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Formation of Extracellular Lipoteichoic Acid by Oral Streptococci and Lactobacilli

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Examination of the culture fluids from a number of strains of oral streptococci and lactobacilli has shown the presence of an erythrocyte-sensitizing antigen with the properties of lipoteichoic acid. The antigen was isolated from the culture fluids of *Lactobacillus casei* and *Lactobacillus fermentum* and characterized chemically and serologically. For other strains, serological evidence for the presence of lipoteichoic acid depends on the reactivity with antiserum specific for the glycerol phosphate backbone. The relative concentrations of the antigen in culture fluids from different organisms, in culture fluids from different stages of growth, and in extracts of organisms was estimated by determining the maximum dilution that fully sensitized erythrocytes; the culture fluid titer, which is the reciprocal of the dilution, varied from 4 to 320. Strains of *Streptococcus mutans* were generally characterized by a high level of extracellular lipoteichoic acid, the amount being greater than that detectable in cell extracts; this conclusion was confirmed by using the quantitative precipitin method. A high-molecular-weight fraction obtained from *S. mutans* BHT culture fluid was effective in sensitizing erythrocytes at a concentration of 1 μg/ml, compared with 2 μg/ml required for cellular lipoteichoic acid from *L. casei*. The detecting procedure depends on the teichoic acid sensitizing erythrocytes but, as shown with *L. fermentum*, low-molecular-weight nonsensitizing teichoic acid may also be present in culture fluid.

Lipoteichoic acids are characteristic membrane components of gram-positive bacteria and consist of a glycolipid joined covalently to a glycerol teichoic acid which carries carbohydrate and D-alanine substituents (19, 39). There is serological evidence that they may function as surface antigens, and the results have been interpreted as indicating that glycerol phosphate chains can penetrate the cell wall (4, 34). As surface antigens they may be useful in the serological classification of bacteria, though the presence of the common glycerol phosphate sequence can lead to considerable cross-reactions (19, 38, 39). There are a number of reports (19, 21, 32) of gram-positive organisms containing a common antigen detectable in cell extracts or culture fluid, generally on the basis of a hemagglutination reaction (9, 27, 29, 30, 33), and the present investigation was undertaken to ascertain whether the results for culture fluid could be accounted for by the presence of lipoteichoic acid. Serological evidence has been obtained for the presence of a component with the properties of lipoteichoic acid in the culture fluid of a number of oral streptococci and lactobacilli; in addition, lipoteichoic acid has been isolated from the culture fluid of *Lactobacillus fermentum* whose cellular lipoteichoic acid has already been characterized (37).

MATERIALS AND METHODS

Organisms. The strains of *Lactobacillus casei* (NCTC 6375), *Lactobacillus plantarum* (NCIB 7220), and *L. fermentum* (NCTC 6991) were those employed in previous studies (16, 17, 18). Cultures of *Streptococcus sanguis* ATCC 10558, *Streptococcus salivarius* ATCC 13419, and *S. salivarius* NCTC 8606 were obtained from the appropriate type culture collection, and *Streptococcus mitior* 439 was obtained from Melbourne University Microbiology Department, Melbourne, Victoria. For the remaining cultures we are grateful for the following donations: *Streptococcus mutans* strains AHT, BHT, OMZ-61, and FA-1R from R. J. Fitzgerald, Veterans Administration Hospital, Miami, Fla., B13 from S. Edwardsson, University of Lund, Malmö, Sweden, Ingbritt from B. Krasse, University of Göteborg, Fack, Sweden, KPSK2 from J. Carlsson, University of Umeå, Umeå, Sweden, and GS 5 from A. S. Bleiweis, University of Florida, Gainesville, Fla.; *S. sanguis* strain 804 from J. Carlsson; and *S. mitior* strain S-3 from R. J. Gibbons, Forsyth Dental Center, Boston, Mass.

Growth conditions. Lactobacilli were grown for 18 h at 37 C in the medium described by de Man et al. (24). Streptococci were grown at 37 C under anaerobic conditions (95% N_2, 5% CO_2); unless otherwise stated a complex medium was used (35), without pH control. Synthetic medium contained the components recommended by Lawson (20) with the omission of pyridoxamine and the addition of sodium phosphate buffer, pH 7.0, to a final concentration of 0.1 M.

