CHARACTERIZATION AND LOCALIZATION OF THE ENZYMATIC DEACYLATION OF LIPOTEICHOIC ACID IN GROUP A STREPTOCOCCI*

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The excretion of lipoteichoic acid (LTA)[1] from Gram-positive bacteria has been clearly established (1–5). Extracellular LTA may consist of fully acylated LTA and deacylated LTA (dLTA) in proportions ranging from almost entirely LTA (1) to entirely dLTA (2, 3). In addition, the precursor:product relationships of intracellular LTA and extracellular dLTA (3), and an enzyme activity appropriate for the conversion (6), have been shown with *Streptococcus faecium.* The enhanced release of LTA and, possibly dLTA, from *Streptococcus sanguis* and a group A streptococcal strain in the presence of penicillin has been reported (4, 5). The temporal and causal relationships of this release with other manifestations of penicillin treatment remain to be established. From a pathobiological viewpoint, the potential involvement of the release of these polymers in the interaction of pathogenic and commensal Gram-positive bacteria with the host may prove interesting.

Recently the observation was made that Triton X-100 extracts of membranes of group A streptococci contained greater amounts of dLTA than LTA (7). This did not appear to be the result of chemical deacylation during extraction because there was no measurable quantitative change in isolated LTA under the same conditions (7). Also, the electrophoretic mobility of the poly(glycerol phosphate) polymer that was obtained by resuspending live streptococci in buffer (8, 9) is probably more consistent with that of dLTA than LTA. Thus, the existence of an enzyme similar to the deacylating enzyme of *S. faecium* (6) was hypothesized. Evidence is presented here for the presence of such an enzyme in group A streptococci. The subcellular location and the cleavage sites on the LTA molecule are also described.

Materials and Methods

Streptococcal Strains and Growth. The group A streptococcal strains used in this study were S43/192/1, T4/95/3, T27A (A variant), S23 Opaque, London Opaque, and London Blue. These strains have all been maintained in our laboratory collection at The Rockefeller University, New York. For most of the experiments, these strains were grown in a Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and yeast extract dialysate medium which has been described in detail elsewhere (10). Growth was measured turbidimetrically in 16-mm tubes using a Coleman 44 spectrophotometer (Perkin-Elmer Corp., Instruments Div., Norwalk,

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[1] *Abbreviations used in this paper:* dLTA, deacylated lipoteichoic acid; Glc, glucose; LTA, lipoteichoic acid; P, phosphorus; XIE, crossed immunoelectrophoresis.

Conn.) at 650 nm. Cultures termed "exponential" were harvested at least one generation before the cessation of exponential growth. Stationary-phase cultures were timed from the onset of deviation from exponential growth.

For the incorporation of radioactive glycerol, S43/192/1 was grown to early stationary phase (~1 h into stationary phase) in Todd-Hewitt broth containing 0.4% glucose and 2 μCi/ml [2-^{3}H]glycerol (500 mCi/mmol, Amersham Corp., Arlington Heights, Ill.).

Incubation of Cells in Buffer. Each of the six streptococcal strains was grown in 300 ml of medium at 37°C. At OD ≅0.2 (exponential phase), 150 ml of each culture was withdrawn, rapidly chilled to 4°C, and centrifuged at 8,000 *g* for 20 min. The remaining 150 ml was kept at 37°C overnight (stationary phase) and then handled in the same manner as the exponential phase sample. After removal of the supernatant medium, the cells were resuspended in 20 ml of cold medium, evenly divided between two 15-ml centrifuge tubes and sedimented at 15,000 *g* for 15 min. The supernatant medium was removed. One cell pellet was immediately frozen at −20°C whereas the other was resuspended in 4 ml of 0.2 M sodium acetate, pH 6 (9) and incubated at 37°C for 30 min. The cells and buffer were separated by centrifugation at 15,000 *g* for 15 min. The buffer was frozen at −20°C and two further buffer resuspensions and incubations were carried out as with the first. After the final centrifugation, the cells were frozen at −20°C. Estimation of the LTA and dLTA contents of cells and buffer were carried out as detailed below.

