A Possible Mechanism for the Cellular Coaggregation between *Actinomyces viscosus* ATCC 19246 and *Streptococcus sanguis* ATCC 10557

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The cells of *Actinomyces viscosus* ATCC 19246 (Av19246) and *Streptococcus sanguis* ATCC 10557 (Ss10557) coaggregated immediately after mixing in 40 mM-Tris/HCl buffer. Optimal conditions were pH 7.5 in the presence of Ca^{2+} at 0.1 mM or higher. Na_2EDTA and its analogues, Na_2MgEDTA and Na_2MnEDTA at 7.5 mM inhibited the coaggregation. Trypsin and heat treatment impaired the reactive site on Av19246 cells, but not on Ss10557 cells. The coaggregates, once formed, dissociated gradually during extended incubation at 37 °C; this was prevented by addition of sufficient Ca^{2+} . The disaggregation appears to be a spontaneous denaturation of the proteinaceous reactive site on Av19246 cell surface. Thus, the coaggregation involves the interaction of a lectin-like substance on the surface of Av19246 with a carbohydrate site on Ss10557. Native Ss10557 cell walls possessed reactivity with Av19246 cells but 5% (w/v) TCA-extracted cell wall residues did not. A carbohydrate moiety extracted from Ss10557 exhibited a high potency in blocking coaggregation, and coaggregates were dissociated upon addition of the carbohydrate. Lactose, galactose and *N*-acetyl-D-galactosamine (the latter two are major constituents of the antigen extract) also significantly inhibited the coaggregation, but the other antigen components, glucose and rhamnose, did not. Relative inhibitory activity, expressed as molar potency, of carbohydrate antigen, lactose, galactose and *N*-acetyl-D-galactosamine respectively, was approximately $26 \times 10^3:16:4:1$. Ss10557 cells and cell walls reacted only with a *Ricinus communis* (castor bean) agglutinin-120 but not with *Glycine max* (soybean) agglutinin, *Arachis hypogaea* (peanut) agglutinin or *Phaseolus vulgaris* agglutinin (phytohaemagglutinin). These results suggest that the reaction site on Ss10557 cells comprises a D-galactose-(1→4)-β-D-glucose- sequence and that *N*-acetyl-D-galactosamine (an immunodeterminant of the streptococcus strain) appears not to be involved in the coaggregation reaction.

INTRODUCTION

Actinomyces viscosus has been implicated in the aetiology of root surface caries and periodontal disease (Jordan & Keyes, 1964; Jordan & Hammond, 1972). *Streptococcus sanguis* is reported to be the most predominant organism in human dental plaque, particularly in the earliest stages of plaque formation (Socransky *et al.*, 1977).

Gibbons & Nygaard (1970) observed specific coaggregations of paired oral bacteria of different species, including a pair of *A. viscosus* and *S. sanguis*, and suggested that the coaggregation plays an important role in the accumulation of oral bacteria on teeth. It has been reported that coaggregations between certain strains of *S. sanguis* and specific strains of *A. viscosus* occur by lectin-carbohydrate interactions of cell surface components (Ellen & Balcerzak-Raczkowski, 1977; McIntire *et al.*, 1978; Cisar *et al.*, 1979; Kolenbrander & Williams, 1981; McIntire *et al.*, 1982). The presence of five complementary pairs of cell surface

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components has been proposed (Kolenbrander & Williams, 1981). Three of these are lactose-non-reversible and two are lactose-reversible. In lactose-reversible cases, the reactive sites of a protein or glycoprotein nature reside on *A. viscosus* and the sites of a carbohydrate nature are on *S. sanguis*.

In the course of studies on the cell surface components of oral streptococci, we found that glucose-grown cells of *S. sanguis* ATCC 10557 and cells of *A. viscosus* ATCC 19246 immediately formed large aggregates under certain conditions but gradually disaggregated during incubation. In this paper we describe the general nature of the interaction and characterization of the surface molecules involved, giving special emphasis to the *S. sanguis* components, and we discuss a possible mechanism for the coaggregation.

