Characterization of a Cell-surface Protein Antigen of Hydrophilic *Streptococcus mutans* Strain GS-5

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Fourteen strains of *Streptococcus mutans* serotype *c* were examined for their cell-surface protein antigens in terms of hydrophobicity, M_r and immunochemical specificities. Thirteen strains were hydrophobic, while strain GS-5 was markedly hydrophilic as compared to the other strains tested. Cell-surface protein antigens were then analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. A protein antigen of M_r 190000 (PAC) was found in cell extracts and culture supernatants of all the hydrophobic strains. Neither culture supernatant nor cell extract of strain GS-5 contained PAC. Strain GS-5, however, produced extracellularly a large amount of a protein of M_r 155000 (PAGS-5) which reacted with rabbit anti-PAC serum. Immunodiffusion analysis showed that PAGS-5 lacked a part of the antigenic moieties in the PAC molecule. SDS-PAGE and radioimmunoassay showed a small amount of PAGS-5 on the cell surface of strain GS-5. These findings suggest that PAGS-5 may correspond to PAC which lacks a region participating in binding of PAC to the streptococcal cell.

INTRODUCTION

Streptococcus mutans has been strongly implicated as a causative organism of dental caries (Hamada & Slade, 1980; Loesche, 1986). The organism possesses various kinds of cell-surface polymers such as proteinaceous antigens, serotype-specific polysaccharide antigens, lipoteichoic acid and peptidoglycan (Hamada & Slade, 1980). Among these cell-surface polymers, much attention has been focused on a high- M_r protein antigen that has been variously designated as antigen B (Russell, 1979), I/II (Russell *et al.*, 1980), IF (Hughes *et al.*, 1980) and P1 (Forester *et al.*, 1983). *Streptococcus sobrinus* also possesses a cell-surface protein antigen that is immunologically and biochemically similar to that of *S. mutans* (Moro & Russell, 1983; Holt *et al.*, 1982; Okahashi *et al.*, 1986).

The high- M_r protein antigen of *S. mutans* serotype *c* (PAC) is an effective vaccine antigen for prevention of dental caries in monkeys (Lehner *et al.*, 1981; Russell *et al.*, 1982). Local passive immunization with monoclonal antibodies raised against PAC prevents the colonization by *S. mutans* of animal and human tooth surfaces (Lehner *et al.*, 1985; Ma *et al.*, 1987). Polyclonal antibodies to PAC are strongly opsonic for *S. mutans* (Scully *et al.*, 1980). McBride *et al.* (1984) suggested that location of PAC on the streptococcal surface is important in forming hydrophobic bonds with hydrophobic regions of salivary pellicle. The organization of PAC on the cell surface, however, is poorly understood.

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Abbreviations: PAC, an M_r 190000 protein antigen of *S. mutans* serotype *c*; PAGS-5, an M_r 155000 protein antigen of *S. mutans* strain GS-5.

In the present study we examined the location of PAc on cell surfaces of various strains of *S. mutans* serotype *c* and their cell hydrophobicity.

METHODS

Bacterial strains. Serotype *c* *S. mutans* strains GS-5, Ingbritt, MT6R, MT12, MT118R, MT8148, MT6801, NG7183, OMZ70, P2, C67-1, C67-25, NCTC 10449R and NCTC 10449S were used in this study. These strains were selected from the stock culture collection in the Department of Dental Research, National Institute of Health, Tokyo, Japan.