After incubation of S43/192/1 in buffers below pH 6.5, increased amounts of LTA were found, but above pH 6.5 the extracellular buffer contained almost entirely dLTA (>99%). All further experiments with S43/192/1 were performed using 0.05 M Tris-HCl, 1 mM $MgSO_4$, pH 6.9.

Temperature Effects on Release of dLTA From Cells in Buffer. S43/192/1 was grown to early stationary phase (1 h), harvested, and resuspended in 1/40 vol (compared to the original culture volume) of 0.05 M Tris-HCl, 1 mM $MgSO_4$, pH 6.9. Equal portions were: (*a*) heat-killed at 65°C for 15 min and then incubated at 37°C for 2 h, (*b*) incubated at 37°C for 2 h, and (*c*) incubated at 0°C for 2 h. The cells were removed by centrifugation and the supernatant buffer of each sample examined for dLTA content as detailed below.

LTA Extraction and Quantitation. LTA and dLTA were extracted from cells with 45% aqueous phenol at 68°C (11) as detailed for LTA elsewhere (3, 12). The aqueous phases were dialyzed against distilled water and lyophilized. After reconstitution in an appropriate volume of distilled water, portions of each were analyzed by crossed immunoelectrophoresis (XIE) using high-titer anti-poly(glycerol phosphate) serum. Buffer samples were analyzed directly without prior extraction. XIE was carried out in the presence of Triton X-100 (13) using a modification (14) of the Laurell technique (15). Barbital HCl buffer (ionic strength, 0.02, pH 8.6) containing 1% Triton X-100 was used throughout. 1% agarose gels were prepared from Seakem HGT (Marine Colloids, Inc., Rockland, Maine) and cast on glass plates (50 × 50 × 0.6 mm) to give a volume to surface area ratio of 0.132 ml/cm^2. Samples were applied to wells of minimal diameter and electrophoresed at 6 V/cm for 40 min in Behring Diagnostic water-cooled immunoelectrophoresis cells (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.). After electrophoresis, an agarose strip (10 × 50 mm) containing the separated antigens was retained on each plate while the rest of the gel was removed and replaced with 1% agarose containing anti-poly(glycerol phosphate) serum. Electrophoresis was then carried out perpendicular to the original direction at 2 V/cm for 16–20 h. Gels were pressed and washed with 0.1 M NaCl. After a final press, they were briefly rinsed with a stream of distilled water and dried at 60°C before staining with Coomassie brilliant blue R-250 (16).

The amounts of LTA and dLTA in each sample were estimated by projecting each immunoplate with a photographic enlarger (~10-fold increase in area), tracing the image, weighing three photocopies of each tracing, and comparing the mean weight of the copies to standard curves for LTA and dLTA. After normalization for phosphorus content, the standard curves for LTA and dLTA were nearly equivalent between 10 and 200 ng with the serum used. Standard LTA and dLTA were prepared as previously described (3, 7, 12).

Preparation of Solubilized Wall Fraction, Protoplasts, Crude Membranes, and Cytoplasm. Protoplasts of S43/192/1 were prepared using the group C phage-associated lysin as previously detailed (10). Briefly, washed streptococci were resuspended in 60 mM sodium-phosphate buffer (pH 6.1) containing 0.5 mM dithiothreitol, 30% raffinose and 10,000 U per ml lysin. After what

appeared to be 100% protoplast formation (1 h at 37°C), the incubation was continued ~30 minutes longer. After sedimentation at 10,500 *g*, the protoplasts were washed once in 0.05 M Tris-HCl, 1 mM $MgSO_4$, 30% raffinose, pH 6.9, and resuspended in the same buffer (fresh). Crude membranes and cytoplasm were prepared by sedimenting the protoplasts at 10,500 *g* and resuspending them in hypotonic buffer (0.05 M Tris-HCl, 1 mM $MgSO_4$, pH 6.9) with 0.1 mg/ml RNase and 0.1 mg/ml DNase (Sigma Chemical Co., St. Louis, Mo.). After incubation at 37°C for 15 min to permit complete lysis, the membranes were removed by centrifugation at 20,000 *g* for 40 min. Small membrane fragments were removed from the supernatant (cytoplasm) fraction by centrifugation at 100,000 *g* for 60 min. This ultracentrifugation step was also carried out on the solubilized wall fraction to insure removal of membrane fragments from any protoplasts that may have burst during wall removal.