METHODS

Preparation of A. viscosus and S. sanguis cells. *Streptococcus sanguis* ATCC 10557 (Ss10557, serotype II; Hamada *et al.*, 1980) and *A. viscosus* ATCC 19246 (Av19246) were kindly provided by Dr S. Hamada (Department of Dental Research, National Institute of Health, Tokyo, Japan). These strains were grown aerobically in Brain Heart Infusion broth (BHI; BBL Microbiology Systems, Cockeysville, Md., USA) at 37 °C for 18 h. Cells were harvested by centrifugation and were washed three times with 40 mM-Tris/HCl buffer, pH 7.5. The cells were suspended in distilled water to give an OD₅₅₀ of 0.75. Since the Av19246 cells aggregated spontaneously in distilled water, their suspensions were homogenized ultrasonically prior to the measurement of turbidity.

A portion of either Av19246 or Ss10557 cells suspended in 40 mM-Tris/HCl buffer, pH 7.5, was heated at 100 °C for 1 min, at 80 °C for 5 min, or incubated with trypsin at 100 µg ml⁻¹ (1:250; Difco), at 37 °C for 3 h. Ss10557 cells were also extracted in 1% (v/v) formalin or 70% (v/v) ethanol solution at 4 °C overnight. The cell residues were collected by centrifugation, washed three times in buffer and uniformly resuspended in the original volume of distilled water for coaggregation testing.

Preparation of S. sanguis cell walls. Cells of Ss10557 were disrupted with the aid of glass beads (0.17–0.18 mm diam.) in a Braun cell homogenizer (Model MSK, B. Braun Apparatebau, Melsungen, FRG). Cell walls were isolated by differential centrifugation at 13000 g for 60 min and then treated with trypsin as previously described (Bleiweis *et al.*, 1964; Okahashi *et al.*, 1983). A portion of the purified cell wall preparation was subjected to treatment with 5% (w/v) TCA at 4 °C for 24 h (Okahashi *et al.*, 1983). After extensive washings with distilled water, these native and extracted cell wall preparations were suspended in distilled water to give an OD₅₅₀ of 0.75.

Assay for coaggregation. The standard mixture consisted of 0.2 ml each of the whole cell or cell wall suspensions of Av19246 and Ss10557 in a total volume of 0.6 ml 40 mM-Tris/HCl buffer, pH 7.5. In some experiments, 4–80 mM-Tris/HCl (pH 7–9) and citrate phosphate buffers (pH 5–7) were used. After the mixture was incubated at 37 °C for 5 min, unless otherwise specified, cellular aggregation was examined macroscopically and scored as – (negative), ± (questionable), 1+ (weak) and 2+ (marked). A control suspension containing either Av19246 cells or Ss10557 cells (or cell walls) was always included.

Effects of various substances on coaggregation. Effects of the following substances on the bacterial coaggregation were examined: calcium chloride, magnesium chloride and potassium chloride at 0.1 to 10.0 mM; Na₂EDTA and its metal-containing derivatives such as Na₂CaEDTA, Na₂MgEDTA and Na₂MnEDTA at 7.5 mM; glucose, galactose, fructose, rhamnose, mannose, lactose, fucose, melibiose, sucrose, maltose, arabinose, xylose, sorbitol, xylitol, mannitol, inositol, glucosamine, galactosamine, mannosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine (all reagent grade), and dextrans T10 and T250 (Pharmacia) at 3 to 600 mM. The serotype II-specific antigen of Ss10557 was also used at final concentrations of from 0.01 to 0.5 mg ml⁻¹. The antigen was extracted from the purified cell walls by cold 5% TCA treatment and purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column followed by gel filtration through a Sephadex G-100 column (Koga *et al.*, 1983). The polysaccharide antigen has been found to be composed of glucose, galactose, rhamnose and *N*-acetyl-galactosamine at a molar ratio of 3:6:3:2, with a molecular weight of 4170 (calculated on the basis of the results obtained by chemical analyses).

