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Characterization of Serological Cross-Reactivity Between Polysaccharide Antigens of *Streptococcus mutans* Serotypes *c* and *d*

S. GROSSI,[1] A. PRAKOBPHOL,[1] R. LINZER,[1]* L. K. CAMPBELL,[2] AND K. W. KNOX[2]

Department of Oral Biology, State University of New York, Buffalo, New York 14226,[1] and Institute of Dental Research, United Dental Hospital, Sydney, New South Wales 2010, Australia[2]

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Immunological assays with antisera prepared against purified *Streptococcus mutans* serotype *c* polysaccharide demonstrated that a cross-reacting determinant on *c* polysaccharide reacted with the wall-associated rhamnose-glucose polysaccharide from *S. mutans* serotype *d*. Studies with 60 antisera prepared against chemostat cultures of *S. mutans* Ingbritt (*c*) demonstrated that the rhamnose-glucose polysaccharide cross-reactive determinant was consistently expressed on *c* antigen under a variety of growth conditions.

Indirect evidence has indicated that *Streptococcus mutans* is a prime etiological agent of dental caries in humans (1, 6). Although seven serotypes of *S. mutans* have been identified (2, 13), epidemiological studies conclude that serotypes *c* and *d* are predominant in human populations (3, 16). Immunochemical studies have reported that the typing antigens of the *c* and *d* strains are wall-associated polysaccharides (9, 11, 17) that are chemically and serologically distinct. However, immunological cross-reactions have been noted between anti-whole cell serotype *c* serum and a second wall polysaccharide, the rhamnose-glucose polysaccharide (RGP) that has been characterized by this laboratory in strains of *S. mutans* serotypes *d* and *g* (14, 15). Because of the importance of serological methods in the identification of *S. mutans* and because of the interest in the potential protective effects of antibodies to surface antigens of *S. mutans* with respect to dental caries, we sought to clarify these observed cross-reactions between serotypes *c* and *d*.

Although *c* antigen is immunogenic on the cell surface, the purified polysaccharide is a poor immunogen. Therefore, antiserum to the purified *c* polysaccharide was prepared by using a lipid-*c* antigen:antibody (L-*c*Ag:Ab) complex. Serotype *c* polysaccharide was prepared by hot acid extraction of *S. mutans* Ingbritt cells and chromatography on columns of DEAE Sephadex A-25 and Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) as described previously (9). The purified *c* antigen had a molar ratio of rhamnose to glucose of 1.7:1.0 and contained less than 1% protein. Purified *c* polysaccharide (0.5 mg) in 1 ml of saline was reacted with rabbit anti-Ingbritt cell serum (1 ml) at 37°C for 30 min and at 4°C overnight. The antigen:antibody (Ag:Ab) precipitate was recovered by centrifugation, washed, and dissolved in saline containing 0.01 N HCl at 4°C. The antibodies were reprecipitated by the addition of lipid-esterified *c* antigen. New Zealand white rabbits were immunized subcutaneously with 1 ml of the L-*c*Ag:Ab complex emulsified in an equal volume of Freund complete adjuvant. Booster injections on days 14 and 70 were in Freund incomplete adjuvant. Each immunogen dose contained approximately 1 mg of carbohydrate, 1 mg of protein, and 0.06 mg of lipid.

The anti-L-*c*Ag:Ab serum was examined for reactivity with the *c* polysaccharide and other glucose-containing polymers by using a passive hemagglutination assay as previously described (15). The additional polysaccharides included (i) RGP, prepared from *S. mutans* B13 (RGP/B13) (15), (ii) lipoteichoic acid, prepared from strain Ingbritt (LTA/Ingbritt) (7), and (iii) dextran T-10 purchased from Pharmacia Fine Chemicals. The purified *c* polysaccharide, RGP/B13, and dextran T-10 were esterified with palmitic acid according to the methods of Hammerling and Westphal (5) to facilitate sensitization of sheep erythrocytes for use in the passive hemagglutination assays.

As shown in Table 1, the lipid-polysaccharide-protein complex elicited production of high-titer antiserum to *c* polysaccharide and demonstrated cross-reactivity with the RGP/B13 antigen, suggesting that the cross-reactive determinant was an integral part of the *c* polysaccharide. This conclusion was supported by agar gel diffusion studies which showed a reaction of partial identity between the purified *c* antigen and RGP/B13 antigen in the presence of anti-L-*c*Ag:Ab serum

TABLE 1. Immunological reactivity of antigens used for the passive hemagglutination assays

Antiserum	Passive hemagglutination titer[a]				
	Control	*c* Ag	RGP/B13	Dextran	LTA/Ingbritt
Normal rabbit serum	≤10	≤10	≤10	≤10	≤10
Anti-L-*c*Ag:Ab	≤10	5,120	640	≤80	≤10
Anti-RGP/B13	≤10	≤10	2,560	≤80	≤10
Anti-dextran T-20	≤10	≤10	≤10	320	≤10
Anti-LTA	≤10	≤10	≤10	≤10	1,280

[a] Reported as the inverse of the highest dilution yielding positive hemagglutination.