Comparison of LTA and dLTA Contents of Triton X-100 Extracts and Aqueous Phenol Extracts of Isolated Membranes. Equal amounts of S43/192/1 membranes (2-h stationary phase) from previous studies (7, 10) were extracted with: (*a*) 45% aqueous phenol (3, 12) and (*b*) 4% Triton X-100 in 0.05 M Tris-HCl, pH 8.6 at room temperature for 1 h (7). The residual membrane from the latter was extracted with 45% aqueous phenol. The LTA and dLTA contents of each extract were estimated as described above.

Quantitation of Deacylase Activity. Based on a previous assay procedure (6) in which the conversion of exogenously added radioactive LTA to dLTA was monitored by agarose gel electrophoresis, as well as the excellent separation of LTA and dLTA achievable by XIE (7), a new assay procedure was devised for quantitation of radioactive LTA and dLTA. In a typical experiment, 15–50 μg/ml of [2-^{3}H]glycerol-labeled LTA (~400 cpm/μg) was incubated in each control or experimental sample. After incubation, protoplasts or membranes were removed by centrifugation in a Microfuge B (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). Each supernate was carefully removed and then dialyzed against distilled water at 4°C with three changes. Buffer controls and any other soluble samples were also dialyzed against distilled water at 4°C. Each sample was lyophilized in conical tubes and resuspended in 50 μl of distilled water. Portions (5–10 μl) of each sample were analyzed by electrophoresis as follows. 1% agarose gels were cast on 50- × 50-mm glass plates as for XIE (see above). Three sample wells were punched 1.5 cm apart in a row 1 cm from one side, with the center well 2.5 cm from the top and bottom edges. Samples (5–10 μl) containing radioactive LTA and/or dLTA were applied to the wells and electrophoresed as for XIE at 6 V/cm for 40 min. The agarose was then cut 0.5 cm to either side of each well in a direction parallel to the current flow. 10 0.5-cm slices were cut perpendicular to the current flow and each 0.5- × 1.0-cm slice was placed in a scintillation vial. After solubilization with 0.5 ml of 90% Protosol (New England Nuclear, Boston, Mass.) at 55°C for 2–3 h, and return of the vials to room temperature, 5 ml of Aquasol-2 (New England Nuclear) was added to each vial. Vials were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Analytical Methods. Phosphorus was measured by the method of Lowry et al. (17) with KH_2PO_4 as the standard. Carbohydrate and fatty acid analyses were carried out by gas-liquid chromatography as previously described (10). The attempted reduction of glucosyl constituents within LTA and dLTA was carried out under the conditions described by McLean et al. (18) with [^{1}H]borohydride rather than [^{3}H]borohydride. Glucose and cellobiose were used as controls. Products of reduction were quantitated using gas-liquid chromatography (10).

Antiserum. The antiserum used in this study was selected from a large group obtained in the course of preparing typing sera for group A streptococci. At the ratio of serum to agarose used for XIE in these experiments (5 μl per ml), 10–200 ng of LTA or dLTA could be quantitated. At lower ratios, as little as 0.1 ng was detectable. Group A carbohydrate was not detectable at this ratio of serum to agarose. In addition, the specificity of the reaction was confirmed using anti-poly(glycerol phosphate) sera kindly supplied by Dr. K. W. Knox of the United Dental Hospital, Surrey, New South Wales, Australia and Dr. B. Rosan, of the School of Dentistry, University of Pennsylvania, Philadelphia, Pa.

