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Structure and Immunological Specificity of the *Streptococcus mutans* Group b Cell Wall Antigen

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The *Streptococcus mutans* group b antigen of strain FA1 has been defined as to chemical composition and immunological specificity. The antigen in cold trichloroacetic acid extracts was fractionated on diethylaminoethyl-Sephadex A-25 at pH 8.5. Two forms were isolated: a polysaccharide and a mucoprotein. The two polymers reacted as a single substance in agar gel diffusion against specific adsorbed FA1 rabbit antisera but were separated by gel immunoelectrophoresis. No reaction with any other *S. mutans* or streptococcal group sera occurred. Galactose composed about one-third and galactosamine about 3% of the total weight of each polymer. Rhamnose was a major component of the polysaccharide (47%) but was present only in traces in the mucoprotein. The protein content of the latter was about 40%. No significant quantities of glycerol, phosphorus, or muramic acid were present in either case. Pepsin and trypsin had no effect on the serological specificity of the mucoprotein. D-Galactose and D-galactosamine were strong inhibitors (70%) of the precipitin reaction, whereas D-glucose, D-glucosamine, and *N*-acetyl-D-glucosamine inhibited between 25 and 35%. The results indicate that the antigen is a major antigenic component of the cell wall and that the specificity of the antigen resides in binding sites which contain both D-galactose and D-galactosamine. Agglutination of whole cells by specific group b antiserum indicates the antibody receptor sites of the polysaccharide antigen are at the surface of the streptococcal cell. The mucoprotein, but not the polysaccharide, was released from the cell by lysozyme. Lysis did not occur. The immunological specificity and other characteristics of the antigen establishes it as the identifying antigen of *S. mutans* group b.

Streptococcus mutans plays a significant role in the development of animal and human dental caries (4, 12, 24). No major differences have been revealed between these strains and noncariogenic streptococci in fermentation end products, or in the amount of acids produced (9, 14). The plaque-forming ability (15) and the special production of dextranase-resistant extracellular dextran (21), however, are considered to be unique properties of cariogenic strains. Further differences among strains of this group, based on serological specificity of acid-extracted antigens (2, 3) and deoxyribonucleic acid (DNA)-DNA reassociation experiments (7, 8), have resulted in the assignment of most strains to four separate groups. Other strains possess the streptococcal group E antigen (2). The composition of this antigen has recently been established as a polysaccharide and found to be located in or on the cell wall (27). However, none of the antigens of the four *S. mutans* groups has yet been purified, nor have their composition and immunological specificity been determined. The present report presents these characteristics on the group b antigen of *S. mutans*, strain FA1.

MATERIALS AND METHODS

Streptococcal strains. We are grateful to A. L. Coykendall, David Edman, and Irving Shklair (Naval Dental Research Institute, Great Lakes, Ill.) and C. E. deMoor for *S. mutans* strains 10449, GS5, FA1, BHT, HS6, OMZ61, AHT, SL1, OMZ176, and B13. The cells for study were grown, harvested, and lyophilized as previously described (28). All the chemical and immunological data presented here (unless otherwise noted) were obtained on cells of strain FA1 (Bratthall group b [2], Coykendall group II [7]).

Antisera. 10449, FA1, HS6, and SL1 cells were used to immunize New Zealand red rabbits (26). The sera were adsorbed before use with streptococcal group A cells (19). Grouping sera were kindly provided by the Center for Disease Control,

Atlanta, Ga., and C. E. deMoor and H. W. B. Engel, National Institute of Public Health, Utrecht, Holland.

Preparation of crude antigens. Crude antigen extracts of FA1 whole lyophilized cells (20 mg) were obtained by the following several methods. The cells were extracted with (i) 1 ml of distilled water at several temperatures for 20 min, (ii) various concentrations of HCl at 100 C for 20 min, (iii) 5% trichloroacetic acid at 60 C for 20 min, or (iv) 10% trichloroacetic acid at 5 C for 16 h. After removing the cells by centrifugation, the extracts were adjusted to pH 7.4 with NaOH to a final volume of 1 ml.