SDS-PAGE. *S. mutans* strains were grown at 37 °C for 18 h in diffusate medium of Brain Heart Infusion (BHI; Difco) broth. The cell-free culture supernatant was collected by centrifugation at 10000 *g* for 20 min at 4 °C. Extracellular proteins were precipitated from the culture supernatant by adding solid ammonium sulphate to 60% saturation. The precipitate was collected by centrifugation, dissolved in distilled water, dialysed against distilled water, and lyophilized. The dried culture supernatant, and whole cells (50 mg dry weight ml⁻¹), were suspended separately in 10 mM-Tris/HCl buffer, pH 6.8, containing 8 M-urea, 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, and heated at 100 °C for 3 min. The cell extract was then clarified by centrifugation. SDS-polyacrylamide slab gels were prepared as described by Laemmli (1970). The culture supernatant (0.5 mg protein) and the cell extract (60 µl) were electrophoresed at 7.5 mA per gel at room temperature for 12 h using a 7.5% (w/v) resolving and a 3% (w/v) stacking gel (14 × 11 × 0.2 cm) containing 0.1% (w/v) SDS. Catalase (*M*_r 60000), bovine serum albumin (67000), phosphorylase *b* (94000) and ferritin (220000) were used as *M*_r standards. Proteins were stained with Coomassie brilliant blue R-250.

Antisera. Anti-PAc serum and anti-PAGS-5 serum were prepared as follows. The concentrated culture supernatants of strains MT8148 and GS-5 were electrophoresed on SDS-polyacrylamide slab gels as described above, and bands corresponding to proteins of *M*_r 190000 (PAc) and *M*_r 155000 (PAGS-5), respectively, were carefully cut out to avoid any contamination. The gel pieces containing each protein were suspended in phosphate-buffered saline (PBS; pH 7.5), and crushed in a porcelain mortar. The crushed gel suspensions (about 0.2 mg protein ml⁻¹) were emulsified with an equal volume of Freund's complete adjuvant (Difco). New Zealand White rabbits were immunized intramuscularly with the emulsions (1 ml), followed 2 weeks later by intramuscular injection of 0.5 ml of the crushed gel suspensions (about 0.2 mg protein ml⁻¹). Blood was taken 3 weeks after the last immunization.

Western blotting. The culture supernatant (0.1 mg protein) and the cell extract (12 µl) were electrophoresed in SDS-polyacrylamide slab gels as described above, and transferred to a nitrocellulose sheet by the Western blotting technique (Burnette, 1981). The sheet was treated with anti-PAc serum. The antibody bound to the immobilized replica proteins on the sheet was detected by solid-phase immunoassay with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Koga *et al.*, 1986).

Radioimmunoassay. *S. mutans* strains were grown at 37 °C for 18 h in BHI broth. The cells were harvested by centrifugation, and washed twice in PBS. The washed cells (5 × 10⁷) were allowed to react with anti-PAc serum (1 µl) or anti-PAGS-5 serum (1 µl) in 0.15 ml PBS containing 1% (w/v) bovine serum albumin for 1 h at room temperature (Kato *et al.*, 1986). After being washed three times in PBS by centrifugation, the cells were allowed to react with 1 µl ¹²⁵I-labelled goat anti-rabbit immunoglobulin G (ICN Radiochemicals) in 50 µl PBS containing 1% (w/v) bovine serum albumin. After incubation for 1 h at room temperature, the cells were washed three times in PBS. The radioactivity associated with the cells was then determined.

Immunodiffusion. Agar gel diffusion was performed in 1% (w/v) agarose gel in 15 mM-barbital hydrochloride buffer, pH 8.6, containing 1% (v/v) Triton X-100. The agar plates were washed with PBS, dried and stained with Coomassie brilliant blue R-250.

Hydrophobicity. *S. mutans* strains were grown at 37 °C for 18 h in BHI broth. The relative surface hydrophobicity of the cells was determined by their adsorption to hexadecane and their aggregation in salt solution as described by Koga *et al.* (1986).

Preparation of PAc. PAc was prepared from the culture supernatant of *S. mutans* MT8148 grown in diffusate medium of BHI broth by ammonium sulphate precipitation, chromatography on diethylaminoethyl-Sephacel (Pharmacia), and subsequent gel filtration on Sepharose CL-6B (Pharmacia) (Russell *et al.*, 1980).

RESULTS

Surface hydrophobicity. Cell-surface hydrophobicity of the 14 serotype *c* *S. mutans* strains was determined by their adsorption to hexadecane and their aggregation in ammonium sulphate. The surface hydrophobicity of strain GS-5 was markedly lower than that of other strains (Table 1).