Results

Loss of LTA and dLTA from Streptococci in Buffer. Various streptococcal strains were incubated in buffer to determine whether both LTA and dLTA were lost from the

TABLE I

Cellular and Extracellular LTA and dLTA after Incubation of Various Streptococcal Strains in Buffer

Strain	Growth phase	Cellular				Extracellular postincubation	
		Preincubation		Postincubation			
		Total*	Percentage of LTA	Total	Percentage of LTA	Total‡	Percentage of LTA
			%		%		%
T4/95/3	Exponential (E)	2.2	75	1.9	100	0.3	5
	Stationary (S)	6.6	78	7.7	83	0.6	2
S43/192/1	E	2.1	95	1.9	100	0.8	13
	S	5.9	70	5.1	75	0.9	6
S23 Opaque	E	2.3	84	1.3	76	1.8	51
	S	4.7	85	5.5	62	0.4	10
London Blue	E	1.3	84	1.6	80	0.9	21
	S	3.2	75	3.0	80	0.7	2
London Opaque	E	0.8	100	1.1	82	0.6	22
	S	4.6	82	4.0	62	0.9	0
T27A	E	2.0	100	2.2	79	1.3	25
	S	8.0	71	5.9	69	3.5	7

* Expressed as μg of total (LTA + dLTA) per ml of original culture.

‡ The amount of total (LTA + dLTA) found extracellularly after three consecutive buffer incubations. Expressed as μg excreted from the cells contained in 1 ml of original culture.

cells and to determine the intracellular and extracellular proportions of each. From this experiment and the effects of heat and cold on the process, it was anticipated that there would be some suggestion of whether the loss from the cells was a result of simple extraction or a biological mechanism. Incubation of live streptococci in 0.2 M sodium acetate buffer pH 6 yielded varying amounts of LTA and dLTA in the extracellular buffer (Table I). Although the cells were resuspended and incubated in buffer three consecutive times, the data for all three samples have been combined for presentation here. In some cases, greater amounts of both polymers were found in the first buffer sample, but in others greater amounts were found in the second sample of the series. Generally, the third sample of each series contained much less of either polymer. However, the relative amounts in each respective buffer sample were not consistent within a given strain; i.e., when comparing cells harvested during the exponential phase of growth and those harvested during stationary phase. It is important to note that the percentage of LTA of total (LTA + dLTA) is much lower in the extracellular buffer than in cells. That is, a greater proportion of dLTA, compared to LTA, was lost from the cells during incubation in buffer.

After resuspension of S43/192/1 in 0.05 M Tris-HCl, 1 mM $MgSO_4$, pH 6.9, at least 99% of the total (LTA + dLTA) was dLTA. Fig. 1 illustrates that the loss of dLTA by S43/192/1 in this latter buffer was apparently linear over the period of time shown (0–3 h). After 3 h of incubation, the rate of loss decreased. The increase

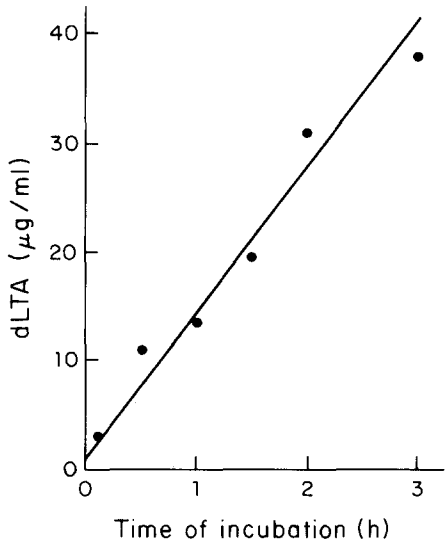

FIG. 1. Increase in dLTA after resuspension of cells in buffer. S43/192/1 was grown to late exponential phase (OD = 0.37), harvested by centrifugation, washed and resuspended in 0.05 M Tris-HCl, 1mM $MgSO_4$, pH 6.9 (concentrated 40 times compared to the original culture volume). Samples were removed at the times shown. After centrifugation, the top 0.5 ml of each 0.6 ml sample was carefully removed and analyzed by XIE.

in dLTA in buffer was negligible after either heat-killing of the streptococci before incubation at 37°C, or incubation at 0°C (Table II).

