. One milliliter of either suspension, prepared just before use, was then added to appropriate Leighton tubes, and each tube was mixed end-over-end (1 rpm) on a tissue culture roller bottle rack. After 40 min at 37°C, nonadherent bacteria were removed by washing each cover slip with PBS (five times). Cover slips were fixed and stained as before, and the attached bacteria per 100 tissue culture cells were counted by using an oil immersion lens. A streptococcal chain was counted as 1 CFU. Data are expressed as the number of

attached bacteria as well as the number of human cells binding bacteria per 100 tissue culture cells. Nonspecific attachment of streptococci was more prevalent when glass cover slips were employed. This nonspecificity was avoided or minimized by extensive washing of all glassware with HCl before sterilization. A successful assay was dependent upon (i) cleanliness of glassware, (ii) viable but subconfluent monolayers of human cells, (iii) appropriate streptococcal cell density, and (iv) adequate washing of tissue cells to remove unattached bacteria.

**Bacterial and tissue cell pretreatments.** Human amnion cells were used to assess the role of LTA in *S. agalactiae* attachment to host cells. Bacteria or subconfluent cell monolayers were treated just before the adherence assay described above. Amnion cells were washed three times with PBS and pretreated for 30 min at 37°C with PBS (control) or with PBS containing 1 mg of TA or LTA per ml, pH 7.2. Excess TA or LTA was removed by washing once with PBS. Streptococcal pretreatments were done with a bacterial suspension (4.5 ml: optical density at 860 nm, 0.4) in Dulbecco PBS with 0.1 g of $CaCl_2$ (GIBCO) per liter diluted to 10 ml with this same PBS. Suspensions were incubated for 30 min at 37°C with the following additions or treatment: none (control), heat treated (56°C, 30 min), 5 mg of trypsin (type I; Sigma Chemical Co., St. Louis, Mo.), 10 mg of pronase (type 14; Sigma), or anti-LTA serum (1:10 final dilution) in a final volume of 10 ml. All suspensions were washed three times with PBS (not Dulbecco) without calcium and resuspended in 4.5 ml of tissue culture medium, and the bacterial adherence assay was performed as above.

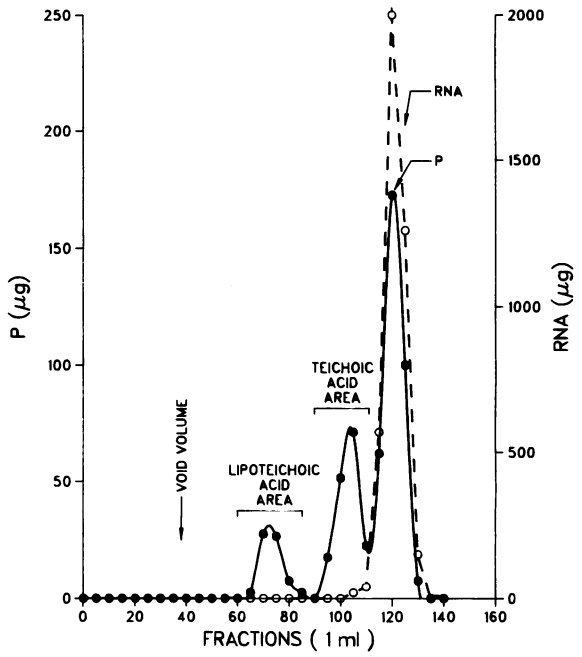



FIG. 1. Sepharose 6B column (1.5 by 81 cm) elution pattern of LTA and TA in crude extract from *S. agalactiae* obtained by hot phenol extraction. The column was loaded with 4.8 mg of phosphorus and eluted with 0.2 M ammonium acetate, pH 6.9, at a flow rate of 14 ml/h at 25°C.