Reversal of coaggregation was examined macroscopically after addition of carbohydrates to previously coaggregated bacteria.

Lectin-induced agglutination of S. sanguis whole cells and cell walls. Reaction mixtures contained 40 µg dry wt of either cells or cell walls of *S. sanguis* and 20 or 200 µg lectin in a total volume of 0.2 ml 0.05 M-potassium phosphate buffer, pH 7.0. After incubation at 37 °C for 2 h, the mixtures were examined macroscopically for agglutination and scored as described above. Sources of the lectins used were as follows: *Triticum vulgaris* (wheat germ) agglutinin (WGA) and *Ricinus communis* (castor bean) agglutinin-120, (RCA-120, one of the ricins) from Vector Laboratories, Burlingame, Cal., USA; *Glycine max* (soybean) agglutinin (SBA) and *Arachis hypogaea* (peanut) agglutinin (PNA) from E.Y. Laboratories, San Mateo, Cal., USA; *Phaseolus vulgaris* agglutinin (phytohaemagglutinin, PHA) from Miles-Yeda, Kiryat Weizmann, Rehovot, Israel; and *Canavalia ensiformis* (jack bean) agglutinin (ConA) from Sigma.

Assay for the degradation of proteinaceous receptor on A. viscosus by cellular protease(s). A portion of Av19246 cell suspension in 40 mM-Tris/HCl buffer, pH 7.5 was incubated at 37 °C for 17 h in the absence or presence of 2 mM-Na₂EDTA or CaCl₂, Na₂EDTA being added to eradicate cell surface-bound Ca²⁺. Cells were removed by centrifugation, then supernatant was assayed for the amounts of proteinaceous cellular components released. Sedimented cells were tested for their ability to coaggregate with Ss10557 as described above.

In order to test for the presence of proteolytic activity in Av19246, cells were incubated with 0.5% casein (Nakarai Chemicals, Kyoto, Japan) under the conditions described above. Unreacted casein was precipitated by the addition of 2.5% TCA and removed, together with cells, by centrifugation. The cell-free liquor obtained was assayed for the presence of digested casein measured as protein by the Lowry method with BSA as standard.

RESULTS

General nature of the coaggregation between the cells of A. viscosus and S. sanguis

Av19246 cells coaggregated markedly with Ss10557 cells in 40 mM-Tris/HCl buffer. The optimal pH for the coaggregation was between 7.0 and 8.0. The degree of coaggregation decreased with decreasing concentrations of the buffer. After standing at 37 °C for more than 3 h in 40 mM-buffer, pH 7.5, the aggregated masses dissociated gradually and almost completely disappeared after 6 h incubation. Even if Ca²⁺ was added at a concentration of 20 mM to the mixture containing the dissociated cells, coaggregation was not restored.

The cells of Av19246 and Ss10557 did not form visible coaggregates in 4 mM-Tris/HCl buffer, pH 7.5. However, the presence of Ca²⁺ at 0.1 mM or higher or Mg²⁺ and Mn²⁺ at 0.5 mM or higher in the buffer induced cellular coaggregation. The coaggregation was completely inhibited by the presence of Na₂EDTA, Na₂MgEDTA or Na₂MnEDTA at a concentration of 7.5 mM in 40 mM-buffer.

Inhibitory effect of Ca²⁺ on the spontaneous dissociation of coaggregated cells

Cells of Av19246 and Ss10557 were separately preincubated at 4 °C or 37 °C for 17 h in 40 mM-buffer, pH 7.5 and then coaggregation activity of these cells was tested. As shown in Table 1, cells of Ss10557 incubated at 4 °C and 37 °C retained the activity to react with untreated *A. viscosus* cells. Preincubation at 4 °C also did not affect the coaggregating activity of Av19246 cells, but after incubation at 37 °C *A. viscosus* cells lost the ability to coaggregate. However, the loss of activity was prevented by the presence of 1 mM-Ca²⁺ in the mixture during the preincubation.