Comparison of Aqueous Phenol and Triton X-100 Extracts of Isolated Membranes. A second indication that an enzyme similar to that in *S. faecium* (6) might account for the release of dLTA was shown by a comparison of Triton X-100 and aqueous phenol extraction of isolated membranes. Because Triton X-100 extraction was carried out at room temperature over 60 min, retention of enzymatic activity during extraction could account for the large amount of dLTA previously observed (7). There was a greater amount of dLTA and a correspondingly lower amount of LTA in the Triton X-100 extract than in the hot aqueous phenol extract (Table III). This could not have been the result of differential extraction of LTA and dLTA by the nonionic detergent because very little additional LTA and dLTA was present in the aqueous phenol extract of cells that were previously extracted with the detergent. In addition, there was close agreement in total (LTA + dLTA) by the two methods.

Demonstration and Localization of Enzyme Activity to the Membrane. Direct evidence for a deacylating enzyme was obtained by adding exogenous [2-^{3}H]glycerol-labeled LTA to protoplasts and examining the extracellular buffer for conversion of LTA to dLTA. Later, various subfractions, including membranes and cytoplasm, were also examined. Fig. 2 illustrates the conversion of LTA to dLTA by crude membranes and the correlation of the one-dimensional electrophoretic assay (Fig. 2A and B) with XIE of LTA and dLTA (Fig. 2C and D). The subcellular localization of enzyme activity is shown in Table IV. The protoplast membrane apparently contained the bulk of the measurable activity, although there was also some activity present in the cytoplasm. There appeared to be no measurable activity in the solubilized cell wall fraction or the spent culture medium. Also the enzyme was not excreted, at least in active form, during incubation of protoplasts in buffer (with osmotic support).

TABLE II

Temperature Effects on Release of dLTA from Cells in Buffer

Treatment of cells	Incubation temperature post-treatment	Time	Fold increase relative to zero time at 37°C and no treatment of cells
	°C	h	
None	37	0	—
		2	9.0
	0	0	0.8
		2	1.2
Heat-killed (65°C, 15 min)	37	0	0.9
		2	1.2

TABLE III

Comparison of the LTA and dLTA Contents of Triton X-100 and Phenol-Water Extracts of Isolated Membranes

Extract	LTA	dLTA
	μg/mg*	μg/mg*
Phenol:water‡	12.1	5.6
Triton X-100§	8.2	10.2
Phenol:water‡ after Triton X-100	0.2	0.1

* μg/mg membrane.
‡ 45% phenol in water, 68°C, 30 min.
§ 4% Triton X-100, 0.05 M Tris-HCl, pH 8.9, 20°C, 60 min.

Characterization of the Cleavage Site(s) within the LTA Molecule. The lipid moiety of group A streptococcal LTA has been determined to be glycerophosphoryldiglucosyl diglyceride (Fig. 3) (19). The presence of fatty acids on LTA and absence of fatty acids on dLTA was confirmed by gas-liquid chromatography. Although quantitative analysis was not done, only trace amounts of fatty acids at levels 20- to 50-fold lower than that in LTA were detected in occasional samples of dLTA. In addition, sensitization of erythrocytes to agglutination by anti-poly(glycerol phosphate) serum was readily accomplished with 10 μg/ml LTA but no sensitization was achieved after incubation of erythrocytes in the presence of 50 μg/ml dLTA. To begin to elucidate whether the cleavage site(s) of the putative deacylating enzyme was (were): (*a*) within this lipid moiety, (*b*) between it and the poly(glycerol phosphate) backbone, or (*c*) at some point along the backbone, the chemical composition of isolated LTA and native dLTA were compared. Native dLTA was isolated by column chromatography of concentrated, dialyzed buffer in which live streptococci had been incubated at 37°C for 90 min (Materials and Methods). The phosphorus and glucose contents and the corresponding ratios of these two components were nearly identical for the two polymers (Table V), indicating that both glucose molecules of the lipid moiety remain intact (Fig. 3). If cleavage occurred at position 2 of Fig. 3, then reduction of dLTA, but not LTA, by sodium borohydride ($NaBH_4$) should yield glucitol. There was no reduction of LTA glucose or dLTA glucose by $NaBH_4$ (Table VI). Under the same

