INFECTION AND IMMUNITY, March 1976, p. 647-652
Vol. 13, No. 3
Printed in U.S.A.
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Formation of Cross-Reacting Antibodies Against Cellular and Extracellular Lipoteichoic Acid of *Streptococcus mutans* BHT

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Received for publication 30 September 1975

Antisera prepared against strains of *Streptococcus mutans,* particularly strain BHT, contain antibodies to the membrane lipoteichoic acid component that will cross-react with lipoteichoic acids from three species of lactobacilli. A more detailed study of the antibodies to strain BHT confirmed that the antibodies are specific for the polyglycerol phosphate component common to the lipoteichoic acids. Sera with a higher hemagglutinating titer could be obtained by injecting disrupted organisms. The partially purified lipoteichoic acid isolated from the culture fluid of *S. mutans* BHT was also immunogenic when injected with Freund complete adjuvant, yielding cross-reactive antibodies; antibodies were also formed against another unidentified component in this extracellular fraction.

Lipoteichoic acids occur as membrane components of most gram-positive bacteria and can frequently be detected as extracellular components (7, 10, 13, 24). In different instances the antibodies formed against lipoteichoic acid have been shown to be specific for the glycerol phosphate backbone or for the attached glycosyl or D-alanine residues (10, 24). Glycosyl-specific antibodies are required if the lipoteichoic acid is to function as a grouping or typing antigen, whereas the formation of "backbone"-specific antibodies will lead to considerable cross-reactions (10, 24). This report describes results indicating that such cross-reacting antibodies are formed against the lipoteichoic acid of *Streptococcus mutans* BHT, a strain used in serological typing of *S. mutans* strains and one defined as belonging to type *b* (1, 2, 15, 21). As in previous studies (12, 17, 23), evidence for the presence of cross-reacting antibodies was sought by examining the ability of antisera to react with a variety of lipoteichoic acids of known and different structure and the inhibition of these reactions with glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2).

MATERIALS AND METHODS

Organisms. *S. mutans* strains were obtained from previously described sources (13); additional strains donated by L. A. Thomson, National Institute of Dental Research, Bethesda, Md., were HS-6, FA-1, 6715-8, LM 7, and V 100. Each strain was characterized as to species and purity by colonial morphology and carbohydrate metabolism (i.e., fermentation reactions and polysaccharide formation from sucrose).

Growth conditions. *S. mutans* BHT was grown in a New Brunswick Microferm fermenter at 37 C under anaerobic conditions (95% N_2, 5% CO_2) in a complex medium (20); the pH was controlled at 6.0 by the automatic addition of NaOH solution. To obtain organisms required for the production of antisera, strain BHT was grown in the above medium without pH control, and other strains were grown in the diffusible component of dialyzed Trypticase soy broth.

Cellular lipoteichoic acid preparations. Lipoteichoic acid was extracted from *S. mutans* BHT with hot aqueous phenol and purified by column chromatography (22). Partial characterization of a high-molecular-weight organic phosphorus fraction (K_d = 0.1 from 6% agarose) as lipoteichoic acid was made on the basis of paper chromatographic identification of acid and alkali degradation products characteristic of conventional lipoteichoic acid (13, 25); these included glycerol, glycerol mono- and diphosphates, fatty acids, and alanine. Various preparations contained a low molar ratio of glucose to phosphorus (<0.1:1.0), indicating that most or all of the glycerol residues of the polyglycerol phosphate portion of the lipoteichoic acid were unsubstituted with carbohydrate; further studies on the structure of the lipoteichoic acid are in progress.

Preparations of lipoteichoic acids from *Lactobacillus casei* NCTC 6375, *L. plantarum* NCIB 7220, and *L. fermentum* NCTC 6991 were those employed in previous studies (10); the teichoic acid component of *L. casei* lipoteichoic acid does not have glycosyl substituents, whereas the substituents on the *L. plantarum* component are α-D-glucose, and on the *L. fermentum* component the substituents are α-1-D-galactosyl $\rightarrow$ 2-D-glucose and α-D-galactose.

Extracellular lipoteichoic acid. Cell-free culture fluid from *S. mutans* BHT was concentrated and fractionated by passing through Diaflo XM 50 and XM 300 filters in an Amicon filter cell at 4 C and a

pressure of 40 and 5 to 10 lb/in^2, respectively (13). The retentate was then fractionated by column chromatography on 6% agarose to give a high-molecular-weight fraction that showed essentially similar degradation products to the cellular lipoteichoic acid.