No significant amount of solubilized protein was detected in the cell-free liquor of the preincubation mixture of Av19246 cells either in the absence or presence of 2 mM-Na₂EDTA or Ca²⁺ (Table 2). In addition, the cell-free liquor of the Av19246 digests showed no ability to inhibit the Av19246-Ss10557 coaggregation reaction, and cell-free and/or cell-bound proteolytic activities were not detected with Av19246 cells under similar conditions (Table 2). These results

Table 1. *Stabilization of the receptor on A. viscosus ATCC 19246 by the addition of Ca²⁺*

Whole cells of both strains were separately incubated at 4 °C or 37 °C for 17 h in 40 mM-Tris/HCl buffer, pH 7.5 in the absence or presence of 1 mM-CaCl₂. They were then washed twice with buffer free from CaCl₂. The reaction for coaggregation was performed in 40 mM-Tris/HCl buffer, pH 7.5. See text for classification of coaggregation.

Pretreatment (17 h)		Coaggregation
<i>A. viscosus</i>	<i>S. sanguis</i>	
4 °C	4 °C	2+
37 °C	4 °C	—
4 °C	37 °C	2+
37 °C	37 °C	—
37 °C + CaCl ₂	4 °C	2+
37 °C	4 °C + CaCl ₂	—
37 °C + CaCl ₂	37 °C	2+
37 °C	37 °C + CaCl ₂	—

Table 2. *Release of proteinaceous cellular substances from and proteolytic activity of A. viscosus ATCC 19246*

The reaction was performed in 40 mM-Tris/HCl buffer, pH 7.5, in the presence of 2 mM-CaCl₂ or Na₂EDTA, with casein, when added, at a concentration of 5 mg ml⁻¹.

Proteinaceous substances	Amount* (mg ml ⁻¹) released or digested in the presence of:	
	2 mM-CaCl ₂	2 mM-Na ₂ EDTA
Cellular proteins released	0.024	0.023
Casein digested	0.018	0.023

* Protein was estimated by the Lowry method with BSA as standard.

Table 3. *Effects of various pretreatments of cells on the coaggregation between A. viscosus ATCC 19246 and S. sanguis ATCC 10557*

The reaction was performed in 40 mM-Tris/HCl buffer, pH 7.5. See text for classification of coaggregation.

Organism	Pretreatment	Coaggregation
<i>A. viscosus</i> ATCC 19246	None	2+
	Heat (100 °C, 1 min)	—
	Heat (80 °C, 5 min)	—
	Trypsin (10 µg ml ⁻¹)	—
	Formalin (1%)	—
	Ethanol (70%)	1+
<i>S. sanguis</i> ATCC 10557	None	2+
	Heat (100 °C, 1 min)	2+
	Trypsin (10 µg ml ⁻¹)	2+

exclude the possibility that the presence of Ca²⁺ inhibits protease activity which might degrade or release a proteinaceous coaggregation receptor on Av19246 cells.

Effects of various pretreatments of cells on the coaggregation

The treatments of Av19246 cells with heat, trypsin and formalin abolished their ability to coaggregate with Ss10557 cells (Table 3), indicating that a protein or glycoprotein on the cell surface of Av19246 may be involved in the coaggregation reaction. In contrast, the reactive site on the cell surface of Ss10557 was resistant to the heat and trypsin treatments.

Inhibition of the coaggregation and dissociation of the coaggregates by sugars

Coaggregation was almost completely inhibited by the presence of 300 mM-*N*-acetyl-galactosamine, 75 mM-galactose, 18.8 mM-lactose and 0.0112 mM-carbohydrate antigen of Ss10557 (minimum concentrations; Table 4). Other carbohydrates tested were without effect even at a concentration of 600 mM. Addition of the D-galactosides at the concentrations indicated above, or higher, induced immediate dissociation of the previously aggregated cellular masses. Glucosamine and galactosamine also inhibited the coaggregation and caused dissociation of coaggregates.