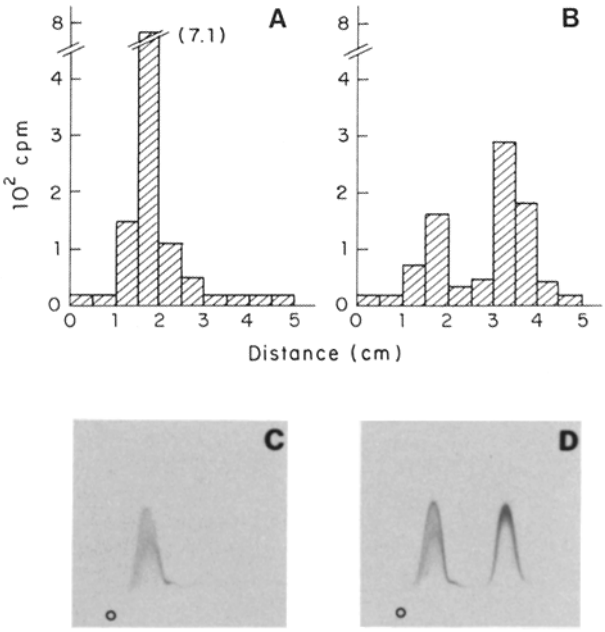

FIG. 2. One-dimensional electrophoresis of ^{3}H-labeled LTA after incubation without (A), or with (B) streptococcal membranes compared to XIE of LTA (C) and LTA plus dLTA (D). First-dimension electrophoresis for all was 6 V/cm for 40 min, anode to the right. In C and D, 0.2 μg of LTA and 0.2 μg each of LTA and dLTA, respectively, were electrophoresed. The second-dimension electrophoresis in C and D was at 2 V/cm for 16 h, anode at the top, against 5 μl/ml anti-poly(glycerol phosphate) serum.

conditions, an equivalent amount of glucose was completely reduced and nearly one-half of the glucose within cellobiose (4-0-beta-D-glucopyranosyl-D-glucose) was reduced (Table VI). The inability to generate glucitol from dLTA suggests that the C-1 position of the internal glucose of the glycolipid remained blocked, presumably by glycerol.

Discussion

The percentage of LTA of the total (LTA + dLTA) in aqueous phenol extracts of whole cells is much higher than the corresponding percentage of LTA in extracellular acetate buffer at pH 6. This difference can be interpreted either to indicate that dLTA is easier to extract than LTA or to be evidence for enzymatic conversion of LTA to dLTA and subsequent loss from the cell. The latter is more likely, considering there was little or no loss of dLTA by cells incubated at 0°C or by heat-killed cells incubated at 37°C. In addition, the loss of dLTA into buffer continued at a steady rate for at least 3 h after suspension. The variation in the percentage of LTA (intracellular) between strains may reflect chemical deacylation during the aqueous phenol extraction or dLTA in transit out of the cell. In some cases, the amount of intracellular LTA post-incubation was higher than the amount of LTA pre-incubation. This may be a result of either experimental error or, more likely, of continued synthesis of LTA at the expense of cellular lipids (3, 20, 21) during the incubation of cells in buffer.

Another piece of indirect evidence for enzymatic release of dLTA is the increased

TABLE IV
Enzymatic Activity of Cellular and Extracellular Fractions

Fraction	Time of incubation with [^{3}H]LTA	Percentage of initial counts per minute recovered*	Percentage of recoverable counts per minute in dLTA
	h	%	%
Protoplasts from exponential phase cells	2	63	32
	17	58	64
Protoplasts from stationary phase cells	2	54	34
	4	61	43
	15	53	64
Membranes	2	75	37
	4	93	63
Cytoplasm	4	95	24
Solubilized wall	4	95	0
Spent culture medium	4	100	0
Extracellular buffer after 1-h incubation with protoplasts	4	98	0–5
Protoplast buffer alone	2	100	0
	17	100	0

* The percentage of initial counts per minute added to the sample that remained soluble after incubation. Does not take into account LTA absorbed to protoplasts or membranes because only the supernate of these samples was analyzed.