## RESULTS

**LTA and TA content of *S. agalactiae*.** Elution patterns of crude cold phenol and trichloroacetic acid extracts were essentially the same as shown previously with similar extracts from *S. pyogenes* (29, 30). However, hot phenol extracts contained, in addition to LTA, a second phosphorus-containing peak (Fig. 1), which was subsequently identified as TA (see below). Table 1 presents the results of the three extraction procedures used to quantitate the LTA and TA content of this streptococcus. As is evident, the amount of LTA obtained by the hot or cold phenol method ranged from 0.58 to 0.98 mg/g (dry weight) of cells, representing at most 0.1% of the dry weight of the organism (Table 1).

The small amount of LTA present in all phenol extracts of this coccus made purification difficult (6). After initial column chromatography, LTA extracted by hot phenol contained less protein (6 to 7%) than that obtained with cold phenol (7 to 15%). Recycling of LTA through the Sepharose column resulted in a product containing approximately 5% protein, by the method of Lowry et al., regardless of the extraction method used (see above). A third passage through the column resulted in the lowest protein values possible for the LTA preparations indicated (see above).

Contaminating RNA in LTA preparations was removed by successive passages through the Sepharose column, yielding a final product almost devoid of this contaminant (<0.8% RNA, Table 1). Finally, there was no difference between the carbohydrate content of purified LTA obtained by hot phenol extraction and that obtained by cold phenol extraction (molar ratios, Fig. 2).

From an equivalent weight of cells, trichloroacetic acid extraction always resulted in yields of crude extractable material and TA in excess of those obtained by hot or cold phenol extraction (Table 1). Also, TA was easier to purify than LTA from *S. agalactiae*, mainly because of the significantly higher concentration of TA relative to its contaminants in these crude extracts. Recycling the isolated TA through the split Sephadex column three times as described previously (29) resulted in a product virtually free of protein (see above) and RNA (Table 1).

Analyses of acid hydrolysates of purified LTA and TA by paper chromatography (solvent system C [29]) failed to show the presence of galactose, rhamnose, or glucosamine. These findings indicate that group B and type III antigens were not present in these preparations.

Analyses of late-stationary (13 h) and logarithmic-stage *S. agalactiae* cells showed that their LTA and TA contents had remained the same.

**Structure of TA.** Purified TA was chosen for structural analysis because of an almost total lack of protein (0.4 ± 0.2%, see above) and RNA (Table 1) contaminants as compared with preparations of LTA. The chemical composition of TA as determined by paper chromatography after acid and alkaline hydrolysis and enzymatic analyses (29) showed glycerol, glycerophosphates, D-alanine, small amounts of D-glucose, and inorganic phosphorus. Alkaline hydrolysates after DEAE-cellulose column chromatography contained glycerophosphate, glycerol diphosphate, and small amounts of diglycerol triphosphate (29). Diglycerol triphosphate was identified by (i) paper chromatography by its characteristic $R_f$ ($R_f$, 0.12; propanol-ammonium hydroxide-water, 6:3:1 [vol/vol/vol]), (ii) the appearance of diglycerol monophosphate ($R_f$, 0.67; same solvent system) after alkaline phosphatase treatment (29), and (iii) molar ratio analysis of diglycerol triphosphate (glycerol-to-phosphorus ratio, 2.00:2.98). The presence of this product is proof of a 1-3 linkage within the TA molecule (15). The average chain length of TA, as determined from the ratio of inorganic to organic phosphorus after phosphatase treatment (8), was 22