Table 1. *Hydrophobicity of strains of S. mutans serotype c*

The relative surface hydrophobicity of *S. mutans* cells was determined by their adsorption to hexadecane and their aggregation in salt. The bacterial suspension (3 ml) was mixed with 300 µl hexadecane. Adsorption was calculated as the percentage loss in optical density relative to that of the initial cell suspension; values are the mean $\pm$ SD of triplicate assays. Bacterial aggregation in salt is expressed as the lowest ammonium sulphate concentration (M) causing bacterial aggregation.

Strain	Hydrophobicity		Strain	Hydrophobicity	
	Bacterial adsorption to hexadecane	Bacterial aggregation (M)		Bacterial adsorption to hexadecane	Bacterial aggregation (M)
GS-5	4.9 $\pm$ 0.3	> 2.0	P2	25.0 $\pm$ 6.3	1.1
Ingbritt	29.0 $\pm$ 2.5	1.1	C67-1	42.5 $\pm$ 3.3	1.0
MT6R	25.9 $\pm$ 1.7	1.2	C67-25	25.7 $\pm$ 2.6	1.2
MT12	27.9 $\pm$ 5.6	1.2	MT6801	23.1 $\pm$ 1.8	1.2
MT118R	27.6 $\pm$ 1.9	1.2	NG7183	32.2 $\pm$ 2.4	1.1
MT8148	24.9 $\pm$ 2.6	1.2	NCTC 10449R	24.2 $\pm$ 1.5	1.2
OMZ70	42.5 $\pm$ 1.7	1.0	NCTC 10449S	43.8 $\pm$ 4.8	1.0

SDS-PAGE analysis of culture supernatants and whole cells. Culture supernatants of the 14 strains of *S. mutans* serotype *c* were analysed by SDS-PAGE and Western blotting. Gels stained with Coomassie brilliant blue showed that culture supernatants of all the strains, except strain GS-5, possessed a protein of M_r 190000 (PAC) (Fig. 1*a*). PAC was not found in the culture supernatant of strain GS-5, but this strain produced extracellularly a large amount of a protein of M_r 155000 (PAGS-5). The band of PAGS-5, as well as that of PAC, reacted with rabbit anti-PAC serum (Fig. 1*b*). SDS-PAGE analysis of urea/SDS extracts from whole cells of *S. mutans* serotype *c* is shown in Fig. 2. Cell extract of strain GS-5 did not contain PAC, which was present in all the other strains, but contained a small amount of the M_r 155000 protein (PAGS-5) that reacted with anti-PAC serum. When culture supernatants and cell extracts of *S. mutans* serotype *c* were analysed by Western blotting, a number of other protein bands with lower M_r , as well as PAC and PAGS-5, reacted with anti-PAC serum. The appearance of these lower- M_r proteins may have resulted from different degrees of proteolysis of PAC and PAGS-5 by endogenous proteases of *S. mutans*.

Immunological comparison of PAC and PAGS-5. Immunological specificity of PAC and PAGS-5 was investigated by immunodiffusion. Immunodiffusion tests revealed that a single precipitin band formed between PAC and anti-PAC serum (Fig. 3*a*). This band was fused with that produced between anti-PAC serum and the culture supernatants of strains MT8148 and Ingbritt, but gave a spur with that produced between anti-PAC serum and the culture supernatant of strain GS-5. On the other hand, the culture supernatants of strains MT8148 and GS-5 formed a precipitin line of identity with anti-PAGS-5 serum (Fig. 3*b*). These results indicated that PAGS-5 lacked a part of the antigenic moieties in the PAC molecule.

