CELL MEMBRANE-BINDING PROPERTIES OF GROUP A STREPTOCOCCAL LIPOTEICHOIC ACID*

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The teichoic acids (TA)[1] of group A streptococci and other gram-positive bacteria are known to adsorb spontaneously to red blood cells (RBC) and a variety of other mammalian cells (1, 2). Such affinity of TA for cell membranes has been of special interest because (a) infections of TA can produce experimental nephritis (3) or arthritis (4) in laboratory animals by binding to tissue membranes and provoking local immunotoxic reactions; (b) it has been postulated (2) that TA may serve as a carrier of other streptococcal antigens and bind them to specific organ tissues where they could produce immunopathological lesions; and (c) small amounts of TA may reside on the surface of virulent streptococci and, thereby, mediate their adherence to mucosal surfaces (5). The component(s) responsible for TA binding to cell membranes, however, have not been clarified.

The chemical composition and cell membrane affinity of streptococcal TA vary according to the method of extraction and purification. Matsuno and Slade (6) extracted TA with trichloroacetic acid (TCA). This extract contained polyglycerolphosphate (PGP) and small amounts of alanine, hexoses, and hexosamines but was not able to sensitize RBC (7). Jackson and Moskowitz (8) extracted TA with phenol. This extract contained PGP with relatively large amounts of ester-linked alanine, but no hexoses or hexosamines. In addition to alanine, Miller and Jackson (9) in preliminary studies observed the presence of small amounts of lipids in such TA preparations. Unlike the TCA extract, however, the phenol extract of TA was able to sensitize RBC spontaneously (8).

Similar observations were reported by Wicken, Knox, and Hewett (10–12) using *Lactobacillus fermenti* TA obtained by TCA and phenol extractions. Chorpenning and Stamper (13) reported that purified *Bacillus subtilis* TA obtained by phenol extraction was devoid of alanine and was capable of sensitizing RBC. In view of these studies, it was of interest to define more precisely the moieties of group A streptococcal TA involved in affinity for cell membranes in order to elucidate its possible role in pathogenesis of streptococcal diseases.

The results of the present investigation indicate that small amounts of fatty acid moieties that are ester-linked to PGP play a major role in the affinity of streptococcal TA for mammalian cell membranes. Moreover, we present evidence to suggest that a portion of these moieties of TA are exposed on the surface

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[1] *Abbreviations used in this paper:* DMF, N,N-dimethyl formamide; FI and FII, fractions 1 and 2; IE, isoelectric; LTA, lipoteichoic acid; PBS, 0.02 M phosphate-buffered 0.9% NaCl; PGP, polyglycerophosphate; TA, teichoic acid; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

of group A streptococci and may be involved in the adherence of these organisms to human oral epithelial cells. Since we found that preparations that exhibited membrane-binding activity contained ester-linked fatty acids, hereafter we shall refer to this active substance as lipoteichoic acid (LTA).

Materials and Methods

Extraction of Lipoteichoic Acid (LTA) from Streptococci. The IRP41 (T type 28, M type 13) strain of group A streptococci was kindly furnished by Dr. Rebecca Lancefield, The Rockefeller University, New York. This strain previously has been shown to yield satisfactory quantities of relatively pure teichoic acid (14). The organisms were grown in 15 liter batches in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h, collected by continuous-flow centrifugation (Ivan Sorvall, Inc., Norwalk, Conn.), and washed three times in distilled water. LTA was extracted by the method of Moskowitz (14) with minor modifications. 5 g (wet-packed weight) of washed streptococci were resuspended in 50 ml of 0.2 N HCl and heated in a boiling (95°C) water bath for 10 min. The cells were then washed six times with ice-cold distilled water and finally resuspended in 10 ml distilled water. 10 ml cold 95% phenol was added and the suspension was gently mixed and centrifuged at 10,000 g for 30 min. The aqueous phase which contained the LTA was removed with a pipette, dialyzed against six changes of distilled water for 3 days, and lyophilized. The yield was 13–15 mg of lyophilized material from 5 g (wet weight) of streptococci.

Assays of RBC-Sensitizing and Antigenic[2] Activities of LTA. The sensitizing activity of LTA was assayed by mixing 0.1 ml of serial twofold dilutions of a 1 mg/ml solution of LTA with 0.05 ml of a washed 2% suspension of group O, Rh-negative human red blood cells (RBC). The mixtures were incubated for 30 min at 37°C. The RBC in each test tube were then washed twice in 0.02 M phosphate-buffered 0.9% NaCl at pH 7.4 (PBS). After the last wash, 0.2 ml of anti-LTA (kindly furnished by Robert W. Jackson, University of Southern Illinois, Carbondale, Ill.) diluted 1:100 was added to each tube and the RBC pellets were resuspended by shaking. The mixtures were incubated for 30 min at 37°C followed by centrifugation at 1,000 g for 5 min. The buttons were gently shaken and agglutination recorded. The minimum amount of LTA (μg:ml) needed to cause agglutination visible to the naked eye was determined. The reciprocal of the amount in μg multiplied by 100 was designated the sensitizing activity.