Crude antigen extract was also obtained from purified cell walls. A 50-mg amount of dry whole cells was broken in a Braun homogenizer (20), centrifuged, and treated with ribonuclease, trypsin, and pepsin as previously described (26). Contaminating lipoprotein from the cytoplasmic membrane was removed by treatment in 5 ml of 2% sodium dodecyl sulfate for 90 min at 37 C. The cell walls were washed three times with distilled water and extracted with 0.5 ml of 0.1% lysozyme (Worthington Biochemical Co.) in 0.02 M phosphate buffer, pH 7.8, for 1 h at 37 C. The extract was used in agar gel diffusion tests against FA1 antiserum to show the presence of the antigen in the cell wall.

Purification of antigen. Two procedures were used. (i) A 10-g amount of lyophilized whole cells (FA1) was extracted twice with 300 ml of 10% trichloroacetic acid as above. The two extracts were combined, neutralized with NaOH, dialyzed four times overnight at 5 C in 5 liters of distilled water, and lyophilized. The material was applied to a column (3 by 55 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 (HCO_3^- form) (18), and eluted with a linear concentration gradient of 500 ml of 0.05 M $(NH_4)_2CO_3$ and 1.0 M $(NH_4)_2CO_3$. Each fraction (15 ml) was collected and analyzed. The fractions which showed a positive precipitin reaction with anti-FA1 serum were combined (total dry weight: first peak, 14.1 mg; last peak, 4.1 mg). The first and last fractions were designated antigen 1 and antigen 2 and further purified with Sephadex G-200 (3 by 55 cm) in 0.05 M $(NH_4)_2CO_3$, pH 8.6. The final yield of antigen 1 was 1.3 mg and antigen 2, 9.3 mg. (ii) A 10-g amount of FA1 cells was extracted with 250 ml of distilled water overnight at 5 C. The extraction was repeated with 250 ml of water. The extracts were combined and lyophilized. The material was applied to a DEAE-Sephadex A-25 column and then to a Sephadex G-200 column as described above. The yields of FA1 antigen 3 and 4 from the water extracts were 1.2 and 0.66 mg, respectively.

Analytical methods. The antigen preparations were hydrolyzed in 4 N HCl at 100 C for 12 h and then examined for reducing sugars, amino acids, ribitol, and glycerol after separation by thin-layer chromatography (18). Total phosphorus (18) and total sugars (10) were measured as previously described. In the cases of analyses of chromatographic fractions, 0.5 ml of each fraction was used. Sugars and amino acids were determined quantitatively by liquid-gas chromatography as previously described (19, 20).

Serological procedures. Agar-diffusion analysis (22) and quantitative and qualitative precipitin reactions, and the inhibition of the former, have been described (25). Agglutination of whole cells by antiserum was determined by mixing 5 μliters of cell suspension (20 mg of dry cells in 3 ml of 0.85% NaCl) and 5 μliters of serum in a capillary tube (no. 3467, A. H. Thomas, Philadelphia). A positive agglutination usually occurred within 20 min at room temperature.

Lability of antigen. A 10-μg amount of antigen was treated with 20 μliters of several concentrations of dilute HCl at 100 C. After several minutes, the solutions were neutralized with NaOH and the volumes were made to 25 μliters. Capillary and quantitative precipitin determinations were performed on the solutions. The degradation of the antigens by enzymes was tested by using 1% solutions of β-glucosidase, lysozyme, trypsin, pepsin, ribonuclease, and deoxyribonuclease as described previously (19, 20).

Release of sugars. A 100-μg amount of antigen 1 was treated with 0.004 N HCl (50 μliters) in a sealed tube at 100 C for 5 to 60 min. After being neutralized with 0.004 N NaOH, the sugars released were obtained by Sephadex G-15 column chromatography (1 by 55 cm) in distilled water (19). The fraction was lyophilized and analyzed by thin-layer chromatography (18) and by liquid-gas chromatography (19, 20).

Molecular weight. The molecular weight determination was made by the gel filtration method as described previously (20).