Reactivity of *S. mutans* cells with anti-PAC serum and anti-PAGS-5 serum. The amount of cell-surface antigens with which anti-PAC serum or anti-PAGS-5 serum reacted was determined by radioimmunoassay. Compared with other strains, the amount of cell-surface antigens of strain GS-5 with which anti-PAC serum and anti-PAGS-5 serum reacted was very small (Table 2). Reactivity of whole cells of *S. mutans* strains used in this study with anti-PAC and anti-PAGS-5 sera and their surface hydrophobicity were not apparently correlated (Tables 1 and 2).

Fig. 1. SDS-PAGE and Western blotting of culture supernatants of serotype *c* *S. mutans* strains. Culture supernatants were concentrated by ammonium sulphate precipitation, and analysed by SDS-PAGE. (a) Gel stained with Coomassie brilliant blue. (b) Anti-PAC serum bound to immobilized antigens on a nitrocellulose sheet transferred by an electrophoretic blotting procedure. The antibody was detected by solid-phase immunoassay with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. The M_r markers used were catalase (M_r 60000), bovine serum albumin (67000), phosphorylase *b* (94000) and ferritin (220000). Lane 1, strain GS-5; lane 2, strain Ingbritt; lane 3, strain MT6R; lane 4, strain MT12; lane 5, strain MT118R; lane 6, strain MT8148; lane 7, strain OMZ70; lane 8, strain P2; lane 9, strain C67-1; lane 10, strain C67-25; lane 11, strain MT6801; lane 12, strain NG7183; lane 13, strain NCTC 10449R; lane 14, strain NCTC 10449S.

DISCUSSION

In this study, we showed the presence of PAC of M_r 190000 in culture supernatants and cell extracts of all the strains, except strain GS-5, of *S. mutans* serotype *c* tested. No PAC was detected in either the culture supernatant or the cell extract of hydrophilic strain GS-5, but this strain produced in the culture supernatant a large amount of PAGES-5 of M_r 155000 reactive

Fig. 2. SDS-PAGE and Western blotting of cell extracts of serotype *c* *S. mutans* strains. Whole cells were suspended in 8 M-urea, 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, and heated at 100 °C for 3 min. The cell extracts were clarified by centrifugation, and analysed by SDS-PAGE. (a) Gel stained with Coomassie brilliant blue. (b) The antigens transferred on a nitrocellulose sheet were allowed to react with anti-PAc serum. For the lanes, see the legend for Fig. 1.

with rabbit anti-PAc serum. A small amount of PAGS-5 was present on the cell surface. Immunodiffusion analysis showed that PAGS-5 lacked a part of the antigenic moieties in the PAc molecule. Ayakawa *et al.* (1987) have recently reported that none of 15 murine monoclonal antibodies directed against antigen P1 (identical with PAc) reacted with whole cells of strain GS-5. The monoclonal antibodies isolated by Ayakawa *et al.* (1987) may be specific for a region of the PAc molecule that PAGS-5 lacks.

PAc is known to exist both in a form apparently closely associated with peptidoglycan of serotype *c* *S. mutans* strains and in their culture supernatants (Russell, 1979; Zanders & Lehner,

Fig. 3. Immunodiffusion of culture supernatants of serotype *c* *S. mutans* strains against anti-PAc serum (a) and anti-PAGS-5 serum (b). Well 1, concentrated culture supernatant of strain MT8148; well 2, concentrated culture supernatant of strain GS-5; well 3, concentrated culture supernatant of strain Ingbritt; well 4, PAc purified from the culture supernatant of strain MT8148; well A, anti-PAc serum; well B, anti-PAGS-5 serum.

Table 2. Reactivity of anti-PAc serum and anti-PAGS-5 serum with whole cells of strains of *S. mutans* serotype *c*

S. mutans cells (5×10^7) were allowed to react with anti-PAc serum (1 μ l) or anti-PAGS-5 serum (1 μ l) for 1 h at room temperature. After being washed three times in PBS by centrifugation, the cells were allowed to react with 125 I-labelled goat-anti-rabbit immunoglobulin G for 1 h at room temperature. The radioactivity associated with the cells was then determined. Values are the mean $\pm$ SD of triplicate assays.