The antigenic activity was assayed by incubating 0.1 ml of similar twofold dilutions of LTA with 0.1 ml of a 1:50 dilution of anti-LTA at 37°C for 30 min. 0.05 ml of 2% RBC which had been previously sensitized with LTA were added and agglutination was recorded after further incubation at 37°C for 30 min as described above. The minimum amount of LTA (μg/ml) needed to inhibit agglutination was determined. The reciprocal of that amount multiplied by 100 was designated the antigenic activity.

Isoelectric Focusing of LTA. 5-mg samples of LTA were dissolved in a 1% ampholyte solution (LKB Produkton AB, Bromma, Sweden). The ampholyte solution chosen was capable of producing a pH gradient between pH 3 and 6. The ampholyte containing the LTA was mixed with a sucrose gradient to prevent later mixing of fractions and was then pumped into a 110 ml isoelectric focusing column (LKB Instruments, Inc., Rockville, Md.). Before the latter, the cathode solution was pumped into the bottom of the column and the anode solution was layered above the gradient (LKB Instruments, isoelectric focusing column instructions). After electrofocusing at 500 V for 24–36 h, the flow of current approached zero. The power source was disconnected and 1.7-ml fractions were collected at the rate of 2 ml per minute. Each fraction was tested for pH, and dialyzed against six changes of PBS for 5 days. 0.1 ml of each fraction was then tested for its ability to sensitize RBC as described above. Serial twofold dilutions of those fractions showing sensitizing activity were prepared and further tested to obtain a sensitizing titer (see Results).

Ammonia Hydrolysis of LTA. Methods were employed to remove and then to restore fatty acids to determine their role in membrane binding of LTA. 10 mg/ml of LTA was extracted with a chloroform:methanol (2:1) solution three times to remove any free lipids. The remaining aqueous phase was lyophilized and redissolved in 1 ml distilled water. To 0.05 ml of this solution, an equal vol of 30% NH$_4$OH (Fisher Scientific Company, Fairlawn, N. J.) was added and incubated overnight at

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[2] In this paper antigenic activity is defined as the capacity of LTA preparations to inhibit passive hemagglutination by anti-LTA of RBC sensitized with LTA.

room temperature. The mixture was then lyophilized and redissolved in 1 ml distilled water. 0.5 ml of this solution was extracted with chloroform:methanol solution three times. The aqueous and chloroform phases were flash-evaporated and redissolved in 0.5 ml PBS.

Esterification of Ammonia-Hydrolyzed LTA. In these experiments, LTA was hydrolyzed under milder conditions in order to preserve optimal integrity of the polyglycerol phosphate antigen while abolishing its sensitizing activity. After trying various concentrations of NH_4OH and various time of hydrolysis, we found that the following procedure produced satisfactory results. LTA, 500 µg, dissolved in 0.5 ml distilled water, was mixed with 0.02 ml of NH_4OH (30% as NH_3, J. T. Baker Chemical Co., Phillipsburg, N. J.) and incubated at 25°C for 1 h. The solution was then bubbled with a nitrogen stream to complete dryness. This material was esterified with palmitoyl chloride by a modification of the method described by Hammerling and Westphal (15). The dried material was dissolved in 0.5 ml of *N,N*-dimethyl formamide (DMF) (Fisher Scientific Co.) and to a 0.25 ml portion of this solution 0.02 ml palmitoyl chloride (Sigma Chemical Corp., St. Louis, Mo.) and 0.02 ml pyridine were added. The other 0.25 ml portion was mixed with pyridine only. After mixing for 1 h at 25°C, 0.25 ml distilled water was added to each mixture and then extracted with an equal volume of chloroform. The aqueous phase was removed with a pipette, dried under nitrogen, and redissolved in 0.25 ml PBS. These solutions were then tested for erythrocyte sensitizing and antigenic activities as described above. A sample of unhydrolyzed LTA dissolved in DMF and extracted with water and chloroform as described above was included as a control.