Ultrafiltration of culture fluid. Cell-free culture fluid was concentrated and fractionated by passing through a Sartorius membrane filter SM12134 (which retains protein molecules of greater than 20,000 molecular weight) or through Diaflo XM 50 (50,000 molecular weight) and XM 300 filters (300,000 molecular weight) on an Amicon filter cell at 4 C and a pressure of 40 and 5 to 10 lb/in², respectively.

Preparation of cell extracts. Saline-washed suspensions of organisms (30 mg [dry weight]/ml) were disrupted with glass beads in a Braun cell homogenizer for 2 to 3 min with CO_2 cooling. The efficacy of the disruption was followed by the Gram-staining reaction. After removal of glass beads by filtration, the suspension of disrupted organisms was centrifuged at 10,000 × *g* for 15 min. The supernatant fraction was reserved for serological estimation of lipoteichoic acid.

Preparation and characterization of teichoic acids. Procedures for the isolation of lipoteichoic acid and teichoic acid from organisms and for studying their structure have been described previously (17, 18, 36, 37).

Fatty acid analyses. Gas chromatographic analyses of fatty acids were kindly performed by D. G. Bishop, CSIRO Division of Food Preservation, Sydney, on columns of 25% diethylene glycol succinate, 2% phosphoric acid on Gas Chrom P (100 to 200 mesh) at 165 C.

Serological methods. Procedures have been described previously for obtaining antisera to lipoteichoic acid and for examining the reaction of teichoic acids (including lipoteichoic acids) by the quantitative precipitin method (16), immunoelectrophoresis (36), and hemagglutination (12).

Antisera employed for detecting extracellular lipoteichoic acid were prepared against lipoteichoic acid from *L. casei* NCTC 6375 since such sera cross-react with different lipoteichoic acids on the basis of the common glycerol phosphate backbone (38); to confirm the specificity of the reaction, glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2) was prepared from cardiolipin (40) and examined for its ability to inhibit the hemagglutination reaction (38).

The relative amounts of lipoteichoic acid in culture fluids and cell extracts were routinely estimated by the sensitive hemagglutination procedure; the cell-free fluids were neutralized (with 6 N NaOH), dilutions were made in phosphate-buffered saline (pH 7.0), and the tubes were heated at 56 C for 15 min to destroy any hemolysins. Where the concentration was sufficiently high, 0.1 to 0.5 ml of culture fluid (after dialysis against 0.85% NaCl) was examined to determine the amount of antibody precipitated from 0.1 ml of antiserum 217 prepared against *L. casei* lipoteichoic acid. From the resultant precipitin curve, the volume of solution required to precipitate approximately half the maximum amount of precipitable antibody was determined, and from this value the amount precipitable by 1 ml of culture fluid or the bacterial cells derived from 1 ml of culture fluid was calculated.

RESULTS

Estimation of extracellular lipoteichoic acid. An estimate of the relative amounts of erythrocyte-sensitizing antigen in the culture fluid of different organisms was obtained by comparing the extent to which serial twofold dilutions retained full sensitizing capacity, as judged by the agglutination of the erythrocytes with the cross-reacting antisera to *L. casei* lipoteichoic acid. The reciprocal of this dilution is called the culture fluid titer and is distinct from the hemagglutination titer, which is the reciprocal of the greatest dilution of serum which still caused visible agglutination of sensitized erythrocytes. The method is illustrated by the results for two strains given in Table 1, where the culture fluid titer for *S. sanguis* strain 804 was 4 compared with 40 for *S. mutans* strain AHT; the table also shows that a concentration of 2 μg of *L. casei* lipoteichoic acid per ml was required for full sensitization.