(Fig. 1). In Table 1, a weak reaction between anti-L-*c*Ag:Ab serum and dextran-sensitized sheep erythrocytes was noted. The *c* antigen antiserum did not react with LTA/Ingbritt, which contains glucose that is β-linked to the 2 position of the glycerol in the polyglycerophosphate backbone.

Immunofluorescent studies were performed to determine the accessibility of the *c* antigen and the RGP/B13 antigen to antibodies at the surfaces of the respective *S. mutans* cells (Fig. 2). With indirect immunofluorescent staining with fluorescein isothiocyanate-labeled goat antirabbit immunoglobulin G (heavy and light chain) sera, the anti-L-*c*Ag:Ab serum gave positive fluorescence with Ingbritt cells (*c*) at serum dilutions of 640 and with B13 cells (*d*) at dilutions of 160. Absorption with Ingbritt cells (*c*) removed all reactivity, whereas absorption of the antiserum with serotype *d* cells reduced the titers to Ingbritt cells (*c*) to 80 and eliminated reactions with serotype *d* cells. This supports the agar diffusion and passive hemagglutination data showing that the *c*-RGP cross-reactive site represents only one of two or more determinants of *c* polysaccharide.

The effects of variations in growth rate, pH, and limiting substrate on the immunogenicity of the RGP cross-reactive determinant of the *c* antigen were examined and related to variations in the immunogenicity of *c* antigen and dextran. A collection of 60 anti-whole cell sera were prepared by intravenously injecting male New Zealand white rabbits with eight populations of chemostat-grown *S. mutans* Ingbritt cells (*c*) as previously described (8) (Table 2). Reactions with RGP/B13 antigen were used to examine titers to the cross-reactive determinant on the *c* polysaccharide; total antibody titers to *c* antigen and dextran were also assayed. All 60 antisera tested reacted with the RGP/B13 antigen, thus demonstrating a consistent expression of the RGP cross-reactive determinant on the *c* antigen. Titers to the cross-reactive determinant did not vary significantly with changes in either culture dilution rate or pH. However, titers to the RGP cross-reactive determinant increased significantly in sucrose- versus glucose-grown cultures at dilution rate (D) = 0.5 h^{-1} (pH 6.0) and D = 0.1 h^{-1} (pH 5.5) (Table 2). These changes were accompanied by increases in titers to the *c* antigen. The most marked effect of limiting substrate variation was on titers to dextran. Cells grown on sucrose versus glucose had mean dextran titer increases of 4.5- to 8-fold. These increases in dextran titers reached significance levels of $P < 0.001$ for cells grown at D = 0.1 h^{-1} (pH 5.5 and 7.5) and $P < 0.02$ for cells grown at D = 0.5 h^{-1} (pH 6.0). Although *S. mutans* is known to accumulate surface glucans and clump when grown in sucrose-supplemented batch culture, no cell clumping was noted, even microscopically, under chemostat conditions when sucrose was the limiting growth nutrient. Examination of antisera for reactivity with dextran by the passive hemagglutination assay pro-

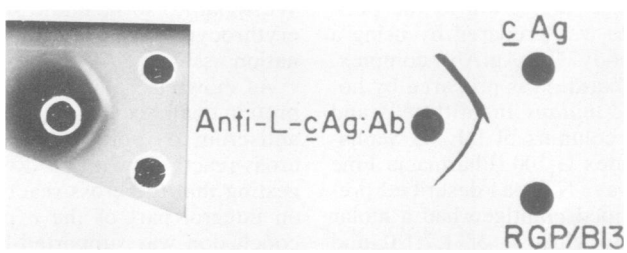

FIG. 1. Cross-reaction between serotype *c* antigen and RGP/B13 on agar gel diffusion. Purified serotype *c* Ag (20 μg) and RGP/B13 (20 μg) were reacted with anti-L-*c*Ag:Ab serum (50 μl). Precipitation patterns show a partial identity between *c* antigen and RGP/B13.