Chemical Analyses. 1 ml of a 1.0 mg/ml solution of LTA and fractions thereof were flash-evaporated, redissolved in 1 ml 6 N HCl, sealed in glass test tubes, and heated overnight at 108°C. This mixture was then flash-evaporated and redissolved in 1 ml distilled water. 5 λ of each hydrolyzed material was spotted on thin-layer chromatography (TLC) plates (Silica Gel EM Lab, Inc., Elmsford, N. Y.). The following solvent systems were used: petroleum ether:diethyl-ether:acetic acid (105:45:3), for lipids and fatty acids, and butanol:acetic acid:water (3:1:1) for amino acids and carbohydrates. The chromatograms were developed by spraying with ninhydrin for amino acids and amino sugars, with $AgNO_3$ reagent for sugars and glycerol, with 2, 7 dichlorofluorescine (Chromato-Spray Research Specialties Co., Richmond, Calif.) for lipids and fatty acids. Glycerol determinations were performed by the method of Chernick (16) and amino acid analyses were performed with a Beckman 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) by a single column technique (17).

Carbohydrate analysis was done in a Perkin-Elmer Model 900 gas chromatograph (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) equipped with dual flame ionization detectors by the procedures described by Katzman and Oronsky (18).

Fatty acids in the chloroform soluble material of hydrolyzed LTA were converted to their methyl ester with anyhydrous methanol-HCl (10%) mixture for 1 h at 70°C. After cooling, the methanol-HCl was removed under a nitrogen stream at room temperature. The residue was dissolved in a few drops of CH_2Cl_2 (methylene chloride) and analyzed in a Hewlett-Packard 402 gas chromatograph (Hewlett-Packard Co., Avondale, Pa.) using columns 4 ft in length containing 15% DEGS on chromasorb WAW (Supelco, Inc., Bellefonte, Pa.) Infra-red spectra were obtained on a Perkin-Elmer Infracord (model 137, Perkin-Elmer Corp.) using 1% LTA in potassium bromide (KBr).

Results

Purification of LTA. 5 mg of LTA were loaded onto a 110 ml isoelectricfocusing (IE) column (LKB Instruments). Two peaks capable of sensitizing RBC were eluted from the column: one at pH 4.65 (fraction I [FI]) and the other at pH 2.95 (FII) (Fig. 1). The sensitizing and antigenic activities of these IE fractions were equal to, or greater (for FI) than, the unfractionated LTA (Table I). Immunodiffusion in agar gel against antiserum to LTA (kindly donated by Dr. R. Jackson, University of Southern Illinois, Carbondale, Ill.) confirmed antigenic identity for the IE fractions and unfractionated LTA. Two or more precipitin lines were observed with FII and LTA, and only one line with FI (Fig. 2).

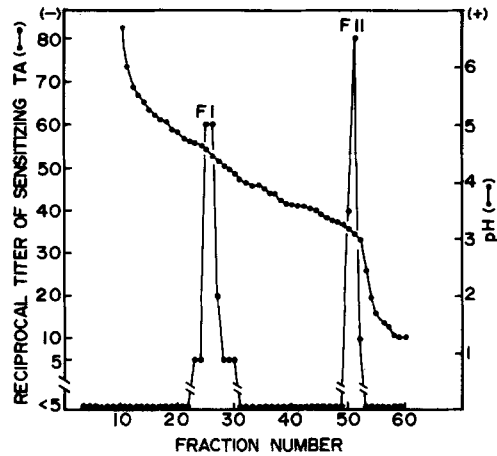*Chemical Composition of LTA and IE Fractions.* Thin-layer chromatography

FIG. 1. Elution profile of RBC sensitizing activity of electrofocused streptococcal lipoteichoic acid. 0.05 ml of washed 2% RBC were incubated with 0.1 ml of serial dilutions of each fraction. After washing the RBC button, 0.2 ml of 1:100 anti-LTA was added and agglutination was recorded after incubation at 37°C for 30 min. Reciprocal of highest dilution of each fraction showing agglutination was recorded in figure.

TABLE I

Sensitizing and Antigenic Activities of Unfractionated and Electrofocused LTA

LTA preparation	Sensitizing activity*	Antigenic activity*
Unfractionated LTA	285	666
Electrofocused LTA		
FI	570	1,332
FII	285	285

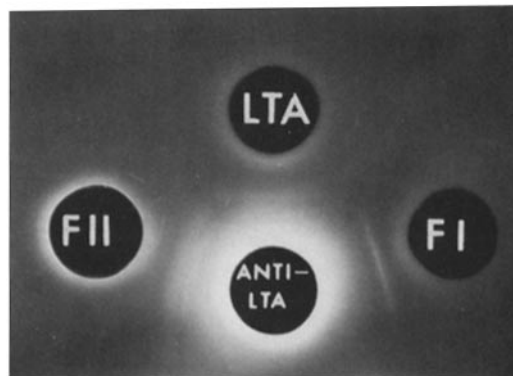* See Materials and Methods for derivation.