To show that the agglutination of sensitized erythrocytes depended on their reactivity with antibodies specific for a glycerol phosphate chain, G_3P_2 was tested for its ability to inhibit the hemagglutination reaction. To serial dilutions of antiserum to *L. casei* lipoteichoic acid was added G_3P_2 (0.5 μmol), followed by erythrocytes fully sensitized with culture fluid from different organisms; the hemagglutination titers are compared with those obtained in the

TABLE 1. *Sensitization of erythrocytes by decreasing concentrations of lipoteichoic acid and culture fluids*

Lactobacillus casei lipoteichoic acid		Culture fluids			
		Streptococcus sanguis 804		*Streptococcus mutans* AHT	
Concn (μg/ml)	Titer	Dilution	HA titer[a]	Dilution	HA titer
10	800	1	400	1	400
5	800	2	400	2	400
2	800	4	400	4	400
1	400	10	200	10	400
0.5	200	20	100	20	400
				40	400
				80	200
				160	50

[a] HA, Hemagglutination.

absence of potential inhibitor in Table 2). For *L. fermentum* where the inhibition was less than for other strains the specificity of the reaction was confirmed by inhibition with *L. casei* lipoteichoic acid, 10 μg, decreasing the titer to <50.

Effect of growth phase. The relative amounts of erythrocyte-sensitizing antigen present in early and mid-log phase and also stationary phase were compared for four cultures. An increase during the growth phase paralleled the increase in cell mass (Table 3).

Evidence on whether the extracellular lipoteichoic acid might be resulting from cell autolysis was sought by growing *S. mutans* strain AHT in a chemically defined medium for 18 h; the yield of cells at the stationary phase was 0.35 mg/ml (compared with 1.0 mg/ml in complex medium) and the culture fluid titer was 10. Rhamnose is a major component of the cell wall of strain AHT (1), but the amount of material estimated as rhamnose (7) in the culture fluid represented less than 5% of that estimated in the cells derived from the same culture fluid.

Comparison of viridans streptococci. Strains of *S. mutans*, *S. sanguis*, *S. salivarius*, and *S. mitior* were grown for 18 h in complex medium and the culture fluids were examined for their ability to sensitize erythrocytes, which were then agglutinated with antiserum to *L. casei* lipoteichoic acid. The results in Table 4 compare the titers for the different culture fluids and also show the corresponding hemagglutination titers for the sensitized erythrocytes. All strains contained in their culture fluid a component with the properties of lipoteichoic acid, with the concentration being relatively high for most strains of *S. mutans*.

For *S. mutans* strains AHT, BHT, and Ingbritt, the amounts of extracellular antigen were compared with the amounts detectable in the soluble fraction from organisms disrupted in the Braun cell homogenizer (model MSK), the soluble fraction being diluted to the same volume as the original culture fluid. For each strain the results (Table 5) suggested that the amount of extracellular lipoteichoic acid exceeded the amount of cellular lipoteichoic acid.

Detection of extracellular lipoteichoic acid by the precipitin method. The concentration of lipoteichoic acid in the culture fluid of *S. mutans* strains is sufficient to detect by the precipitin method. Using antiserum to *L. casei* lipoteichoic acid (217) and 0.1 to 0.5 ml of cul-

TABLE 2. *Inhibition of hemagglutination by G_3P_2*

Culture fluid	Hemagglutination titer	
	Control	G_3P_2 (0.5 μmol)
Lactobacillus casei NCTC 6375	200	<50
L. fermentum NCTC 6991	400[a]	200
Streptococcus mutans AHT	400	<50
BHT	400	<50
Ingbritt	200	<50

[a] Decreased to <50 by 10 μg of *L. casei* lipoteichoic acid.

TABLE 4. *Serological reactivity of streptococcal culture fluids*

Species	Serotype	Strain	CF titer[a]	HA titer[b]
mutans	*a*	AHT	40	400
		OMZ-61	20	400
	b	BHT	320	400
		FA-1R	40	400
	c	K2	20	200
		GS 5	10	400
		Ingbritt	20	200
	d	B13	4	200
sanguis		804	4	400
		10558	4	200
salivarius		8606	40	800
		13419	4	200
mitior		S3	4	200
		439	10	200

[a] CF, Culture fluid.
[b] HA, Hemagglutination.