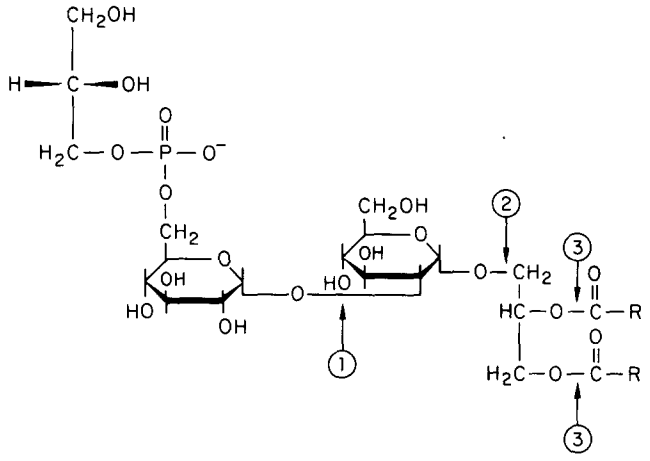

FIG. 3. Structure of the glycerophosphoryldiglucosyl diglyceride portion of LTA from *Streptococcus pyogenes* (19).

amount of dLTA found in Triton X-100 extracts of isolated membranes when compared to an aqueous phenol extract of the same membrane. The latter extraction rapidly denatures proteins and probably inactivates most enzymes, whereas the former procedure may allow continued enzymatic activity. The aqueous phenol extraction as

TABLE V

Comparison of the Phosphorus and Glucose Contents of LTA and dLTA

	Phosphorus	Glucose	P:Glc
	μmol/mg	*μmol/mg*	
LTA*	3.37 ± 0.09	0.314 ± 0.015	11:1
dLTA‡	3.44 ± 0.13	0.306 ± 0.022	11:1

* Mean of four preparations, each analyzed in duplicate (glucose) or triplicate (phosphorus), ± SEM.

‡ Mean of two preparations, each analyzed in duplicate (glucose) or triplicate (phosphorus), ± SEM.

TABLE VI

Absence of Glucosyl Reducing Groups in LTA or dLTA

	Treatment	Glucose	Glucitol
		nmol	*nmol*
LTA*	None	63.1 ± 1.3	<1.0
	$NaBH_4$§	64.7 ± 1.0	<1.0
dLTA‡	None	61.2 ± 0.9	<1.0
	$NaBH_4$	63.9 ± 0.6	<1.0
Glucose	None	70.1 ± 0.5	<1.0
	$NaBH_4$	<1.0	70.6 ± 1.0
Cellobiose	None	68.0 ± 0.5	<1.0
	$NaBH_4$	33.5 ± 3.0	28.2 ± 0.6

* Mean of three preparations ± SEM.

‡ Mean of two preparations ± SEM.

§ Sodium borohydride reduction (Materials and Methods).

a control is less than ideal, however, as a result of the possibility of chemical deacylation during heating at 68°C. Despite this drawback, the data is interpreted to suggest enzymatic release of dLTA during incubation of isolated membranes in buffer containing Triton X-100. It is important to note that incubation of isolated LTA in the same buffer does not result in any measurable deacylation (7).

As with *S. faecium* (6), incubation of [2-^{3}H]glycerol-labeled LTA with protoplasts of S43 can be shown to result in conversion of the LTA to dLTA. The enzyme in S43/192/1 appears to be located in the membrane, although activity is also detectable in the crude cytoplasm. Comparison of the relative amounts of enzyme and/or enzyme activity within the membrane and cytoplasm is difficult because assay of the cytoplasm involves a soluble system whereas the assay of membranes is a two-phase system. In the latter, the adsorption of LTA to the membrane may be a rate-limiting step before deacylation. Also, the membrane contains residual LTA that may compete with the exogenous radioactive LTA in the enzyme assay. Thus, without analysis of adsorption kinetics as well as enzyme kinetics or selective solubilization of the enzymes from the membrane, it is difficult to determine the subcellular distribution of the enzyme. Certainly, the apparent membrane localization of LTA (22) makes a membrane location more likely than a cytoplasmic location.