Serological methods. Antisera to whole organisms of *S. mutans* BHT were prepared by injecting rabbits at 2- to 3-day intervals with increasing doses (0.2 ml, 0.5 ml, 1.0 ml, and 1.5 ml) of a suspension of formalinized cells (optical density at 600 nm in 1 cm cell = 1.0) followed by two injections (1.0 ml and 1.25 ml) of a stronger suspension (optical density = 2.0); rabbits were then bled after a further 6 to 10 days. Antisera to disrupted organisms were prepared by intravenous injection (18, 25), and antisera to extracellular material were prepared by subcutaneous injection with Freund complete adjuvant (8).

Antisera were also prepared (by Helen Agus) to strains from serotypes *a* (strain HS 6), *b* (FA-1), *c* (JC-2, GS-5), and *d* (6715-8, B-13), using organisms heated at 60 C for 1 h, and to type *e* strains (LM 7, V 100) with live suspensions; in each case the turbidity of the suspension in saline was adjusted to give a turbidity equivalent to standard 9 on a Macfarland scale. Rabbits received a total of 13 injections at 2- to 3-day intervals of increasing amounts (0.25 ml to 2.0 ml) of suspension and were bled 7 and 10 days after the last injection.

Typing antisera to *S. mutans* strains AHT, BHT, GS 5, and B 13 were prepared by A. S. Bleiweis in his laboratory by a previously described method (19, 21).

Procedures have been described previously for examining lipoteichoic acids by the quantitative precipitin method (8), immunoelectrophoresis (22), and hemagglutination (6). In all cases where the quantitative precipitin method was used, the amount of antigen required for precipitation of the maximum amount of antibody was determined; this provided the basis for determining the percentage of cross-reaction of antisera with different lipoteichoic acids and also for calculating the amount of lipoteichoic acid to be added to precipitate specific antibodies in antisera to extracellular material (i.e., absorbed sera).

To confirm that antibodies were specific for the glycerol phosphate backbone of lipoteichoic acid, G_3P_2 was prepared from cardiolipin and examined for its ability to inhibit the precipitin reaction (23).

RESULTS

Detection of cross-reacting antibodies to cellular lipoteichoic acid of S. mutans BHT. During a survey (with A. S. Bleiweis) of antisera employed in the serological classification of *S. mutans* strains, it was observed that the antisera (designated 4 and 5) to whole cells of strain BHT (serotype *b*) agglutinated erythrocytes sensitized with lipoteichoic acid from *S. mutans* BHT and also from *L. plantarum*, *L. casei*, and *L. fermentum* (Table 1). This cross-reactivity was confirmed with antisera produced in our laboratory (Table 1). By the quantitative precipitin method the amount of antibody reacting with strain BHT cellular lipoteichoic acid was very low—0.53 and 0.46 mg/ml, respectively—for antisera 4 and 5.

Previous studies (9, 25) had shown that more reactive sera may be obtained by injecting disrupted organisms. Antisera obtained against disrupted *S. mutans* BHT cells were no more reactive in the quantitative precipitin method, the amount of antibody being 0.57, 0.15, and 0.57 mg/ml for rabbits 357, 358, and 359, respectively. However, the two more active sera did have a higher hemagglutinating activity than antisera to whole organisms (Table 1); these two antisera reacted strongly with *L. plantarum* and *L. casei* lipoteichoic acids but only weakly with *L. fermentum* lipoteichoic acid (Table 1). The weak reaction with *L. fermentum* lipoteichoic acid was confirmed by the quantitative precipitin method (Table 2).

The reaction of the extracellular lipoteichoic acid fraction (XM 300 retentate) with antisera 357 and 359 was also examined by the quantitative precipitin method; with each serum the

TABLE 1. *Antibody titers for sera against S. mutans BHT using erythrocytes sensitized with different lipoteichoic acids*

S. mutans prepn injected	Antiserum	Sensitizing lipoteichoic acid			
		S. mutans BHT	*L. plantarum*	*L. casei*	*L. fermentum*
Whole organisms	4[a]	400	800	800	800
	5[a]	400	800	400	800
	265	200	800	400	800
	267	200	200	50	50
Disrupted organisms	357	1,600	3,200	3,200	100
	359	3,200	6,400	6,400	50
Extracellular fraction	363	1,600	200	800	400
	364	800	200	200	100

[a] Sera from A. S. Bleiweis.