RESULTS

The capillary precipitin reaction of crude antigen extracts from whole cells of FA1 against anti-FA1-adsorbed serum showed that extraction with distilled water at 100 C for 20 min or with 10% trichloroacetic acid at 5 C for 16 h was effective. However, the quantity released by water at 100 C was only about one-fifth that released by trichloroacetic acid. HCl (0.1 to 0.5 N) at 100 C destroyed a large part of the immunological specificity of the antigens; however, lower concentrations (0.01 to 0.05 N) of HCl were also effective as extracting agents. It can be seen in Fig. 1 that 0.02 N HCl extracts as FA1 and BHT, both belonging to *S. mutans* b group (2), produce a positive reaction with FA1 antiserum. This result would appear to indicate a single substance; however, it will be shown later that two substances with a single serological specificity were present.

Extracts of other *S. mutans* strains, HS6, OMZ61 and AHT (group a), 10449 and GS5 (group c), SL1, OMZ176, and B13 (group d) (2), did not contain a significant amount of any anti-

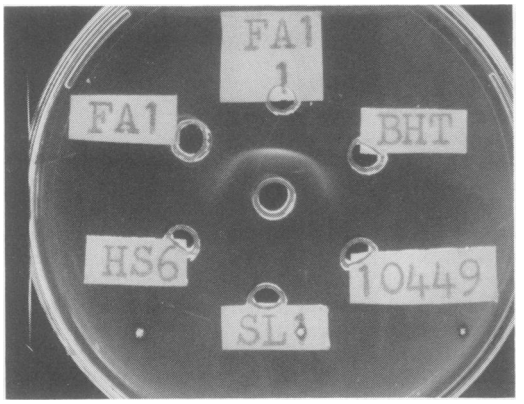

FIG. 1. *Reaction of HCl extracts with FA1 antiserum, FA1-1 contained 15 μg of purified antigen from a trichloroacetic acid extract of FA1 cells. Each outer well contained 0.02 N HCl extracts of whole cells from 1 ml of broth. Center well contained 10 μliters of anti-FA1 serum. The extracts of OMZ61, AHT, GS5, OMZ176, and B13 as well as HS6, SL1, and 10449 did not react with anti-FA1 serum.*

gen which reacted with anti-FA1 serum. Similar results have been reported (2, 3). Figure 1 also shows that the antigen in the HCl extract of FA1 and BHT possessed a reaction of identity with the antigen purified from a trichloroacetic acid extract of FA1.

Figure 2A shows the chromatographic purification of a 10% cold trichloroacetic acid extract of FA1 whole cells. The extract contained large amounts of proteins, sugars, and phosphorus; however, only a small quantity of these substances was present in the fractions which contained the antigens. The two fractions were eluted at 0.37 M and 0.67 M of $(NH_4)_2CO_3$, and designated as 1 and 2. Each fraction that gave a positive precipitin reaction was collected and further purified on Sephadex-G200 in carbonate buffer. The pattern for antigen 2 is shown in Fig. 2B. The sugar and protein remained essentially the same as in Fig. 2A; however, the phosphorus content was markedly reduced. The technique employed has been found (19) to remove protein not bound to polysaccharide. The high protein and sugar content of fraction 2, consequently, indicates the presence of a mucoprotein. The molecular weight of fraction 1 was 52,000 and fraction 2 was 77,000. A water extract of FA1 whole cells was purified by the same procedures as described above. Two fractions which correspond to 1 and 2 were obtained and designated as 3 and 4.

Figure 3 shows that the antigen extracted from whole cells by cold trichloroacetic acid (WC-TCA) and the fractions 1 and 2 purified from the trichloroacetic acid extract possess a reaction of identity with the antigen extracted from cell walls (CW-Lys) by lysozyme. Fractions 3 and 4 (from a water extract) also possess the same antigen. These results indicate, based on agar gel diffusion, that the fractions from the FA1 cells possessed a single immunological specificity and that both fractions were located in the cell wall. However, it was found by electrophoresis in gel (Fig. 4) that the antigen in fraction 2 possessed a faster mobility than that in fraction 1. The mobility of fraction 3 did not differ greatly from 1, and likewise 4 from 2.