TABLE 3. *Detection of erythrocyte-sensitizing antigen during different stages of growth*

Organism	Growth phase					
	Early log		Mid-log		Stationary	
	Dry wt (mg/ml)	CF titer[a]	Dry wt (mg/ml)	CF titer[a]	Dry wt (mg/ml)	CF titer[a]
Lactobacillus casei NCTC 6375	0.23	1	0.60	2	2.3	4
L. plantarum NCIB 7220			0.40	2	1.7	4
L. fermentum NCTC 6991	0.25	4	0.51	4	1.8	4
Streptococcus mutans AHT	0.10	4	0.60	10	1.0	40

[a] CF, Culture fluid.

ture fluid from strains AHT, BHT, and GS 5, the results confirmed those obtained by hemagglutination, namely a greater reactivity for the BHT culture fluid. The values (Table 6) are expressed as the amount of antibody precipitable by 1 ml of culture fluid. For strain BHT grown in medium containing sucrose, and therefore synthesizing extracellular polysaccharide(s), the amount of antibody precipitable by 1 ml of culture fluid was 810 μg. By comparison with the culture fluid values, 10 μg of *L. casei* lipoteichoic acid precipitated 240 μg of antibody.

To compare the amounts of cellular and extracellular lipoteichoic acid for each strain, the cells recovered from 500 ml of culture were extracted with hot aqueous phenol, dilutions of the extract were tested by the quantitative precipitin method, and the results were expressed, as before, as the amount of antibody precipitable by the cells derived from 1 ml of culture (Table 6). For strain BHT, the comparison was extended (Table 6) by concentrating 100 ml of culture fluid to 20 ml, extracting with hot phenol, and determining the reactivity of the recovered aqueous phase after dialysis against 0.85% NaCl.

Absorption of extracellular lipoteichoic acid to hydroxyapatite. Teichoic acids, including lipoteichoic acid, will absorb to hydroxyapatite (unpublished observations and personal communication from D. C. Ellwood). Culture fluid (pH 6.0) from *S. mutans* strain BHT was shaken at room temperature for 1 h with an equal volume of a saline suspension of hydroxyapatite (Bio Gel HTP, Bio-Rad Laboratories, Richmond, Calif.), to give a final concentration of 5 and 10 mg/ml. The amount of lipoteichoic acid remaining in the supernatant was estimated by reacting 0.2 ml with 0.1 ml of antiserum 217 and determining the amount of antibody precipitated. At 5 mg of hydroxyapatite per ml there was a 30% decrease in antibody precipitated, and at 10 mg/ml there was a 47% decrease.

TABLE 5. *Comparison of cellular and extracellular lipoteichoic acid by hemagglutination method*

Streptococcus mutans strain	Dilution retaining full activity	
	Cellular fraction	Extracellular fraction
AHT	10	40
BHT	40	320
Ingbritt	10	20

TABLE 6. *Determination of relative amounts of lipoteichoic acid by precipitation of antibody to Lactobacillus casei lipoteichoic acid*

Fraction	Antibody precipitated (μg/ml of culture)		
	Strain BHT	Strain AHT	Strain GS 5
Culture fluid (a)	1,100	188	170
Phenol extract of culture fluid	930	ND[a]	ND[a]
Phenol extract of organisms (b)	96	76	106
Ratio a/b	11.5	2.5	1.6

[a] ND, Not determined.

Membrane filtration of culture fluid from S. mutans strain BHT. *S. mutans* strain BHT was grown in the New Brunswick Microferm fermentor with pH control (pH 6.0) for 18 h. Compared with the culture previously grown without pH control, the yield of cells had increased from 0.76 to 2.4 mg/ml and the amount of antibody to *L. casei* lipoteichoic acid precipitable by 1 ml of culture fluid increased from 1.10 to 2.04 mg. A portion of the culture fluid (150 ml) was concentrated by ultrafiltration on an XM 50 membrane (yield 112 mg) and further fractionated (105 mg) on an XM 300 membrane to yield 31 mg of retentate and 67 mg of diffusate. Several ultrafiltrations with this culture fluid and also culture fluids from organisms grown without pH control showed that at least 95% of serological activity detectable by the precipitin method was retained by the XM 50 membrane; the amount of serologically active material passing through the XM 300 membrane was low and variable, the diffusate for the reported culture fluid being inactive. The XM 300 retentate (5 mg/ml) was examined by immunoelectrophoresis; it showed a single component reacting with antiserum 217, which had the same mobility as lipoteichoic acid (2 mg/ml) from *L. casei* NCTC 6375 (Fig. 1). By the quantitative precipitin method, 10 μg of retentate precipitated 58 μg of antibody from antiserum 217, compared with 240 μg of antibody precipitated by 10 μg of *L. casei* lipoteichoic acid. However, the preparation showed a greater activity in the hemagglutination assay, 1 μg/ml being sufficient to sensitize erythrocytes, compared with the required concentration of 2 μg/ml for *L. casei* lipoteichoic acid (Table 1).