TABLE 2. *Detection of cross-reacting antibodies to S. mutans BHT lipoteichoic acid by precipitin method*

S. mutans prepn injected	Antiserum	Cross-reaction (%)			
		S. mutans BHT	*L. plantarum*	*L. casei*	*L. fermentum*
Disrupted organisms	357	100	65	44	9
	359	100	57	57	19
Extracellular fraction	363	100	67	86	70
	364	100	68	98	78

amount of antibody precipitable was greater than that precipitated by cellular lipoteichoic acid (Table 3). Absorption of antiserum 359 with BHT cellular lipoteichoic acid reduced the amount of antibody reacting with the extracellular lipoteichoic acid (Table 3); the incomplete removal of antibody indicated the presence in the extracellular fraction of at least one other antigen. Confirmation of this was obtained by immunoelectrophoresis, where, in addition to lipoteichoic acid, a slower-moving negatively charged component was detected.

Column chromatography of the extracellular lipoteichoic acid fraction on 6% agarose (13) failed to remove the additional antigen, nor have other chromatographic methods previously employed for purifying lipoteichoic acid (22) proved successful (unpublished observations with Merilyn Hewett). Column chromatography did, however, lead to a partial purification as shown by the reactivity of fractions with antiserum to *L. casei* lipoteichoic acid (13, 23); this antiserum cross-reacts with different lipoteichoic acids on the basis of the common polyglycerol phosphate backbone. Figure 1 shows that the fraction obtained from 6% agarose chromatography was more reactive than the unfractionated material, although it was still less reactive in terms of the amounts required for maximum antibody precipitation than the cellular lipoteichoic acid.

Detection of cross-reacting antibodies to extracellular lipoteichoic acid. The extracellular fraction from *S. mutans* BHT, which is retained by ultrafiltration through a Diaflo XM 300 membrane, was injected with Freund complete adjuvant into three rabbits (363, 364, 365). The presence of antibodies specific for the lipoteichoic acid component was shown by examining the reaction of the antisera with the purified cellular lipoteichoic acid from strain BHT. By the quantitative precipitin method the amount of antibody in antisera 363, 364, and 365 was 1.1, 1.8, and 0.3 mg/ml, respectively. The two more active antisera were examined further for the presence of cross-reacting antibodies, and positive results were obtained by both the hemagglutination (Table 1) and precipitin methods (Table 2). In this case there was a strong cross-reaction with the *L. fermentum* lipoteichoic acid as well as with the *L. casei* and *L. plantarum* lipoteichoic acids.

TABLE 3. *Reactivity of cellular and extracellular lipoteichoic acid preparations from S. mutans BHT*

S. mutans prepn injected	Antiserum	Antibody protein (mg/ml)		
		Cellular	Extracellular	Absorbed[a]
Disrupted cells	357	0.6	1.6	NT
	359	0.6	1.6	0.8
Extracellular fraction	363	1.1	2.2	NT
	364	1.8	2.6	0.7

[a] Absorbed with cellular lipoteichoic acid and then tested for reactivity with extracellular fraction. NT, Not tested.

The agglutination of erythrocytes sensitized with the extracellular lipoteichoic acid fraction was effectively inhibited by purified cellular lipoteichoic acid; the titer of both antisera 363 and 364 decreased from 800 to <50 in the presence of the inhibitor (10 μg/10 μl in each well). This result indicated that lipoteichoic acid is probably the only immunogen in the extracellular fraction that is capable of sensitizing erythrocytes. However, examination of antisera by the quantitative precipitin reaction indicated the presence of at least one other extracellular immunogenic component (Table 3). The results of immunoelectrophoresis using antisera before and after absorption with cellular lipoteichoic acid indicated that antibodies had been formed against the slow-moving component previously detected with antisera to disrupted organisms.

Antisera with similar properties were also obtained by the injection with Freund adjuvant of the extracellular lipoteichoic acid fraction prepared by chromatography on 6% agarose.

Inhibtion of antibody reaction with G_3P_2. The ability of G_3P_2 to inhibit the quantitative precipitin method was examined, as this pro-

vides an indication of the presence of antibodies capable of reacting with the glycerol phosphate backbone of lipoteichoic acid. With antisera 357 and 359 (against disrupted organisms), the reaction between strain BHT cellular lipoteichoic acid (20 μg and 15 μg, respectively) and 0.2 ml of serum was inhibited 60 and 47%, respectively, by 1 μmol of G_3P_2. With 0.1 ml of antisera 363 and 364 (against extracellular material) and 10 μg of strain BHT cellular lipoteichoic acid, the comparable values were 40 and 67%, respectively.