Table 1 shows the chemical composition of fractions 1 and 2. Fraction 1 is principally composed of sugars (77%), whereas fraction 2 is composed of both sugars (38%) and amino acids (42%). The chemical compositions of fractions 3 and 4 are similar to those of fractions 1 and 2, respectively. It is evident that the electrophoretic mobility of fractions 2 and 4 (Fig. 4) is due in large measure to their amino acid content. It is of interest (Table 1) that the principal sugars of fraction 1 are rhamnose and galactose, whereas fraction 2 is mainly galactose.

Figure 5 shows the quantitative precipitin curve of fractions 1 and 2 from a cold trichloroacetic acid extract of FA1 whole cells. The equivalence point was reached with 20 to 30 μg of fraction 1 and with approximately 20 μg of fraction 2 per 25 μliters of antiserum. It is apparent that fraction 2 possessed more activity than 1 and was therefore used in subsequent studies. To obtain the same intensity of reaction in agar gel diffusion, only one-fourth as much fraction 2 as fraction 1 was required.

Each of the sugars present in the fractions, and α-glycerophosphate, was tested for its ability to inhibit the precipitin test. It can be seen (Fig. 6) that D-galactose (78%) and D-galactosamine (66%) were strong inhibitors, and that *N*-acetyl-D-galactosamine, D-glucose, and D-glucosamine inhibited between 25 and 35% at a concentration of 3 mg per 40 μliters. Rhamnose, *N*-acetyl-D-glucosamine α-glycerophosphate, D-alanine, and glycerol inhibited less than 10%. As derivatives of galactose, the inhibition abilities of lactose (*O*-β-D-galactopyranosyl-[1,4]-β-D-glucopyranoside), melibiose (*O*-α-D-galactopyranosyl-[1,6]-α-D-glucopyranoside), and stachyose {(*O*-α-D-galactopyranosyl-[1,6])$_2$,α-D-glucopyranoside} were tested. However, none of these derivatives inhibited more than 10%.

To distinguish which sugars (galactose or galactosamine) were responsible and to determine if there were one or two serologically specific sites for the precipitin reaction, both sugars were mixed at different ratios with a concentration of