Column chromatography of the XM 300 retentate on 6% agarose indicated the presence of typical micellar lipoteichoic acid, as well as low-molecular-weight nonmicellar teichoic acid.

Isolation of cellular and extracellular li-

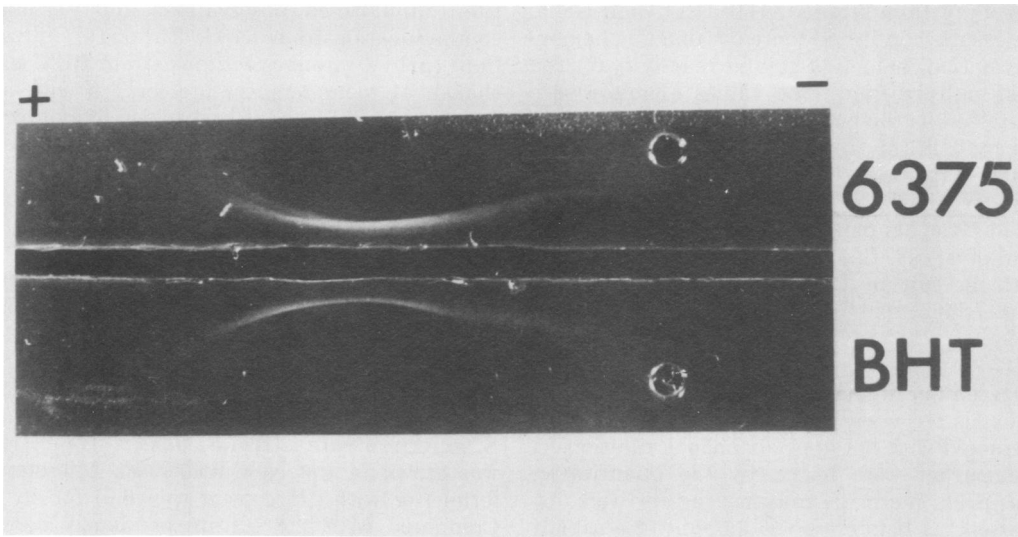

FIG. 1. *Immunoelectrophoretic comparison of extracellular teichoic acid fraction from Streptococcus mutans BHT with lipoteichoic acid from Lactobacillus casei NCTC 6375. Electrophoresis in 0.01 M potasium phosphate buffer, pH 7.0, performed for 75 min at 6 V/cm and antiserum to L. casei lipoteichoic acid then added.*

poteichoic acid and teichoic acid from L. fermentum cultures. For a comparison of cellular and extracellular lipoteichoic acid, *L. fermentum* NCTC 6991 was chosen for further study as the structure and properties of its cellular lipoteichoic acid have been reported (37). A culture (10 liters) of *L. fermentum* NCTC 6991 was grown to the stationary phase in the New Brunswick Microferm fermentor without pH control. The culture fluid was concentrated to approximately 700 ml by membrane filtration (Sartorius membrane filter SM12134), dialyzed, further concentrated to 90 ml by rotary evaporation, and then extracted with hot aqueous phenol (36). The aqueous phase, after dialysis, was adjusted to pH 7.0 and incubated with deoxyribonuclease and ribonuclease (36) for 4 h at 37 C; 4 volumes of cold ethanol was then added and, after standing overnight at 4 C, the precipitate was collected by centrifugation, washed with ethanol, and dried (yield = 2.417 g). Chromatography of 100 mg of extract on 6% agarose in 0.2 M ammonium acetate at pH 6.9 gave a major organic phosphorus peak with K_d = 0.1 and a minor component with K_d = 0.5, the ratio in terms of organic phosphorus being 6.4:1.0. Studies on several organisms (19) have shown that a K_d of 0.1 on 6% agarose is typical of a lipoteichoic acid, whereas a K_d of 0.5 is obtained for low-molecular-weight teichoic acid.