Figure 2 summarizes the evidence for cross-reacting antibodies in antiserum 364, showing each of the precipitin reactions with different lipoteichoic acid preparations expressed relative to the reaction with BHT cellular lipoteichoic acid (which is shown as 100%) and the percentage of inhibition of each of these reactions by 1 μmol of G_3P_2.

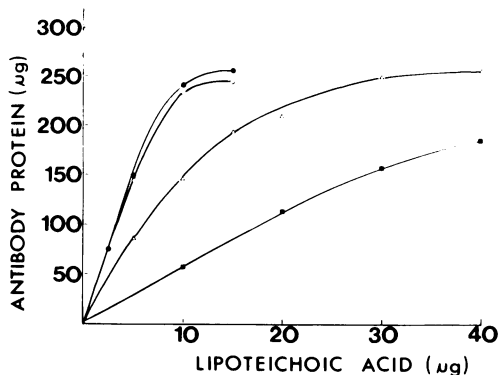

FIG. 1. *Detection of lipoteichoic acid in fractions from culture of S. mutans BHT by their reactivity with cross-reacting antiserum (0.1 ml) to L. casei NCTC 6375 lipoteichoic acid. Symbols: ●, L. casei lipoteichoic acid; ○, S. mutans cellular lipoteichoic acid; ■, extracellular fraction from S. mutans retained by XM 300 membrane; △, extracellular lipoteichoic acid fraction from S. mutans eluted from 6% agarose.*

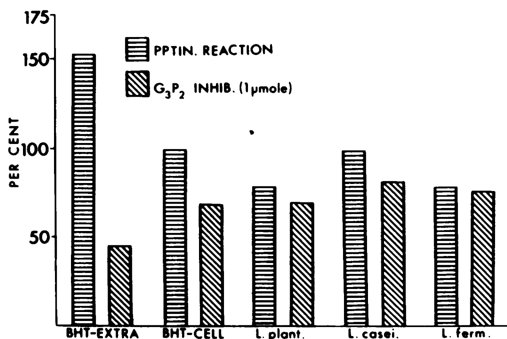

FIG. 2. *Detection of cross-reacting antibodies to lipoteichoic acid in antiserum to S. mutans BHT extracellular fraction by comparing the reaction of antiserum 364 (0.1 ml) with the extracellular fraction, S. mutans BHT cellular lipoteichoic acid (expressed as 100%), and with lipoteichoic acids from L. plantarum, L. casei NCTC 6375, and L. fermentum; the percentage of inhibition of each of the reactions by 1 μmol of G_3P_2 is also shown.*

Formation of cross-reactive antibodies by other S. mutans strains. As indicated previously, the initial observation with *S. mutans* BHT antiserum was made during a survey of typing antisera. Other antisera examined (with A. S. Bleiweis) had lower titers but also cross-reacted with lipoteichoic acids from *L. plantarum*, *L. casei*, and *L. fermentum* (Table 4). Antisera prepared against representative strains of serotypes *a* to *e* confirmed that cross-reactive antibodies could be formed, with strain FA-1 providing the only exception (Table 4).

DISCUSSION

Because of its implications as a causative organism of dental caries, *S. mutans* has been subjected to detailed physiological, chemical, and serological examination (see reference 5). Bratthall (2) defined five serological groups, designated *a* to *e*, by immunoelectrophoresis of acid extracts of cells, and subsequently (3) employed the typing sera in a study on the identifi-

TABLE 4. *Antibody titers for sera against S. mutans strains using erythrocytes sensitized with different lipoteichoic acids*

Serotype	Strain	Sensitizing lipoteichoic acid		
		L. plantarum	*L. casei*	*L. fermentum*
a	AHT[a]	40	20	40
	HS 6[b]	40	20	20
		640	320	640
b	FA-1[b]	<20	<20	<20
		<20	<20	<20
c	GS-5[a]	20	160	160
	JC-2[b]	160	160	80
		320	320	20
d	B-13[a]	80	40	80
	6715-8[b]	320	640	320
		80	40	<20
e	LM 7[b]	160	80	40
	V 100[b]	640	640	320
		80	160	40

[a] Sera from A. S. Bleiweis.