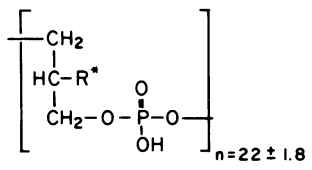
TABLE 1. TA and LTA yields from *S. agalactiae* by various methods[a]

| Extraction method and material(s) obtained | Crude extract | | LTA or TA content | | % RNA in purified preparations |
|---|---|---|---|---|---|
| | mg of P/g (dry weight) of cells | mg/g (dry weight) of cells | mg of P/g (dry weight) of cells | mg/g (dry weight) of cells[b] | |
| Cold phenol | | | | | |
| LTA | 1.87 ± 1.30 | 34.6 ± 0.7 | 0.10 ± 0.02 | 0.58 ± 0.12 | 0.62 ± 0.21 |
| Hot phenol | | | | | |
| LTA | | | 0.17 ± 0.06 | 0.98 ± 0.35 | 0.76 ± 0.04 |
| | 2.18 ± 1.05 | 48.5 ± 23.4 | | | |
| TA | | | 0.28 ± 0.10 | 1.62 ± 0.58 | 0.68 ± 0.45[c] |
| Trichloroacetic acid | | | | | |
| TA | 5.71 ± 0.03 | 124.0 ± 15.5 | 1.38 ± 0.40 | 8.01 ± 2.32 | 0.50 ± 0.01 |

[a] Results from 10 g (dry weight) of early-stationary-phase cells. Average of two determinations ± standard deviation of the mean.
[b] Calculated based on a conversion factor of 1 mg phosphorus = 5.81 mg of teichoic acid.
[c] Duplicate determination of a sample from the Sepharose column after one passage through a stacked Sephadex column.

glycerol phosphate units long. Figure 2 presents the proposed structure of TA and the molar ratios of TA and LTA from *S. agalactiae*. As is evident, molar ratio analyses ($n$ = 2) showed the presence of small amounts of D-glucose. Although no proof was obtained that D-glucose was covalently bound to TA, the fact that it was always detected in preparations almost free of protein and nucleic acid and not contaminated by other carbohydrates (type and group antigens) strongly suggests that glucose is a component of this anionic polymer. From the molar ratios obtained, TA of *S. agalactiae* is substituted with one D-alanine per 1.7 glycerophosphate units and probably one D-glucose per 2.3 TA chains.

The chemical composition of LTA as determined by both phenol extraction procedures was identical and similar to that of purified TA except that glucose was more abundant. Molar ratio analyses showed fivefold more glucose in LTA ($n$ = 2) than in TA of *S. agalactiae*. Likewise, fatty acid studies (26) established the presence of covalently linked lipid in LTA by showing the presence of 10 major fatty acids. Normal, straight chain, saturated fatty acids made up most (57.9%) of the total lipid content of LTA, with unsaturated acids (mostly *cis*-vaccenic and palmitoleic acids) and *cis*-9,10-methylenehexadecanoic acid adding to this (38.3 and 3.8%, respectively). Tridecanoic acid constituted 21.9% of all the fatty acids detected.




Molar Ratio of Purified Teichoic Acid

| Phosphorus | Glycerol | D-Alanine | Glucose |
|---|---|---|---|
| 1.00 | 1.02 ± 0 | 0.58 ± 0.13 | 0.02 ± 0.01 |

R*
D-Alanine every 1.7 units
D-Glucose every 2.3 teichoic acid chains

Molar Ratio of Purified Lipoteichoic Acid

| Phosphorus | Glycerol | D-Alanine | Glucose |
|---|---|---|---|
| 1.00 | 1.05 ± 0.07 | 0.48 ± 0.09 | 0.11 ± 0 |

FIG. 2. Proposed structure of TA and comparative molar ratios of TA and LTA from *S. agalactiae*.

**Cytotoxicity of streptococcal LTA.** A wide range of cell types was tested for their susceptibility to group B streptococcal LTA. Human amnion, brain, kidney, heart, and HeLa cells in media with 1% FBS were incubated with 250 μg of purified LTA from *S. agalactiae* per ml for 48 h. Cytotoxicity was related to the percentage of monolayer destruction by visual observation: 1+, 0 to 25%; 2+, 25 to 50%; 3+, 50 to 75%; 4+, 75 to 100%; 5+, 100%. Cells were considered nonviable when they no longer attached to cover slips and were incapable of excluding Erythrocin B dye. Although exposure to LTA produced marked destruction (3+ to 4+) of human amnion and brain cells (Fig. 3) and human embryonic kidney cells (not shown), established cell lines (Giardi heart and HeLa) were more drastically affected (5+). Cytotoxicity was not observed when TA (250 μg/ml) replaced LTA. Because of this greater sensitivity, HeLa cells were used to quantitate the toxicity of lesser amounts of LTA.