Reactivities of the S. sanguis whole cells and cell walls with various lectins and lectin-like substances

The purified cell walls of Ss10557 showed Av19246-coaggregating activity similar to the whole streptococcal cells. However, the 5% TCA-extracted cell wall residues lost the activity.

Cells and cell walls of Ss10557 were tested for their ability to react with various lectins. It was found that of the lectins tested only RCA-120 produced agglutination of the cells and cell walls, but not of the extracted cell wall residue. The other lectins, PNA, PHA, SBA, WGA and ConA, did not react with Ss10557.

Table 4. *Effects of sugars and sugar derivatives on the coaggregation and disaggregation between A. viscosus ATCC 19246 and S. sanguis ATCC 10557*

Negative results are recorded as —, and positive results as +. The minimal concentration that induced significant inhibition is given in parentheses.

Sugars and sugar derivatives*	Coaggregation inhibition	Disaggregation
Distilled water	—	—
<i>S. sanguis</i> carbohydrate antigen†	+ (375 µg ml ⁻¹)	+ (188 µg ml ⁻¹)
Glucose	—	—
Galactose	+ (75 mM)	+ (150 mM)
Lactose	+ (18.8 mM)	+ (37.5 mM)
Rhamnose	—	—
Fucose	—	ND
Glucosamine	+ (75 mM)	+ (150 mM)
Galactosamine	+ (75 mM)	+ (75 mM)
Mannosamine	—	—
<i>N</i> -Acetylglucosamine	—	—
<i>N</i> -Acetylgalactosamine	+ (300 mM)	+ (300 mM)
<i>N</i> -Acetylmannosamine	—	—

ND, Not determined.

* Other sugars and sugar derivatives listed in Methods were negative in both assays.

† Comprises glucose, galactose, rhamnose and *N*-acetylgalactosamine at a molar ratio of 3:6:3:2 (mol. wt 4170 — see Methods).

DISCUSSION

The results shown in Table 3 suggest that the coaggregation of Av19246 and Ss10557 involves the interaction of a proteinaceous substance on the cell surface of the actinomycete with a carbohydrate site on the streptococcus cell.

The essential role of Ca²⁺ in the lectin-carbohydrate cell-cell coaggregations between *A. viscosus* and *S. sanguis* has previously been reported (McIntire *et al.*, 1978; Cisar *et al.*, 1979). Our results show that the coaggregation between the Av19246 cells and Ss10557 cells is also Ca²⁺-dependent. Complete inhibition of the coaggregation reaction by Na₂MgEDTA seems to eliminate a possible Mg²⁺ dependency of the reaction. The coaggregates of Av19246 cells and Ss10557 cells gradually dissociated and completely disaggregated after extended incubation at 37 °C. Addition of Ca²⁺ at 20 mM to the mixture did not restore coaggregation of the dissociated cells. This phenomenon is due to the spontaneous inactivation of a lectin-like binding site on the surface of Av19246 cells during the extended incubation at 37 °C (Table 1). The presence of sufficient amounts (1 mM) of Ca²⁺ repressed the denaturation of the reactive site, suggesting that the metal ion is sufficiently incorporated into, or in very close proximity to, the reactive site and thereby facilitates the preservation of structural integrity of the site. As shown in Table 2, neither activity of cell-free and cell-bound proteases nor the release of coaggregation-inhibitory proteinaceous substance(s) were detected with Av19246 cells during extended incubation at 37 °C. Thus, the detailed mechanism of the activity loss is obscure and remains to be elucidated.