Strain	125 I associated with cells (c.p.m.)		Strain	125 I associated with cells (c.p.m.)	
	Anti-PAc serum	Anti-PAGS-5 serum		Anti-PAc serum	Anti-PAGS-5 serum
GS-5	1092 $\pm$ 177	673 $\pm$ 126	P2	7475 $\pm$ 535	5809 $\pm$ 356
Ingbritt	6035 $\pm$ 718	6181 $\pm$ 731	C67-1	7262 $\pm$ 394	6782 $\pm$ 1098
MT6R	6096 $\pm$ 130	4061 $\pm$ 183	C67-25	9295 $\pm$ 369	6371 $\pm$ 698
MT12	4090 $\pm$ 221	3529 $\pm$ 520	MT6801	6214 $\pm$ 752	4712 $\pm$ 506
MT118R	7234 $\pm$ 331	6181 $\pm$ 626	NG7183	6602 $\pm$ 935	5860 $\pm$ 665
MT8148	6845 $\pm$ 872	5360 $\pm$ 511	NCTC 10449R	6524 $\pm$ 1325	6438 $\pm$ 421
OMZ70	6444 $\pm$ 739	5876 $\pm$ 457	NCTC 10449S	6767 $\pm$ 721	6557 $\pm$ 355

1981; McBride *et al.*, 1984). In strain GS-5, PAGS-5 was predominantly found free in the culture supernatant. Hollingshead *et al.* (1986) have shown that type 6 M protein of *Streptococcus pyogenes* is attached to the streptococcal cell through a proline-rich wall stabilization region and a membrane anchor region at the carboxyl-terminal end of the protein. It is possible that PAGS-5 lacks a region participating in linking to the cell wall of *S. mutans*. For verification of this hypothesis, we are presently attempting to clone the genes coding for PAc and PAGS-5 into *Escherichia coli* and to determine the nucleotide sequences of the genes.

There is currently great interest in the role of cell-surface hydrophobicity in mediating bacterial adherence to teeth and oral mucosal surfaces (Gibbons, 1984). Westergren & Olsson (1983) isolated variant strains LK36 and GW36, with decreased hydrophobicity, by repeated subculture of freshly isolated strains LK and GW, respectively, of *S. mutans* serotype *c*, and

showed that the variant cells adhered less well to saliva-coated hydroxyapatite than did the parent cells. The variant strains were agglutinated to a lower extent by parotid saliva or salivary agglutinin than the parent cells (Rundegren & Olsson, 1987). Moreover, McBride *et al.* (1984) have shown that cell extracts of hydrophobic strains LK and GW contain a number of high- M_r (mainly M_r 190 000) proteins which are not present on cells of strains LK36 and GW36 with decreased hydrophobicity. Russell & Smith (1986) have reported that the protein of M_r 190 000 altered in the LK strains is indeed antigen B. We confirmed that PAc also was immunologically identical to antigen B kindly supplied by Dr R. R. B. Russell (unpublished data). We have shown in this study that hydrophilic strain GS-5 did not produce PAc of M_r 190 000. These results support the suggestion of McBride *et al.* (1984) that the M_r 190 000 protein is important in surface hydrophobicity of *S. mutans*. In this connection, we should note that the amount of cell-associated PAc and surface hydrophobicity of serotype *c* *S. mutans* strains used in this study were not apparently correlated. Russell & Smith (1986) similarly found that the hydrophobicity of *S. mutans* could not be correlated solely with PAc, but involved various other surface molecules such as other surface proteins and lipoteichoic acid. Therefore, it is difficult to rule out the possibility that other antigens not detected in this study may be altered in strain GS-5.

In conclusion, an M_r 190 000 protein antigen was present on cell surfaces of hydrophobic strains of *S. mutans* serotype *c*. Hydrophilic *S. mutans* strain GS-5 did not produce the high- M_r protein antigen, but produced extracellularly an M_r 155 000 protein antigen that lacked a region participating in binding to the streptococcal cell.

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