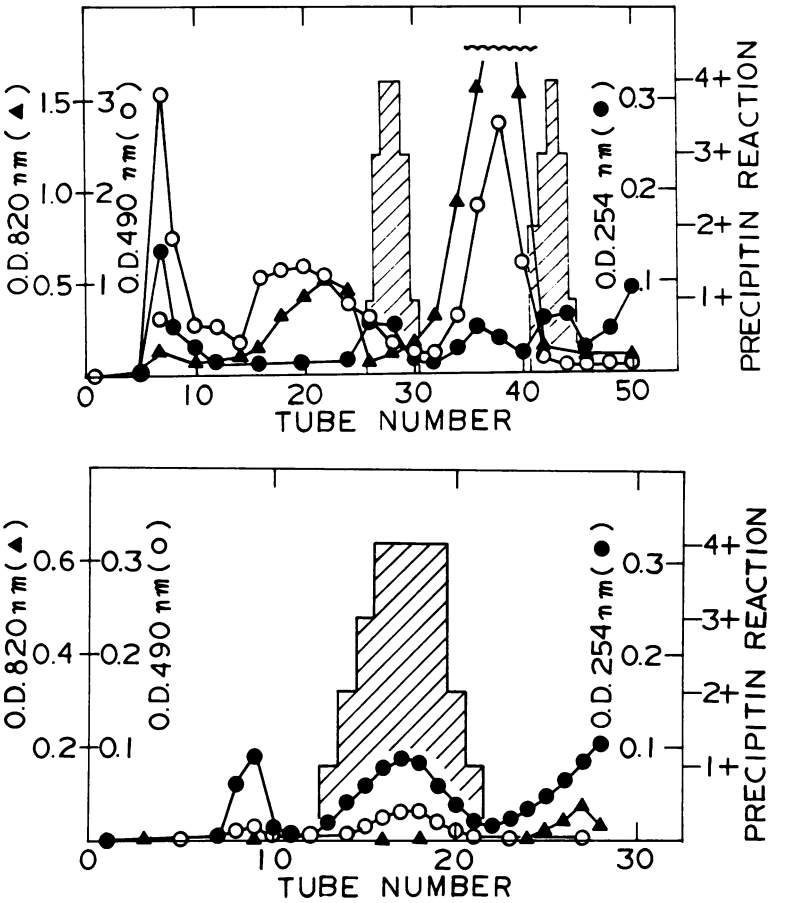

FIG. 2. *Top, Purification of S. mutans (Fa1) antigen on DEAE-Sephadex A-25 column. The 10% cold trichloroacetic acid extract of 10 g of dry cells was dialyzed against distilled water, lyophilized, and added to the column. Column length, 55 by 3 cm; starting buffer, 500 ml of 0.05 M $(NH_4)_2CO_3$; final buffer, 500 ml of 1.0 M $(NH_4)_2CO_3$; flow rate, 4.5 ml/min; each tube, 15 ml; Protein-nucleic acids (254 nm), ●; phosphorus (820 nm), ▲; sugars (490 nm), ○; shaded area, precipitin reaction against FA1 antiserum. The first fraction which showed positive precipitin reaction was designated fraction 1 and the last, fraction 2. Bottom, Elution of fraction 2 on Sephadex G-200 column. The fraction was collected, lyophilized, and added to the column. Buffer, 0.05 M $(NH_4)_2CO_3$, pH 8.6; column length, 3.0 by 55 cm; flow rate, 0.75 ml/min; each tube, 7.5 ml. Legend is the same as in A.*

total sugars at 1.6 mg per 40 μliters (Fig. 7). Glucose plus galactose exhibited the same effect as either one alone (Fig. 6). However, the inhibition obtained with galactose plus galactosamine was about one-half of the sum of each sugar alone. For example, galactose at 0.8 mg per 40 μliters showed about 25% and galactosamine 15% inhibition (Fig. 7), and, if both had inhibited the reaction independently of each other, the total inhibition of the mixture would have been 40%. Considering that either sugar alone could inhibit the reaction either 65 or 78% (Fig. 6), this result indicates that there is only one serologically specific site in the antigen which could be inhibited by either galactose or galactosamine and that such inhibitors were competitive with each other.

All enzymes tested did not destroy fractions 1, 2, 3, or 4. Heating of the fractions at 100 C for 30 min in distilled water or in 0.85% NaCl did not change their serological activity. However, heating of the fractions at 100 C in concentrations of HCl higher than 0.005 N destroyed most of the activity within 20 min. The serological activity of fraction 2 decreased to about 25% of the original after 40 min in 0.004 N HCl at 100 C (Fig. 8). Analyses by thin-layer chromatography and by liquid-gas chromatography showed that when a negative precipitin reaction was reached, one half of the galactose and galactosamine had been re-

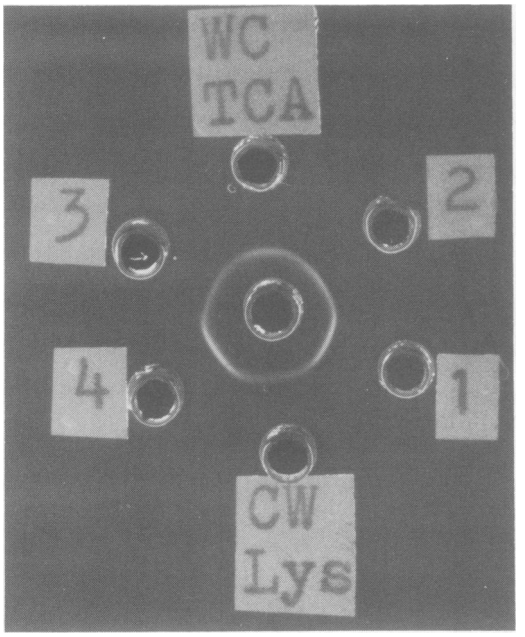

FIG. 3. *Reaction of whole cell extract, cell wall extract, and purified fractions with FA1 antiserum. WC-TCA: material from 0.38 mg of dry whole cells extracted by 10% trichloroacetic acid at 5 C for 16 h; CW-Lys: material released by lysozyme from cell walls (from 1 mg of dry whole cells); 1 and 2: 10 μg and 2.5 μg of purified fractions 1 and 2 from a trichloroacetic acid extract of whole cells (Fig. 2A); 3 and 4: 10 μg of each of the precipitin-positive fractions from the water extract of whole cells (Sephadex separation same as in Fig. 2). Inner well contained 10 μliters of FA1 antiserum.*