FIG. 2. Immunodiffusion tests of lipoteichoic acid (LTA) and its electrofocused fractions (FI and FII) against antiserum to LTA (anti-LTA).

of LTA and its IE fractions revealed that glycerol-phosphate and unidentified spots of lipids were present in all preparations (Table II). Sugars were not detected in any of the preparations and alanine was detected only in the unfractionated LTA. Quantitative amino acid analysis by automatic amino acid analysis revealed that alanine was present at levels of 0.52 μm/mg in LTA and was absent in the IE fractions. There were no other amino acids or amino sugars detected in the LTA and its IE fractions. The gas chromatography profile for carbohydrates indicated absence of any detectable sugars in 5-mg samples of LTA. Quantitative determination of glycerol showed 5.5 μm/mg glycerol in LTA. This value corresponds to alanine:glycerol molar ratio of approximately 1:10 in the unfractionated LTA. The low content and absence of alanine and sugars, respectively, are reflected in the infra-red analysis (Fig. 3) which showed little if any ester-linked components (absorbency at 1,700–1,600 cm^{-1}). These analyses are similar to those of hot acid-extracted PGP obtained by McCarty (19) and differ from those observed by Jackson and Moskowitz (8) for phenol-extracted LTA, which possessed higher amounts of alanine. The treatment of the

TABLE II

Chemical Analysis of LTS and its IE Fractions by Thin-Layer Chromatography

LTA preparation	Lipids	Alanine	Carbohydrate	Glycerol PO$_4$
Unfractionated LTA	+*	+	--	+
Electrofocused LTA				
FI	+	--	--	+
FII	+	--	--	+

* +, Presence of stainable spots; --, absence of stainable spots after spraying with the appropriate stains as described in Materials and Methods.

streptococci with hot acid before the phenol extraction in the present study probably accounts for the latter difference.

Role of Lipids in RBC Sensitizing Activity. In view of the sensitizing activity of the IE fractions, both of which lacked alanine (Table II), it appeared that in spite of its presence in the unfractionated LTA, alanine must not be involved in the sensitizing activity of LTA. Since lipids were present in the IE fractions and LTA, their possible role in sensitization of RBC was further investigated. Mild ammonia-hydrolysis has been used to release ester-linked substances by several investigators (11, 12). Therefore, we employed ammonia hydrolysis to study the possible role of ester-linked lipids in RBC sensitization by LTA.

Ammonia hydrolysis of LTA completely abolished its sensitizing activity (from 285 to 1) and partially its antigenic activity (from 570 to 142) (Table III). This partial loss of antigenic activity was evident also from immunodiffusion in agar gel, where only one precipitin line was produced by the ammonia-treated LTA.

The possibility that the ammonia hydrolysate, which lacked sensitizing activity, might contain moieties which could still bind to RBC and thereby block the sensitizing activity of unhydrolyzed LTA was investigated. 1 ml of 2% washed RBC was incubated for 30 min at 37°C with 0.1 ml of ammonia-hydrolyzed LTA

solution, or with solutions obtained after chloroform extraction (see Materials and Methods).

Preincubation of RBC with ammonia-treated LTA markedly reduced the sensitizing activity of unhydrolyzed LTA as compared to the buffer control (Table IV). This effect resided in the chloroform-methanol-soluble material but not in the water-soluble material suggesting that ester-linked lipids play a major
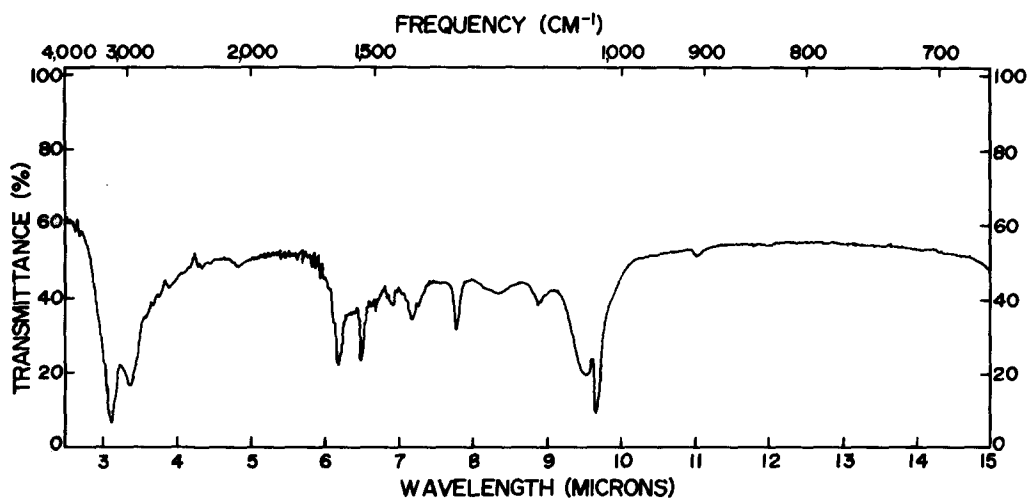

FIG. 3.   Infra-red spectrum of LTA.