Changes in HeLa cell morphology were evident within 6 h in the presence of 100 μg of LTA per ml. Initially, adjacent cells in the monolayer separated and then rounded up. Total detachment occurred after 48 h. Also, as is evident from Table 2, as little as 25 μg of LTA resulted in a 94% detachment after 48 h. The negating effect of serum on LTA cytoxicity was noted during these experiments. Increasing the serum content of tissue culture medium from 1 to 10% produced a fivefold increase in HeLa cell numbers. However, under these conditions, 100 μg of LTA was unable to produce an observable cytotoxic effect. A higher concentration of LTA (200 μg/ml) resulted in only a 35% decrease in cell numbers as compared with untreated controls after 48 h. No difference in the toxicity of the LTA obtained by either phenol extraction method was observed.

**Adherence of *S. agalactiae* and *S. pyogenes* to host cells.** Maximum streptococcal binding was only obtained with subconfluent monolayers. As is apparent in Table 3, this group B streptococcus adhered more avidly to human amnion (14-fold) than to HeLa cells and to a larger proportion of these cells (5-fold), suggesting a possible tropism for this primary cell line rather than for the established cell line. In contrast, *S. pyogenes* showed a threefold greater binding to HeLa over amnion cells. Also, a greater percentage of HeLa cells was able to bind this streptococcus. Finally, more *S. pyogenes* than *S. agalactiae* cells attached to both cell lines. A similar percentage of amnion cells accepted both streptococci (Table 3).