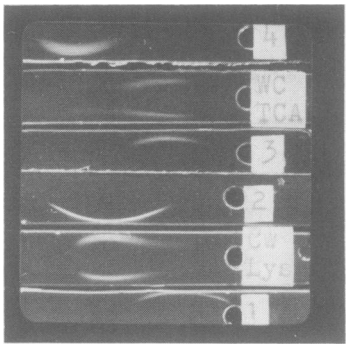

FIG. 4. *Immunoelectrophoresis of crude extracts and purified antigens from FA1. Symbols used are the same as in Fig. 3. A 5-μg amount of purified antigen or 5 μliters of extract was used in each well. Separation was carried out at 80 V for 1 h. In the four trays, 50 μliters of FA1 antiserum was used in each case. Positive electrode at left side.*

leased from the antigen. No significant quantity of the other sugars or amino acids were released at the same time.

DISCUSSION

The present results show that the serological specificity of *S. mutans* (group b) antigen is dependent on D-galactose and D-galactosamine. The optical form of the D-galactose is not known. α-D-Galactose was reported to be the terminal sugar of the streptococcal group R antigen (28), pneumococcal capsular polysaccharides (23), and *Enterobacteriaceae* somatic and capsular polysaccharides (17), whereas β-D-galactose is the terminal sugar of the streptococcal group F type 4 (29) and the group B type II antigens (13). However, the *S. mutans* group b antigen did not react with group R or group F type 4 antisera, and the precipitin reaction was not inhibited with α-D-galactose derivatives (melibiose and stachyose) or a β-D-galactose derivative (lactose). Therefore,

TABLE 1. *Chemical composition of FA1 antigens*[a]

Chemical	Percent composition	
	Antigen 1	Antigen 2
Sugar		
Rhamnose	47.4	0.3
Galactose	27.4	29.3
Galactosamine	1.9	3.0
Glucose	0.5	5.1
Total sugars	76.9	37.7
Glycerol	1.9	0.1
Total phosphorus	2.3	1.3
Amino acid		
Alanine		4.8
Valine		3.3
Isoleucine		0.6
Threonine	1.3	2.1
Glycine	0.4	2.3
Leucine		2.7
Serine		0.5
Proline	0.7	1.3
Aspartic acid	0.3	1.3
Methionine		0.3
Tyrosine	0.8	7.6
Phenylalanine	0.5	2.5
Glutamic acid	0.5	3.5
Lysine	0.4	1.6
Histidine	0.8	1.1
Cysteine		3.5
Total amino acids	5.4	38.8
Recovery	86.5	76.9

[a] Less than 1% muramic acid present in each; no ribitol present.

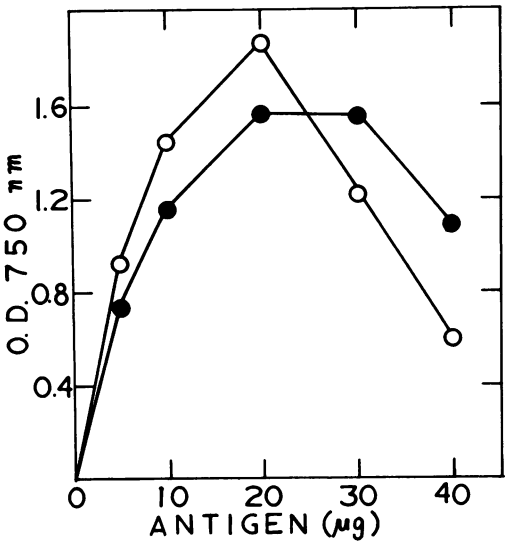

FIG. 5. *Quantitative precipitin curves of fractions 1 and 2 against FA1 whole cell antiserum. A 25-μliter antigen solution and 25 μliters of serum were used. Fraction 1, ●; fraction 2, ○.*