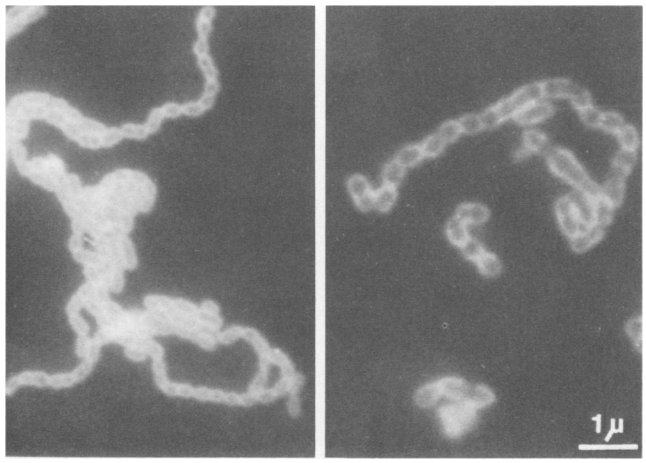

FIG. 2. Indirect immunofluorescent staining of *S. mutans* cells. Cells of *S. mutans* Ingbritt (*c*) (left) and *S. mutans* B13 (*d*) (right) were reacted with anti-L-*c*Ag:Ab serum and subsequently stained with fluorescein isothiocyanate-labeled goat antirabbit immunoglobulin serum.

vided a sensitive means for detecting antibodies to surface-associated glucans in amounts that were not evident in earlier studies (7, 8).

The immunological specificity of both the *c* polysaccharide and the RGP antigen have been related to the presence of α-linked glucosyl residues in the molecules (14, 18). The absence of cross-reactivity between the anti-L-*c*Ag:Ab serum and LTA/Ingbritt may be related to the fact that the glucose moieties in the LTA polymer are β-linked. The agar diffusion data, passive hemagglutination assays, and immunofluorescent staining studies all suggested that the *c* polysaccharide possessed antigenic determinants with at least two specificities, a *c*-specific site and a cross-reacting *c*-RGP site. The presence of multiple antigenic specificities on one polysaccharide molecule has previously been suggested to describe the cross-reactions between the serotype *c* and *e* (18) and the serotype *a* and *d* (4, 10) antigens of *S. mutans*. Multiple immunodominant components have also been well characterized among the polysaccharide O antigens of *Salmonella* species (12). Interesting-

TABLE 2. Variations in immunogenicity of chemostat-grown cultures with respect to variations in substrate

Growth conditions		pH	No. of rabbits	Geometric mean antibody titers[a]		
Dilution rate (h^{-1})	Carbohydrate source (0.5%)			*c* Antigen	RGP/B13	Dextran
0.05	Glucose	6.0	5	1,860 ± 1,980	100 ± 36	38 ± 31
	Sucrose		7	2,820 ± 1,710	316 ± 315	251 ± 222
				NS[b]	NS	NS
0.5	Glucose	6.0	4	1,350 ± 887	120 ± 32	56 ± 23
	Sucrose		8	2,880 ± 1,550	269 ± 74	446 ± 253
				NS	$P < 0.01$	$P < 0.02$
0.1	Glucose	5.5	10	3,390 ± 1,500	204 ± 82	72 ± 31
	Sucrose		8	4,570 ± 2,120	537 ± 221	331 ± 179
				NS	$P < 0.001$	$P < 0.001$
0.1	Glucose	7.5	10	3,890 ± 756	302 ± 176	66 ± 45
	Sucrose		8	2,140 ± 1,810	302 ± 232	416 ± 249
				$P < 0.02$	NS	$P < 0.001$

[a] Titers were determined by the passive hemagglutination assay.

[b] NS, Not significant as determined by *t* test for independent geometric means. Antisera to organisms grown with the same pH values and dilution rate but different carbohydrate substrate were compared.

ly, although the cross-reactive site was consistently expressed and immunogenic on the surface of serotype *c* cells, cross-reacting antibodies to *c* antigen were very rarely found during examination of anti-RGP/B13 sera. This same type of one-sided immunogenicity was observed between serotype *c* and *e* sera (18). In those studies, cross-reacting antibodies to serotype *e* were consistently found by using serotype *c* sera, but cross-reactions with anti-*e* serum and various extracts of serotype *c* cells or cell walls were never detected. This may suggest that the cross-reacting determinant is more favorably oriented to elicit an immune response in the serotype *c* polysaccharide or on the serotype *c* cell wall or both. That the *c*-RGP cross-reacting site is accessible on the serotype *d* cell wall was demonstrated by the immunofluorescence studies. Future structural studies should clarify the nature of the cross-reactive site.

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