Fractionation of the hot aqueous phenol extract from one-half of the organisms derived from 10 liters of culture on 6% agarose also gave two organic phosphorus peaks with K_d = 0.1 (72.5 μmol of P/g of cells) and K_d = 0.5 (8 to 10 μmol of P/g). When the remaining portion of the organisms was incubated in 0.05 tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) at 37 C for 10 min and the extract was fractionated by column chromatography on 6% agarose, low-molecular-weight teichoic acid (32.4 μmol of P/g of cells) was isolated but no lipoteichoic acid fraction was detectable. Subsequent extraction of these cells with hot aqueous phenol yielded a lipoteichoic acid fraction (50.3 μmol of P/g of cells) but only a trace of low-molecular-weight teichoic acid.

Products with the properties of lipoteichoic acid and teichoic acid were also isolated from *L. casei* NCTC 6375 culture fluid but were not studied further.

Characterization of cellular and extracellular teichoic acid fractions from L. fermentum. The high- and low-molecular-weight fractions from the cells and from culture fluid were hydrolyzed with acid and alkali and examined for the presence of teichoic acid components by paper chromatography (37). Acid hydrolysates of each preparation contained glucose, galactose, D-alanine, and glycerol, glycerol monophosphate, and diphosphate; alkaline hydrolysis followed by treatment with phosphomonoesterase gave, in each case, glycerol,

diglycerol phosphate, and the previously identified glycerol glycosides obtained from cellular lipoteichoic acid (37), namely 2-*O*-α-D-galactosyl-glycerol and *O*-α-D-galactosyl-1 → 2-*O*-α-D-glucosyl-1 → 2-glycerol from the main chain of the teichoic acid moiety, and *O*-α-D-galactosyl-1→2-*O*-α-D-glucosyl-1→1 glycerol from the glycolipid.

Table 7 compares the relative proportions of phosphorus, glucose, and galactose in the teichoic acid preparations and Table 8 gives the results for fatty acid analysis. The results for cellular and extracellular low-molecular-weight teichoic acid were similar, and only the results for the extracellular fraction are recorded.

As shown by the quantitative precipitin method, both the extracellular lipoteichoic acid and teichoic acid reacted strongly with antiserum prepared against *L. fermentum* lipoteichoic acid (16) and also antiserum 217 to *L. casei* lipoteichoic acid. The cellular and extracellular lipoteichoic acids were indistinguishable by immunoelectrophoresis. The extracellular lipoteichoic acid but not the teichoic acid sensitized erythrocytes in the hemagglutination procedure.

DISCUSSION

There are numerous reports in the literature of the presence in extracts of gram-positive organisms and also in culture fluids of erythrocyte-sensitizing antigens (19, 39). Frequently it was assumed that the antibodies were detecting an antigen common to all the reactive organisms, and hence the use of such terms as nonspecies specific (29), heterophile (22), and heterogenetic antigen (3). The possibility that the "common antigen" in certain cells was teichoic acid, as suggested by Salton (see 9), received support from the observation that polyglycerol phosphate inhibited the agglutination of erythrocytes sensitized with lysates of *Staphylococcus aureus* (9). The same experimental procedure enabled Stewart (33) to conclude that the "Hickey antigen" in the culture fluid of some streptococci was also a teichoic acid. It is now known that the sensitization of erythrocytes depends on the lipid moiety of lipoteichoic acid (19, 39) so that a lipoteichoic acid would have been the component detected in each of the above studies.

TABLE 7. *Ratio of components in teichoic acids from Lactobacillus fermentum*

Fraction	Phosphorus	Glucose	Galactose
LTA-extra[a]	1.00	0.21	0.14
LTA-intra[a]	1.00	0.06	0.12
Teichoic acid	1.00	0.15	0.23

[a] Extracellular and intracellular lipoteichoic acid fraction, respectively.

TABLE 8. *Fatty acid components of teichoic acid fractions from Lactobacillus fermentum*

Fatty acid	Fraction		
	LTA-extra[a]	LTA-intra[a]	Teichoic acid
Total (μmol/10 μmoles of P[b])	0.76	0.76	0.03
Component (% total)			
14:0	21.0	1.3	46.8
16:0	35.3	37.4	21.3
16:1	5.8	2.3	16.6
18:0	Trace	6.2	Trace
18:1	23.6	25.4	10.2
19:0Δ	13.5	27.3	3.9

[a] Extracellular and intracellular lipoteichoic acid fraction, respectively.