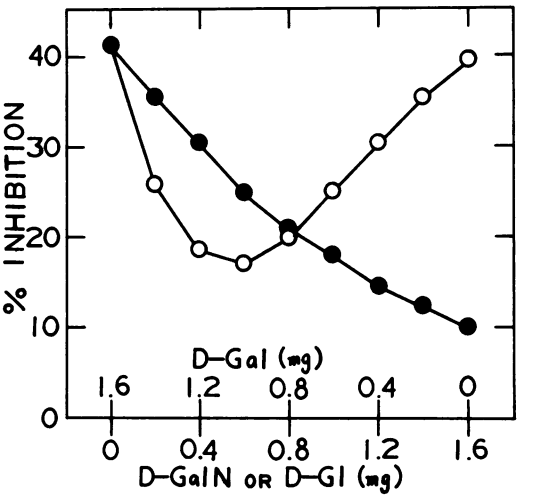

FIG. 7. *Inhibition of precipitin reaction between fraction 2 and whole cell serum by a mixture of sugars. Antiserum (20 μliters) and 8 μliters of sugar were incubated at 37 C for 30 min, and 5 μliters (10 μg) of fraction 2 was added to the solution and incubated at 37 C for 40 min.* D-*Gal* + D-*GalN*, ○; D-*Gal* + D-*Glu*, ●

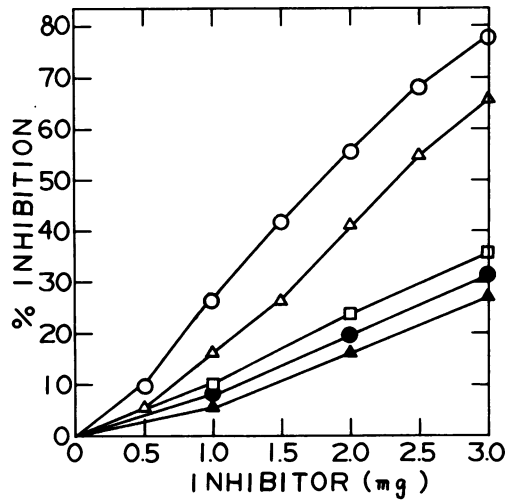

FIG. 6. *Inhibition of precipitin reaction between antigen 2 and FA1 whole cell antiserum. To 20 μliters of antiserum, 15 μliters of inhibitor in saline was added and preincubated for 30 min at 37 C, and then 5 μliters (10 μg) of fraction 2 was added and incubated. Symbols:* D-*galactose*, ○; D-*galactosamine*, △; *N-acetyl-*D-*galactosamine*, □; D-*glucose*, ●; D-*glucosamine*, ▲.

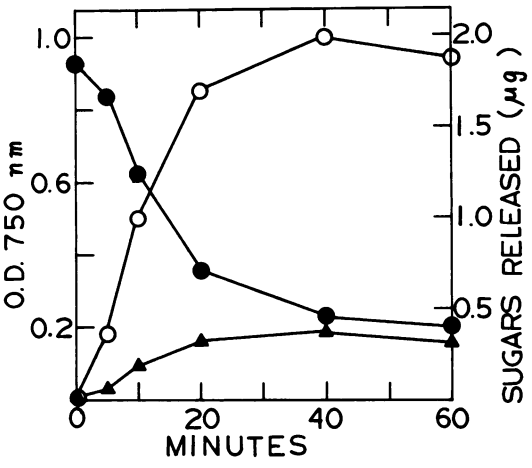

FIG. 8. *Release of sugars from 3 μg of fraction 2 by hydrolysis in 0.004 N HCl at 100 C. Sugars were separated on a Sephadex G-15 column (1 by 30 cm). Methylation and silation procedures were applied to the samples, and the derivatives were analyzed by gas chromatography. For each experiment 20 μliters of reaction mixture (10 μg) and 20 μliters of serum were used. Quantitative precipitin reaction against FA1 antiserum, ●; galactose released, ○; galactosamine released, ▲.*

a galactosamine-galactose terminal location would be more likely according to the results shown in Fig. 6 to 8.