**Effect of host cell or coccal treatment on adherence.** Pretreatment of amnion cells with TA or LTA did not negate the

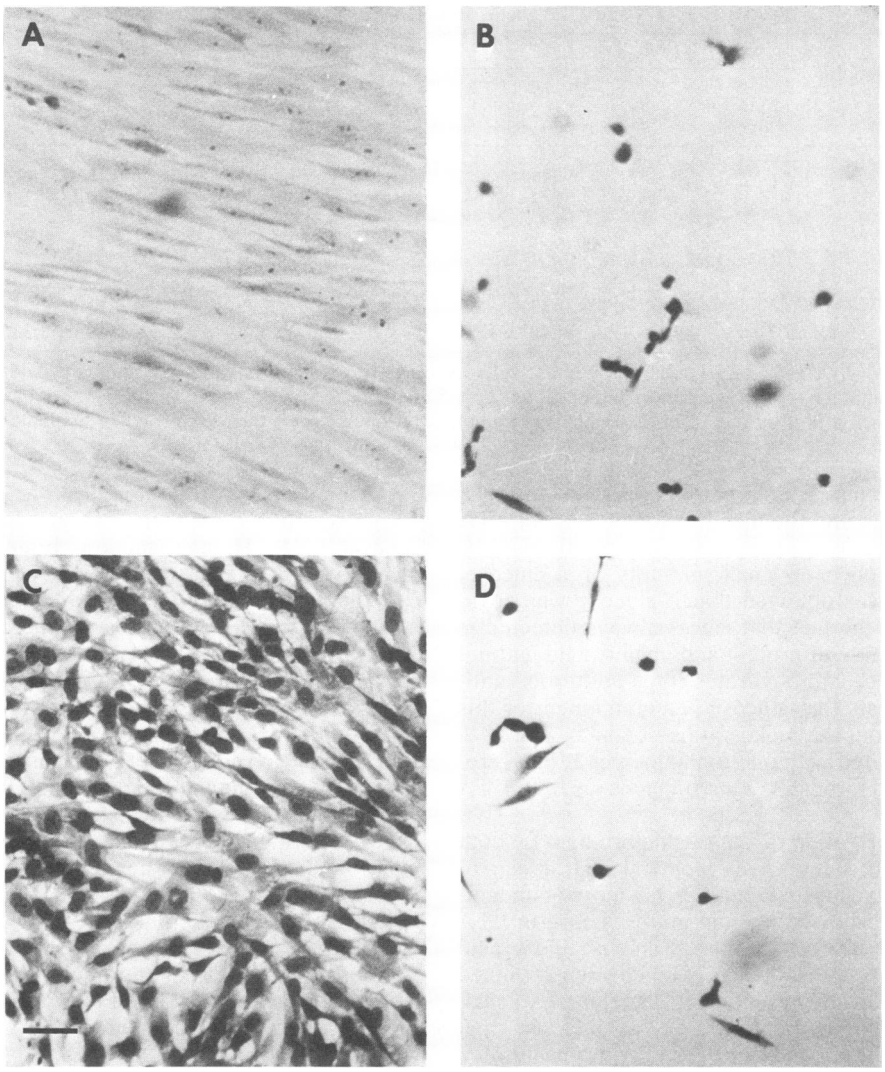



FIG. 3. Primary human embryonic amnion (GM472) and brain cells with and without exposure to LTA (250 μg/ml) from *S. agalactiae* for 48 h in MEM with 1% FBS. Amnion cells: A, control; B, exposed. Brain cells: C, control; D, exposed. Cells were washed with PBS, fixed, and stained. Bar equals 25 μm.

adherence of this strain of *S. agalactiae* to human amnion cells (Table 4). Also, prior addition of anti-LTA serum to the streptococcus did not inhibit coccal binding to this host cell line. However, coccal pretreatment with trypsin or pronase significantly reduced its binding ability, with pronase treatment causing an almost 90% reduction in coccal adherence. Heat treatment almost entirely abrogated the ability of this organism to attach to this human cell line (Table 4).

## DISCUSSION

The ratio of TA to LTA in this strain of *S. agalactiae* type III was at least 8:1, showing that TA is the most abundant form of this polymer in this organism. Only hot phenol extracts yielded a mixture of LTA and TA of which the latter predominated, confirming the dominance of TA. Finally, although it had been shown that this extraction method was efficient in extracting LTA from intact cells (24), the yields of LTA obtained by both phenol procedures, after purification, were similar.

The structure of TA from this strain of *S. agalactiae*, with the exception of its D-glucose content, was nearly identical to that detailed for *S. pyogenes* (29). This included the presence of 1–3-linked polyglycerol phosphate units, now known to be in most gram-positive bacteria (17), and a similar polyol chain length, 22 and 25 glycerol phosphate units for *S. agalactiae* and *S. pyogenes* (29), respectively. Likewise, the only substitutions on the C-2 of this polyol polymer were D-alanine and, probably, a very small amount of D-glucose. This similarity in structure and composition of TA from these two cocci was supported by the finding that antisera against purified group B LTA cross-reacted to the same extent with LTA from *S. pyogenes*.

The paucity of LTA recovered from this group B streptococcus precluded an extensive structural analysis. However, the increased glucose content of LTA over that of TA is probably due to a glucose-containing lipid comprising the complex lipid portion of this amphipathic molecule. Although the nature of this lipid was not established, bacterial glycerophosphoglycolipids are known to be anchors for membrane TA (14). The complex lipid portion of LTA from

TABLE 2. Cytotoxic effect of LTA on HeLa cell viability in tissue culture[a]

| LTA in medium (μg/ml)[b] | Mean no. of HeLa cells per 10 fields[c] | % of control | Cytotoxicity[d] |
|---|---|---|---|
| 0 | 112 ± 4 | 100.0 | 0 |
| 12.5 | 78 ± 11 | 70.0 | 2+ |
| 25.0 | 7 ± 2 | 5.8 | 4+ |
| 50.0 | 2 ± 0 | 2.2 | 4+ |
| 100.0 | 0 | 0 | 5+ |
| 200.0 | 0 | 0 | 5+ |

[a] In MEM with 1% FBS after 48 h. Results are averages of duplicate determinations.
[b] Obtained by extraction with hot phenol.
[c] Oil immersion lens.
[d] See text.