[b] Sera from Helen Agus; where two results are given they represent values for antisera from different rabbits.

cation of *S. mutans* strains by immunofluorescence; a considerable degree of cross-reaction of the fluorescein-conjugated sera was observed with strains of other serotypes and also with viridans streptococci, although for three of the serotypes, namely *a*, *b* and *d*, specific sera could be obtained by appropriate cross-absorptions. The extent of the cross-reactions when whole organisms were reacted with antisera indicated that there were surface antigens detectable by this procedure that were not detectable by immunoelectrophoresis of acid extracts. The nature of these cross-reacting surface antigens has not been defined, but the present results indicate that lipoteichoic acid could be contributing.

Lipoteichoic acid is a membrane component that can also be detected as a surface component (10, 24). The extent to which it occurs as a surface component and also functions as an immunogen is variable, depending on the organism and on the injected rabbit (10, 24). Further, the extent to which the antibodies to a lipoteichoic acid will cross-react with other lipoteichoic acids is also variable, depending on the structure and conformation of the lipoteichoic acid, the preparation injected, and the individual rabbit variation (10, 24). Evidence for cross-reacting antibodies to lipoteichoic acid can be obtained by showing that sera react with lipoteichoic acids carrying different glycosyl substituents and also by inhibiting the reaction with G_3P_2. By such procedures evidence has been obtained that the lipoteichoic acid component of *S. mutans* BHT cells does induce the formation of such cross-reacting antibodies. Previous studies have shown (10, 24) that their formation is more likely when the polyglycerol phosphate component is devoid of or has a very low degree of substitution with glycosyl residues, and the *S. mutans* BHT lipoteichoic acid would appear to be in this category from the partial characterization described above. With other strains of *S. mutans,* including ones employed by Bratthall (2) in his classification, the results indicate that cross-reacting antibodies to the lipoteichoic acid component can also be formed. The reactivity of these antisera with lipoteichoic acids from lactobacilli is generally low and variable, and the results did not include a comparison with homologous lipoteichoic acid. However, where such sera are being used in the classification of *S. mutans* strains, the extent of reactivity is sufficient to suggest that (i) an examination of sera by this procedure could aid in eliminating individual sera with a high titer of cross-reactive antibodies, and (ii) strain FA-1 is probably preferable to strain BHT for producing serotype *b* antiserum. It should also be possible to remove these cross-reactive antibodies by absorbing antisera with lipoteichoic acid.

Whereas these cross-reacting antibodies to lipoteichoic acid are a problem in the reaction with whole cells, they may not be detected by the less sensitive procedures using the precipitin reaction, such as immunoelectrophoresis. This had been noted by Bratthall (2) and would relate to both the relatively low concentration of lipoteichoic acid compared with the amount of a cell wall carbohydrate antigen and also the degradation of lipoteichoic acid during acid extraction. For each of the serotypes *a* to *d* of *S. mutans* a cell wall carbohydrate has been shown to be serotype specific (11, 14, 15, 21; H. D. Slade and A. S. Bleiweis, private communications), although for type *a* strain AHT the membrane lipoteichoic acid also reacts with the typing sera (19).

A number of studies have shown that human sera may contain a significant titer of antibodies to lipoteichoic acid, with the evidence in some instances being indicative of their being cross-reactive antibodies (10, 12, 24). Unequivocal evidence for an organism inducing these cross-reacting antibodies in humans has not been obtained, but the results of the present study do indicate that *S. mutans* is a possible candidate. It may also be of significance that antisera to *S. sanguis* (16) also contain antibodies cross-reacting with a variety of lipoteichoic acids (unpublished observations with B. Rosan).

Besides a general antibody response to lipoteichoic acid, a specific localized reaction in oral tissues would also be of significance, as it is likely that immunopathological mechanisms are involved in certain diseases of the periodontium (4). The possibility of such a reaction would also be increased should the extracellular lipoteichoic acid diffuse into gingival tissue and there invoke an immunological response or react with antibodies already present. As has been observed, there are other, although unidentified, extracellular immunogens formed by *S. mutans* BHT, and these could also contribute to immunological reactions in tissues.

ACKNOWLEDGMENTS

We wish to thank A. S. Bleiweis, University of Florida, and B. Rosan, University of Pennsylvania, for their contributions to this study, Merilyn Hewett for her assistance in preparation of lipoteichoic acids, and Helen Agus for providing antisera to *S. mutans* serotypes.

This work was supported by a grant from the Australian National Health and Medical Research Council.

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