It was shown by gel electrophoresis that hot acid extracts of FA1 cells contained two antigens when tested against unadsorbed anti-FA1 serum (2, 30). Trichloroacetic acid extracts of FA1 contain two fractions (Fig. 2, 4), which possess differ-

ent chemical compositions (Table 1), although gel diffusion shows a reaction of identity between them (Fig. 3). These two antigens are very likely the same as those considered characteristic of group b (2).

Group b (strain BHT) cell walls were reported to contain material which was composed of glycerol, phosphate, alanine, glucose, and galactose (1). Incomplete data on the composition of the material did not provide a complete description; however, the presence of glycerol phosphate indicated a teichoic acid (1). It is clear, however, that it is a different type of antigen than that reported in the present study. It is most probable that the immunological specificity of group b depends on a polysaccharide structure rather than a teichoic acid. Fraction 2 did not react with staphylococcal or group A streptococcal teichoic acid antisera. Fraction 2 is the first example of a cell wall mucoprotein as a major antigen of the streptococci. The presence of hexosamine satisfies the definition of mucoproteins, although the total sugar content is considerably higher than that commonly found in mammalian cells.

FA1 antiserum was found to produce a rapid agglutination of whole cells, and at the same time the anti-group b antibodies were removed (results not shown). A rapid reaction of this type indicates a surface location for the polysaccharide portion of the antigen. The absence of any immunological specificity for the protein portion of the mucoprotein and the strong possibility that it represents a part of the cell wall protein also support a surface location for the polysaccharide portion of the mucoprotein.

The results indicate that the group b antigen is the only major antigen in the FA1 cell wall. The quantity of galactose in the cell wall antigen was 5.7% of the total galactose of the cell wall. Figure 2A shows that three polysaccharide-containing materials, in addition to the group b antigen, were present in the trichloroacetic acid extract. The absence of antibodies to these polysaccharides in FA1 whole cell antiserum (Fig. 3) shows that they possess no significant antigenicity. In addition, no other antigens were present in Pronase, lysozyme, ribonuclease, trypsin, water, or 0.01 to 0.5 N HCl extracts at 5 or 100 C. The surface location and the antigenicity of this antigen indicate an important role for the polymer in the surface activities of the streptococcus.

The streptococcal cell wall has long been known to contain protein as well as polysaccharide polymers. The FA1 mucoprotein may represent a general type of complex present in the wall of *S. mutans* and possibly other streptococci. Lysozyme was highly active in releasing this complex but the cell walls were not completely solubilized. Lysozyme did not produce lysis. The complex possessed a reaction of identity (Fig. 1) and the same mobility as the mucoprotein fraction 2 (Fig. 4). Electron micrographs have illustrated a destructive effect of lysozyme on group b (strain BHT) cells (1) and the release of 20% of the total hexosamine from *S. lactis* (16). Incubation of lysozyme-digested cells with 0.2% sodium lauryl sulfate (6, 16) or 0.5 M NaCl (16) resulted in lysis. It appears that the removal of the surface polysaccharide and the underlying protein (or a portion thereof) by lysozyme, facilitated the rupture of ionic bonds by both sodium lauryl sulfate and NaCl so that additional protein was solubilized. This action would facilitate the penetration of lysozyme to the peptidoglycan and lysis of the cell. The type of bond attacked by lysozyme and which resulted in the release of mucoprotein is unknown and appears to differ from the action of this enzyme on cell wall peptidoglycan. Trypsin and pepsin did not release the mucoprotein.

Antigens 1 and 2 of FA1 are specific for *S. mutans* group b. They did not react with groups a, c, and d *S. mutans* antisera, and antisera to streptococcal groups A to H and K to T. Adsorption of FA1 whole cell antiserum by either antigen 1 or 2 removed all activity against either one. No additional precipitating activity remained in the serum.

These results demonstrate the immunological specificity of group b. Their cariogenic ability (11), genetic constitution (7), and formation of ammonia from arginine (5) should contribute to our recognition of this group as a distinct group of *S. mutans*.

ACKNOWLEDGMENTS

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