TABLE III

Biological Activity of LTA Before and After Ammonia Hydrolysis

LTA	Sensitizing activity	Antigenic activity	No. precipitin lines against anti-LTA
Unhydrolyzed	285	570	2
Hydrolyzed	1	142	1

TABLE IV

Inhibition of Sensitizing Activity of LTA by Pretreatment of RBC with Various Agents

Agents used for pretreatment of RBC	Sensitizing activity of LTA with pretreated RBC	Antigenic activity of agents
PBS	285	0
NH₄OH hydrolyzed LTA (1 mg/ml)	2	142
Chloroform-extracted* hydrolysate of LTA		
A. H₂O soluble	142	142
B. Chloroform soluble (10×)‡	1.3	0

* Extracted with chloroform:methanol as described in Materials and Methods.
‡ Concentrated to one-tenth the original volume of the NH₄OH-hydrolyzed LTA.

role in the sensitizing activity of LTA. The water-soluble material retained antigenic activity of 142 whereas the chloroform-methanol-soluble material lacked any detectable antigenic activity. Gas-liquid chromatography of the chloroform-soluble material (Table V) revealed that fatty acids of chain length C14, C16, and C18, both saturated and unsaturated, were present. Palmitic acid, however, was the major fatty acid (58%).

Restoration of Sensitizing Activity of Ammonia-Hydrolyzed LTA. It has previously been shown that bacterial polysaccharides, which are unable to sensitize RBC, gained such activity after esterification with fatty acids (15, 20). Since palmitic acid was the major fatty acid in our LTA preparations, an attempt was made to restore the sensitizing activity which was lost after ammonia hydrolysis by esterification of the hydrolyzed antigen with palmitoyl chloride. Since the palmitoyl chloride will react with any free hydroxyl group, and since the content of fatty acid in LTA is less than 1%, we derived empirically a method (see Materials and Methods) to esterify hydrolyzed LTA with palmitic acid that

TABLE V

*Fatty Acid Composition in Chloroform Extract of Ammonia Hydrolyzed LTA**

Fatty acids	Percent of total
	%
14:0	2.45
14:1	0.82
16:0	47.7
16:1	10.4
18:0	14.7
18:1	16.1
18:2	7.9
20:0	0

* LTA was chloroform extracted before hydrolysis to remove free lipids.

resulted in minimal loss of antigenic activity. Table VI shows, as predicted, that the esterification of hydrolyzed LTA with palmitoyl-Cl_2 restored the sensitizing activity from 8 to 87 without altering the antigenic activity, supporting the hypothesis that LTA can bind to cell membranes via ester-linked lipid moieties.

It should be stressed that the sensitizing activity of 87 represents the highest degree of restoration we could achieve in six repetitive experiments. The restored sensitizing activity in these experiments ranged from 22 to 87. The reasons for such variability and for the failure to restore a greater proportion of the original sensitizing activity to hydrolyzed LTA remain unclear.

Effect of Hydrolyzed and Untreated LTA on Adherence of Streptococci to Human Oral Epithelial Cells. Since LTA has great affinity for mammalian cell membranes and recently some of it has been shown to reside on the surface of certain streptococci (21), we investigated the possibility that LTA might inhibit adherence of streptococci to human oral epithelial cells. Adherence experiments were performed by the method of Gibbons and Van Houte (22) using scrapings of buccal mucosal cells and a strain (SF 42) of type 12 group A streptococci.

Table VI

Restoration of the Sensitizing Activity of Hydrolyzed LTA by Conjugation with Palmitic Acid

LTA preparation	Sensitizing activity	Antigenic activity
Untreated	250	ND
NH₄OH-hydrolyzed*	8	125
NH₄OH-hydrolyzed, conjugated with palmitoyl chloride	87	125

* LTA hydrolyzed as described in Materials and Methods.

To test for the inhibitory effect on adherence by LTA and its hydrolyzed fractions, epithelial cells were washed twice in PBS, then resuspended in PBS containing 1 mg/ml of various LTA preparations. After incubating at 37°C for 30 min, the cells were washed twice in PBS, resuspended in PBS, and mixed with an equal volume of a standardized suspension (22) of PBS-washed streptococci that had been grown in Todd-Hewitt broth (Difco) for 16 h. The mixtures were incubated for 1 h at 37°C in a constantly shaking water bath.

The epithelial cells were then washed on a Millipore filter (pore size, 14 μ) to remove nonadherent streptococci. Smears were prepared and stained with Wright's-Giemsa and examined by bright-field microscopy.

Untreated LTA and the chloroform-methanol-soluble, but not the water-soluble, fraction of the ammonia-hydrolyzed LTA markedly reduced adherence of streptococci to epithelial cells (Fig. 4 and Table VII). Moreover, treatment of streptococci with antibody to purified LTA also inhibited adherence of the organisms to epithelial cells (Table VII).