*S. agalactiae* may be identical to that from *S. pyogenes*, which is glycerophosphoryldiglucosyl diglyceride (30). Since this lipid is present in the membrane of *S. agalactiae* (13), it may comprise the complex lipid portion of the LTA of this group B coccus as well.

The finding of a relatively large amount of tridecanoic acid in the LTA of *S. agalactiae* was unexpected, since short-chain fatty acids are generally regarded as minor constituents of the membrane lipids of bacteria. LTA from *S. pyogenes* contains mostly fatty acids of a chain length greater than 16 carbon atoms (30). The significance of this difference is unknown.

The rapid morphological changes (and eventual destruction) in HeLa cell monolayers by a small quantity of LTA suggests a possible role for this amphiphile in group B streptococcal infections. It has been stated that the group B streptococci secrete LTA after exposure to penicillin (33). Thus, large numbers of organisms metabolizing on the meninges or mucosal surfaces and secreting LTA could aid in host tissue destruction by secreting this polymer directly onto the cell surface. Under these conditions, the nullifying effect of serum on LTA cytotoxicity (20; I. Ginsberg, personal communication) is minimized or negated and the effective concentration of LTA per cell possibly is increased beyond that tested for in these in vitro studies.

It seems unlikely that the cytotoxicity of LTA is due to the small amount of contaminating protein remaining since (i) very small amounts (<15 μg/ml) of LTA always produced significant cell damage after 48 h, and (ii) heat treatment (30 min, 56°C) did not negate cytotoxicity. Therefore, the destructive property of LTA is seemingly related to the complex lipid portion of the molecule; TA was nontoxic. However, Simpson et al. (28) have indicated that the disaccharide moiety of the lipid of LTA is responsible for toxicity. Their LTA from *S. pyogenes* strain LRP41 was more than twice the length (56 glycerol phosphate units) of that from this strain of *S. agalactiae*, and glucose was only present in the lipid portion of the molecule. However, their inability to (i) completely nullify LTA (or deacylated LTA) cytotoxicity by periodate oxidation, (ii) the extremely low glucose content of their long polymer, and (iii) the massive amount of LTA (and deacylated LTA) required for maximal cytotoxicity (1 mg/ml) of human cells make it difficult to ascribe their cytotoxic results to glucose alone. In our study, a very small amount of LTA (<15 μg/ml) from *S. agalactiae* destroyed a human cell monolayer even though its total glucose content was infinitesimal. In addition, earlier results with LTA from *S. pyogenes* type 12 are not in accord with those of Simpson et al. (28). The LTA from this group A coccus was shorter in length (25 glycerol phosphate units) and its polyglycerol phosphate backbone contained alanine and significantly more glucose, results which, in most respects, are similar to those obtained with the LTA from this strain of *S. agalactiae*. When this LTA was deacylated it still contained the diglucosyl component of its complex lipid plus the glucose unit(s) in the TA portion of the molecule, yet it was nontoxic (10). At present, what effect, if any, major differences in the chain length of the TA portion of LTA may have on the cytotoxic capability of LTA is unknown.

The increased toxicity of LTA for established versus primary human cell lines was unexpected. An explanation for this may reside in membrane differences between transformed (established) and normal (primary) cells (19). There is precedence for this. For example, a related amphiphile, an atypical lipopolysaccharide, has been shown to bind with greater affinity to transformed rather than to normal rat embryo fibroblasts (1). Also, LTA binds tenaciously to osteosarcoma cells and is not easily displaced from the surface of these cells (25).