Characterization of the cleavage site(s) within the glycolipid portion of LTA was approached by comparing the chemical compositions of LTA which was extracted

from cells and dLTA which was isolated from the extracellular fluid after incubation of cells in buffer. Assuming the average poly(glycerol phosphate) chain length of LTA and dLTA to be equal, a higher phosphorus (P) to glucose (Glc) ratio (P:Glc) in dLTA than LTA would suggest cleavage by a glycosidase between the two glucoses of the glycolipid moiety (arrow 1, Fig. 3). This change in P:Glc would be readily detectable because group A streptococcal LTA appears to contain very little, if any, glucose outside of the glycolipid. That is, if one assumes all of the glucose to be contained within the glycolipid, then the P:Glc of 11:1 shown in Table V would give a calculated chain length of 22 glycerol phosphate units which agrees well with a previously published estimate for LTA from another strain of group A streptococcus (19). It should be noted that the calculation of chain length based on the ratio of total phosphorus available to phosphomonoesterase would be inappropriate unless the cleavage of LTA that results in dLTA occurred within the chain or at the phosphodiester bond linking the chain to the glycolipid. In any event, the P:Glc, P:dry weight and Glc:dry weight ratios for dLTA were nearly equal to the corresponding ratios for LTA. This is interpreted to indicate that both glucoses remain intact on dLTA.

If the cleavage occurred between the glycerol of the diglyceride and the internal glucose of the glycolipid (arrow 2, Fig. 3), then the internal glucose of the glycolipid (which would be the external glucose on dLTA) should contain a reducing group in dLTA but not in LTA. Treatment of dLTA, but not LTA, with $NaBH_4$ would result in generation of D-glucitol after acid hydrolysis. The absence of D-glucitol after treatment of dLTA with $NaBH_4$ suggests the C-1 position of the internal glucose of the glycolipid remains blocked. Therefore, the cleavage sites are most likely at position 3 in Fig. 3. Thus, it appears that the conversion of LTA to dLTA takes place by true deacylation. Whether a single lipase or two lipases are responsible for deacylation remains to be determined.

The relative effects of release from group A streptococci of dLTA, as well as LTA, on host defense mechanisms or adherence remain to be assessed. For example, chemically deacylated LTA has been shown to fail to inhibit the binding of streptococci to mucosal cells (23) and to fail to inhibit platelet aggregation induced by collagen or its denatured alpha-1 chain (24). However, the mild base conditions used to hydrolyze fatty acids from LTA may alter the molecule itself or, perhaps, some minor, but important, constituent that remains intact on native dLTA. Thus, comparisons of the effects of LTA and dLTA may require use of native dLTA rather than that obtained by base hydrolysis. In addition, the amount of released dLTA relative to extracellular and intracellular LTA may be important in evaluating the impact of release after deacylation on the interactions of streptococci and their hosts.

Summary

Protoplasts of a group A streptococcal strain were shown to contain enzymatic activity capable of converting lipoteichoic acid (LTA) to deacylated lipoteichoic acid (dLTA). The enzyme(s) appear to be located mainly in the membrane, although activity was also found in the cytoplasm. Determination of the sites of cleavage within the LTA molecule was approached by comparing the chemical composition of LTA and native dLTA. Native dLTA, as distinguished from chemically deacylated LTA, was isolated from buffer in which live streptococci had been resuspended and incubated. The chemical data suggest that the enzyme(s) was(were) lipolytic in

nature; that is, the conversion of LTA to dLTA was the result of cleavage of the ester linkages between the fatty acids and the remainder of the LTA molecule.

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