In preliminary studies, we found that rabbit antisera against various serotypes of whole group A streptococci were similarly capable of inhibiting adherence. These sera have included absorbed and unabsorbed typing and grouping antisera. So far, however, only those sera which contained anti-LTA antibody have been inhibiting at dilutions of 1:100 or greater. Absorption of these antisera with LTA-modified erythrocytes abolished their inhibitory effects on adherence even though antibodies to C carbohydrate and the respective M antigens were still present. These results suggested that LTA is exposed on the surface of group A streptococci and, therefore, may be involved in adherence and colonization by these human pathogens.

Further evidence that LTA resides on the surface of group A streptococci was obtained from preliminary experiments in which LTA-sensitizing activity was extracted from intact organisms by pepsin (1 mg/ml at pH 2.0 for 20 min at 37°C) digestion. The pepsin extract dialyzed against PBS at pH 7.4 exhibited the presence of LTA by its ability to sensitize RBC which agglutinated in the presence of anti-LTA. The sensitizing activity detected in this extract was relatively low (2 as compared to 285 for purified LTA).

Discussion

Several investigators have implicated alanine in the spontaneous adsorption of streptococcal lipoteichoic acid to mammalian cell membranes (7, 8). The
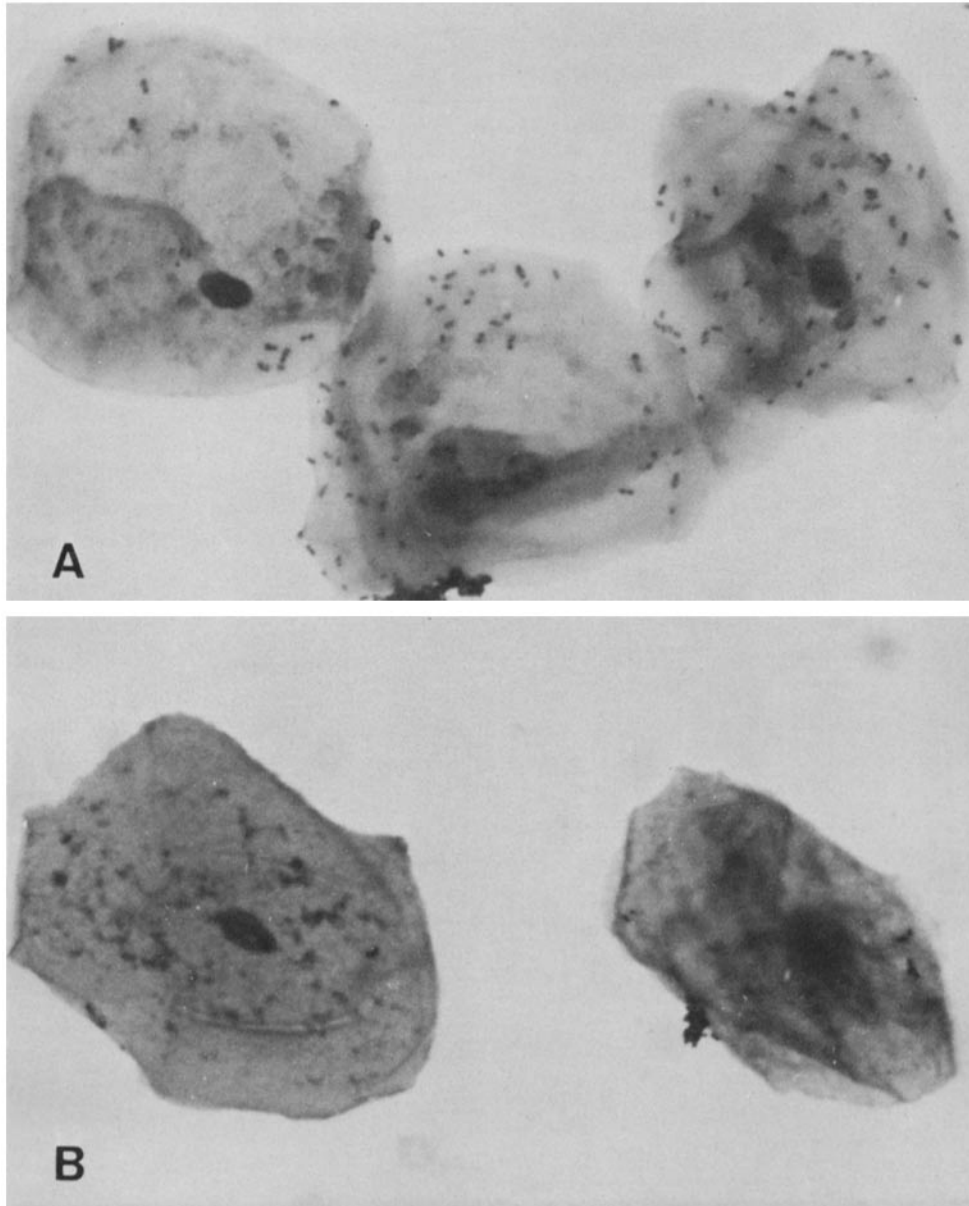
Fig. 4. Inhibition of adherence of type 24 streptococci to buccal epithelial cells by LTA. (A), control cells treated with PBS: (B), epithelial cells pretreated with 1 mg/ml purified LTA.