[b] Assuming average molecular weight of 250.

In the present investigation the use of antibodies specific for the polyglycerol phosphate "backbone" of lipoteichoic acid enabled the detection of a component with the properties of lipoteichoic acid in the culture fluids of a variety of oral streptococci and lactobacilli. Supporting evidence is provided by the inhibition of the reaction with G_3P_2, the immunoelectrophoretic mobility of the component in the culture fluid from *S. mutans* BHT, and more particularly the isolation of lipoteichoic acid from the culture fluid of *L. fermentum* and *L. casei*. With the lactobacilli and *S. mutans* BHT, evidence was also obtained for a lower-molecular-weight non-erythrocyte-sensitizing teichoic acid, so that using the hemagglutination method to survey culture fluids does not necessarily detect all of the extracellular polyglycerol phosphate-containing material.

The use of antibodies specific for the polyglycerol phosphate backbone to detect different lipoteichoic acids, whose carbohydrate substituents are unknown, is based on the premise that such lipoteichoic acids would react. A number of lipoteichoic acids carrying different carbohydrate substituents have been shown to react with antiserum of this specificity (38) but, should a negative result have been obtained, it would not necessarily have indicated lack of a lipoteichoic acid. In fact, all culture fluids did contain a reactive component, though the different hemagglutination titers probably indicate varying degrees of cross-reactivity. The

various terms used by earlier workers to describe erythrocyte-sensitizing components implied that the antigen was the same in each case. If by "antigen" was meant the entire structure, there is no evidence to support such a conclusion, though the evidence is consistent with a common antigenic determinant—the glycerol phosphate backbone—in lipoteichoic acids of different structure.

Whereas different hemagglutination titers are indicative of the differences in the reactivity of sensitized erythrocytes with antibodies, the culture fluid titer, defined as the dilution retaining full sensitizing capacity, presumably is a measure of the relative amount of sensitizing antigen in the culture fluid or cell extract. On this basis, the concentration of extracellular lipoteichoic acid is relatively low for the lactobacillus strains tested but frequently high for streptococci, particularly *S. mutans* strains. To quantitate the culture fluid titer in terms of the amount of lipoteichoic acid present requires knowing the amount of material needed to sensitize erythrocytes. For the isolated *L. casei* lipoteichoic acid, this value is 2 μg/ml, though for the culture fluid fraction from *S. mutans* BHT a concentration of 1 μg/ml is sufficient. Assuming that for *S. mutans* strain AHT a concentration of 1 to 2 μg/ml is required for full sensitization, then from the results in Tables 3 and 5 it can be calculated that the amount of cellular lipoteichoic acid is 1 to 2% of cell mass. This would be the expected value (19) and would suggest that the method is giving a reasonable estimate of lipoteichoic acid concentration.

Comparative analyses with *S. mutans* strains of the amounts of cellular and extracellular lipoteichoic acid by both the hemagglutination and precipitin reactions indicate that the amount of extracellular material can exceed the amount associated with the cells in stationary phase. Assuming that, say, 1 μg of lipoteichoic acid per ml is required for sensitization, the amount of extracellular lipoteichoic acid for the *S. mutans* strains in Table 4 would be 10 to 320 μg/ml, with 20 to 40 μg/ml being the most frequent. Should the lipoteichoic acids from other strains not be as efficient as that from BHT in sensitizing erythrocytes, so that, say, 2 μg/ml would be required as with *L. casei* lipoteichoic acid, then the concentration for these strains would be even higher. The very high titer for strain BHT may relate in part to the efficacy of its lipoteichoic acid in sensitizing erythrocytes, but the high ratio of extracellular to cellular lipoteichoic acid found by the precipitin method does point to this strain producing considerably more extracellular lipoteichoic acid than the others examined.