The coaggregation between Av19246 and Ss10557, therefore, involves a Ca²⁺-dependent, lectin-carbohydrate cell-cell interaction which is lactose-reversible (Table 4). Mechanisms of coaggregation of this type have been studied with *A. viscosus* T14V (AvT14V) and *S. sanguis* 34 (Ss34). The properties of the protein counterpart on the cell surface of AvT14V have been elucidated biochemically, immunologically and morphologically (Cisar & Vatter, 1979; Cisar *et al.*, 1980; Revis *et al.*, 1982) and a carbohydrate counterpart on Ss34 cell walls probably contains β-D-galactosides (McIntire *et al.*, 1978; Cisar *et al.*, 1979).

The native cell walls of Ss10557 coaggregated with Av19246 cells, but the 5% TCA-extracted cell wall residues did not. Moreover, the coaggregation was inhibited by the carbohydrate antigen extracted with 5% TCA from the cell walls of Ss10557 (Table 4). Experiments reported elsewhere (Koga *et al.*, 1983) showed that the immunochemical reaction of the antigen extract with anti-serotype II antiserum is markedly inhibited by *N*-acetylgalactosamine but little

affected by galactose or lactose (Gal-(1→4)-β-Glc). In contrast, lactose and galactose most effectively inhibited the coaggregation between Av19246 and Ss10557 cells and disaggregated the preformed coaggregates (Table 4). *N*-Acetylgalactosamine also exhibited an inhibitory activity but only at a higher concentration (16 times the concentration of lactose and 4 times that of galactose). These results indicate that the site of Ss10557 cells reactive to Av19246 cells is part of the antigenic polysaccharide, but that the active region primarily involved in the coaggregation reaction is distinct from the immunodeterminant region.

McIntire *et al.* (1982) demonstrated that Gal-(1→3)-β-GalNAc-α-*O*-CH₂C₆H₅ and [Gal-(1→3)-β-GalNAc-α-*O*-Thr-Ala-Ala]₄ exhibited remarkably high inhibitory activity (roughly 10- to 20-fold the activity of lactose) towards the coaggregation between AvT14V and Ss34, suggesting that the active portion of the coaggregation site on *S. sanguis* cells comprises a Gal-(1→3)-β-GalNAc- sequence. Only one of the lectins tested, RCA-120, which has a specific affinity for the β-D-galactose residue of the Gal-(1→4)-β-GalNAc- sequence, agglutinated the Ss10557 whole cells and cell walls (Table 4). Other lectins which recognize the terminal D-galactose residue of the Gal-(1→3)-β-GalNAc- sequence (PNA), or possess a specific affinity for β-GalNAc (SBA) and for GalNAc or Gal-(1→4)-β-GlcNAc-(1→2)-β-Man (PHA), did not react with cell surface component(s) of Ss10557. Therefore, based on these data, the active site on Ss10557 cells for the coaggregation reaction appears to contain the sequence Gal-(1→4)-β-Glc-.

In addition, D-fucose (6-deoxy-D-galactose) did not inhibit the coaggregation between Av19246 and Ss10557 (Table 3), in contrast to the findings that D-fucose and D-talose as well as D-galactose each inhibit equally the coaggregation of AvT14V and Ss34 (McIntire *et al.*, 1982). Thus, detailed mechanisms of the cellular coaggregation must be different between these two pairs of *A. viscosus* and *S. sanguis*.

The inhibition of the coaggregation by glucosamine and galactosamine (Table 4) may be non-specific, because these hexosamines were not detected in the carbohydrate antigen extract from Ss10557 cell walls (Koga *et al.*, 1983). However, there is sufficient similarity between GalNAc (one of the antigen components) and galactosamine for 'specific' inhibition to occur. Indeed, the inhibition by these hexosamines is more efficient than that by GalNAc (Table 4). It should be noted here that these hexosamines inhibit the reaction of the carbohydrate antigen with anti-Ss10557 antiserum. Similar 'non-specific' inhibition by these hexosamines has been observed with the reactions of *S. sanguis* b antigen and *Lactobacillus plantarum* cell wall teichoic acid with their homologous antisera (Knox & Wicken, 1973; Appelbaum & Rosan, 1978).

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