evidence for this was primarily based on the observation that mild hydrolysis of teichoic acid, which removed ester-linked alanine, also abolished sensitizing activity. Hewett et al. (12), however, demonstrated that such hydrolysis of *L. fermenti* LTA cleaved ester-linked lipids as well as alanine, and they suggested that the lipid moieties which are capable of forming hydrophobic bonds with cell membranes might be involved in spontaneous adsorption. Our data clearly

TABLE VII

*Inhibition of Streptococcal Adherence to Human Buccal Cells by
LTA and by Antiserum to LTA*

Epithelial cells treated with:	Inhibition of adherence
PBS	0
LTA	78
Lipid fraction of hydrolyzed LTA*	91
H_2O soluble fraction of hydrolyzed LTA	0
Streptococci treated with anti-LTA, 1:100	40
Streptococci treated with NRS‡, 1:100	0

* Chloroform:methanol soluble material from ammonia hydrolyzed LTA.
‡ NRS, normal rabbit serum.

demonstrated that the purified fraction of streptococcal lipoteichoic acid which lacked any detectable alanine retained full red cell sensitizing activity. This finding confirms the observations of Chorpenning and Stamper (13) using LTA from *B. subtilis*.

Evidence presented in the present study which indicates that lipid moieties, rather than alanine, were involved in the erythrocyte-sensitizing activity of group A streptococcal LTA is summarized as follows: (a) Purified fractions of lipoteichoic acid which lacked any detectable alanine were capable of spontaneously sensitizing RBC. The presence of the ester-linked alanine in the unfractionated LTA did not appear to have any added sensitizing effect. Furthermore, our data suggest that the fractions(s) in our LTA preparations which contained the ester-linked alanine had no sensitizing activity at all, since an alanine-containing fraction capable of sensitizing RBC was not detected in the isoelectric-focusing column. Alternatively, hydrolysis of the labile ester linkages of alanine may have occurred during extraction and purification, as suggested by Archibald et al. (23). This interpretation is supported by the observation of McCarty (19) who obtained from group A streptococci hot acid-extracted PGP which lacked ester-linked alanine and by Chorpenning and Stamper (13) who reported that the alanine content of *B. subtilis* LTA was considerably reduced during purification procedures. (b) Mild ammonia-hydrolysis abolished the sensitizing activity of LTA without abolishing antigenic activity. The ammonia-hydrolyzed LTA contained a chloroform-methanol-soluble material which was capable of inhibiting sensitization of RBC by unhydrolyzed LTA. The water-soluble material retained antigenic activity but failed to sensitize or inhibit sensitization of RBC. In agreement with other investigators (21), the chloroform-methanol-soluble material consisted of a mixture of unsaturated and saturated fatty acids with a predominance of palmitic acid. (c) Esterification of the antigenic fraction of hydrolyzed LTA with palmitic acid restored RBC-sensitizing activity. These findings strongly suggest that the lipid moieties, and not alanine, are responsible for the spontaneous adsorption of streptococcal LTA to cell membranes.

It should be noted that with repetitive experiments we were able to restore at best only one-third of the original sensitizing activity of hydrolyzed LTA. Our results, however, do not indicate whether or not the range of fatty acids present in

minute amounts in LTA play a role in cell membrane affinity. Previous studies (15, 20) have shown that the degree of in vitro esterification of fatty acids with various polysaccharide antigens is probably more important than the species of fatty acid employed. Whether or not this is also true for naturally occurring ester-linked fatty acids has not been clarified. In this regard, the possibility exists that the location and distribution of various fatty acids on or among PGP molecules may play a role both in cell membrane affinity and antigenic activity of LTA. Until these issues are resolved, it is too early to speculate on the relative importance of the various fatty acids in the biological activity of LTA.