Recent studies employing labeled glycerol have provided evidence for high- and low-molecular-weight glycerol phosphate polymers in the culture fluids of *S. sanguis* (6), *S. mutans* FA-1, and *Streptococcus faecalis* (13); in the latter study confirmatory serological evidence supported the presence of lipoteichoic acid. The studies on *S. faecalis* also showed that 90% of the cellular teichoic acid was detectable in the medium after valine starvation; similar results were obtained with *S. mutans* FA-1 after overnight culture (13). This release was not the result of cell lysis or wall turnover, as determined by the lack of release of protein, nucleic acid, and [^{14}C]lysine-labeled peptidoglycan. In the present study, the lack of cell wall rhamnose in culture fluid is also taken as providing evidence that release of lipoteichoic acid is not dependent on cell lysis. Thus, the lipoteichoic acid in the culture fluid qualifies to be under Pollock's restrictive definition of extracellular (28).

Although lipoteichoic acid is a membrane component, it can function as a surface antigen (4, 34) and can be readily released from cells by mild procedures (36). Further, cell wall preparations may contain lipoteichoic acid (2, 18). Thus, lipoteichoic acid released from cell membrane may occur as a transient cell wall component before its release into the culture fluid, where the molecules being amphipathic would aggregate to form the native high-molecular-weight micelles detectable in *S. mutans* BHT culture fluid.

In the present study the release of lipoteichoic acid from cells, as measured by hemagglutination, increased during the growth phases. However, the detailed investigations by Joseph and Shockman (13) with *S. faecalis* and *S. mutans* FA-1 have shown that a moderate release of teichoic acid during the exponential phase is followed by a massive release of accumulated cellular teichoic acid and lipoteichoic acid during the stationary phase. From the studies on *L. fermentum* stationary-phase organisms, the fraction released into the medium has been shown to differ markedly with respect to 14- and 19-carbon fatty acids from the fraction still retained by the cells.

L. fermentum cells have also been shown to contain variable quantities of a readily extractable low-molecular-weight teichoic acid, with a similar fraction being detectable in culture fluid. The low fatty acid content suggests that it is deacylated lipoteichoic acid, similar to the partially deacylated product detectable in *S. sanguis* wall preparations (2). Thus, deacylation by enzymatic or other means may contribute to the release from the cell membrane of

part of the extracellular lipoteichoic acid; such products do not sensitize erythrocytes and thus would not be detected in the hemagglutination reaction.

The formation of relatively high concentrations of extracellular lipoteichoic acid by oral streptococci may contribute to the pathogenic potential of these organisms. Should the lipoteichoic acid diffuse into gingival tissue, as will lipopolysaccharides (31), it may induce an immunological response or react with antibodies already present. It has been observed that human sera frequently contain antibodies reacting with different lipoteichoic acids (25).

Lipoteichoic acid may also be implicated in the resorption of bone in periodontal disease. Streptococci have been shown to induce alveolar bone loss in rats (15), and lipoteichoic acid at a concentration of ≥ 10 μg/ml will stimulate bone resorption in organ culture (10). Although this concentration is greater than that required for lipopolysaccharide to induce bone resorption (10, 11), it is still within the range detectable in culture fluid. The effect in each case apparently depends on an amphipathic molecule as deacylated lipopolysaccharide or lipoteichoic acid is ineffective (10).

Teichoic acids, by virtue of their ionized phosphate groupings, also have the potential to react with tooth enamel (5), and supporting evidence for this has been obtained by the absorption of the lipoteichoic acid in *S. mutans* BHT culture fluid to hydroxyapatite. Studies on another polyphosphate, phytic acid, indicate that its salts have cariostatic properties and that these may be related to the ability of phytates to lower the solubility in acid of enamel (23). The potential ability of lipoteichoic acid to bind to the tooth surface could therefore have beneficial effects. Balancing this, though, is the possibility that the adherence of bacteria to the tooth surface and other tissues, a factor given considerable ecological importance (8), could be similarly aided by cell surface lipoteichoic acid. As extracellular lipoteichoic acid was still present in large amounts when *S. mutans* BHT was grown in sucrose, and therefore forming glucans, it might be expected that the glucan, which is believed to be involved in the adherence of *S. mutans* to the tooth surface (8), would also be permeated by lipoteichoic acid. This could account for reports on the presence of phosphate in the glucans from *S. mutans* strains (14, 26).

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