The greater affinity of *S. agalactiae* for amnion versus HeLa cells may reflect a selectivity for more primitive embryonic receptors. It has been shown that group B type III strains adhere better to neonatal than to adult oral epithelial cells (5). By comparison, *S. pyogenes* attached in greater numbers to a cell line (HeLa) which was more sensitive to LTA treatment, suggesting that maximum binding and increased cytotoxicity may be mediated by a common set of receptors. Finally, attachment to only a portion (40%) of the amnion cells by both streptococci indicated that there may be a limited and cyclic expression of receptors on primary human cells in vitro. It had been suggested that streptococcal adherence to a particular subpopulation of a cell line may be a property of all human cells growing in vitro (2).

TABLE 3. Variable adherence of *S. agalactiae* and *S. pyogenes* to selected human cells in tissue culture

| Cell line | *S. agalactiae* type III | | *S. pyogenes* type 12 | |
|---|---|---|---|---|
| | Mean CFU bound per 100 host cells[a] | % Receptive host cells[b] | Mean CFU bound per 100 host cells[a] | % Receptive host cells[b] |
| Established | | | | |
| HeLa | 11.9 ± 6.6 | 8.2 ± 5.5 (4) | 1,201.8 ± 228 | 89.5 ± 8.8 (10) |
| Primary | | | | |
| Amnion | 172.6 ± 44.6 | 40.6 ± 6.6 (10) | 333.7 ± 42 | 42.1 ± 4.7 (10) |

[a] With use of oil immersion lens, 1 CFU = 1 to 10 individual coccal cells per chain.
[b] Only those binding streptococci. The number of cover slips counted (two to five experiments performed in duplicate) is shown in parentheses.

TABLE 4. Cell treatments[a] and changes in bacterial adherence

| Cells | Pretreatment | Mean CFU bound per 100 host cells | % Receptive host cells[b] |
|---|---|---|---|
| Amnion | None | 151.8 ± 35 | 38 ± 4.1 (5) |
| | TA (1 mg/ml) | 143.3 ± 3 | 36 ± 3.1 (4) |
| | LTA (1 mg/ml) | 162.9 ± 14 | 36 ± 0.8 (4) |
| *S. agalactiae* | None | 193.4 ± 46 | 42 ± 7.3 (5) |
| | Anti-LTA serum (1:9 dilution) | 171.6 ± 42 | 41 ± 4.5 (4) |
| | Trypsin (0.5 mg/ml) | 91.8 ± 27 | 27 ± 4.8 (4) |
| | Pronase (1 mg/ml) | 19.4 ± 6 | 16 ± 3.3 (4) |
| | Heat | 2.5 ± 1 | 2 ± 1 (4) |

[a] Duration of treatments was 30 min in PBS at 37°C except for heat treatment. Untreated cells were held in PBS for the corresponding time period.

[b] The total number of cover slips counted from separate experiments is shown in parentheses.

LTA has been shown to inhibit the adherence of another type III strain of *S. agalactiae* to oral epithelial cells (7). Also, Nealon and Mattingly have shown that virulent group B streptococci contain five times more LTA than avirulent strains and that their binding to human embryonic and fetal epithelial cells is LTA mediated (24). In the present study, we showed that a different strain of *S. agalactiae* with only a very small amount of LTA adheres to a human cell line, but not by an LTA-mediated mechanism, suggesting instead a protein-mediated attachment. Therefore, these collective data indicate that different adherence mechanisms for virulent and avirulent strains may exist. It is known that the content of LTA in streptococci grown in vitro is influenced by the growth conditions of the culture (18). However, the effect of such changes in vivo is unknown. Nevertheless, the adherence of *S. agalactiae* to human cells by an LTA-mediated mechanism (and the resulting cytotoxic effects of this amphiphile) or by a protein-mediated mechanism may be a deciding factor in the initiation of group B streptococcal pathogenesis.

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