The role of LTA and its lipid moiety(ies) in host-bacterial interactions, which require cell membrane affinity, was given special consideration in this report. Only recently (24) have investigations been made on the role of epithelial adherence mechanisms in the virulence of various bacteria that are pathogenic for man. Adherence is now recognized to be a prerequisite for colonization and invasion of mucosal surfaces by virulent bacteria including group A streptococci (25). Several lines of evidence presented in this study suggest that streptococcal adherence might be mediated by LTA via its lipid moiety. First, both the LTA and its lipid moiety prevented adherence of streptococci. Second, streptococci preincubated with anti-LTA lost their ability to adhere to epithelial cells. Moreover, the inhibitory effects of antisera against whole group A streptococci was attributable to the presence of anti-LTA that appears widespread among antisera which contain antibodies to a variety of streptococcal products (19). Since bacterial adherence is a surface phenomenon, these findings imply that at least some LTA resides on the surface of streptococcal cells.

Several direct and indirect observations suggest the possible location of some LTA on the bacterial surface. Although it has been suggested (25) that M protein associated with surface "fimbriae" might be responsible for adherence to epithelial cells we recently demonstrated that removal of M protein by mild peptic digestion at suboptimal pH did not abolish either surface fimbriae or adhering ability (26, footnote 3). The complete removal of these surface structures by digestion with trypsin or pepsin at optimal pH, however, did abolish adhering ability.[3] In fact, as shown in the present study the pepsin extract contained LTA activity. This is consistent with the observation reported by McCarty (19) that trypsin extract of group A streptococci contains PGP. Since such enzymatic treatment had no apparent effect on the integrity and viability of streptococcus cells, it can be implied that LTA was released from the surface of these organisms. Moreover, several studies (7, 27, 28) have suggested that LTA may be covalently bound or closely associated to certain purified M protein preparations. One might postulate, therefore, that LTA may be found in a complex with the surface M protein of group A streptococci. On the other hand, this would not exclude the possibility that LTA may reside on the surface of M-negative group A streptococci (5). Finally, the presence of LTA on the surface of other streptococci has been recently reported (21). The amount relative to the total LTA that resides on the group A streptococcal surface, however, remains to be determined. It has already been demonstrated that mesosomes of group A

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[3] Beachey, E. H., G. L. Campbell, and I. Ofek. Unpublished data.

streptococci are rich in LTA (29). The mesosomes might be the origin of the surface lipoteichoic acid, since these structures have been suggested to be intimately associated with the synthesis of the bacterial cell wall and its components (30).

The possible role of LTA, and especially its lipid moieties in the pathogenesis of tissue injury has only recently been recognized (2). In preliminary studies, Miller and Jackson (9) reported that LTA may alter the immune response to sheep red blood cells and lipopolysaccharides of gram-negative bacteria in mice and that its lipid moieties play a major role in such activities. The role of the lipid moiety in other toxic reactions mediated by LTA such as experimental nephritis (3), delayed hypersensitivity (31), or experimental arthritis (4) remains to be elucidated. The possible implications of the molecular association of LTA with streptococcal protein antigens with respect to the pathogenesis of nonsuppurative sequelae of streptococcal infections has been discussed recently by Knox (2). Perhaps LTA, with its great affinity for cell membranes, plays a central role not only in the initial event (epithelial adherence) of a streptococcal infection, but also in the pathogenesis of the tissue injury in the nonsuppurative sequelae of certain group A streptococcal infections.

Summary

Lipoteichoic acid (LTA) was extracted from group A streptococci, previously treated with hot HCl, by the phenol method. The extracted LTA was loaded on an isoelectric (IE) focusing column and two fractions were collected: one at pH 4.65 and the other at pH 2.95. Chemical analysis demonstrated that the unfractionated LTA contained alanine and glycerolphosphate at molar ratio of 1:10, and ester-linked lipids, but no detectable sugars or amino-sugars. The two IE fractions contained lipids but lacked alanine.

The LTA and its IE fractions spontaneously adsorbed to human erythrocytes (sensitization) causing them to agglutinate in the presence of rabbit anti-LTA. The RBC-sensitizing and antigenic activities of IE fractions were equal to, or greater (for IE fraction at pH 4.65) than the unfractionated LTA, indicating that alanine is not involved in the sensitizing activity of LTA.

Mild ammonia-hydrolysis abolished the RBC-sensitizing activity of LTA and its IE fractions. Chloroform-methanol-soluble material of the ammonia-hydrolysate lacked antigenic activity but blocked sensitization of erythrocytes by LTA. The water-soluble material of the hydrolyzed LTA retained antigenic activity, was not able to block sensitization by LTA, and its sensitizing activity was restored after esterification with fatty acids.

These experiments indicate that ester-linked fatty acids (palmitic acid being the major one) are involved in the spontaneous adsorption of LTA to erythrocytes. The LTA, its lipid moiety, and anti-LTA blocked adherence of group A streptococci to human epithelial cells, suggesting that small amounts of LTA may reside on the streptococcal surface to mediate attachment and colonization of these organisms on mucosal surfaces in vivo.

References

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