Independence of water-insoluble glucan synthesis and adherence of *Streptococcus mutans* to smooth surfaces

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1. INTRODUCTION

Water-insoluble glucans (WIG) produced from sucrose by *Streptococcus mutans* glucosyltransferases (GTases) are of major importance in the adherence and colonization of the organisms on tooth surfaces and the subsequent development of dental caries [1,2]. In fact, mutants of *S. mutans* which lack the ability to synthesize WIG do not stick to glass surfaces and do not induce smooth-surface-caries formation in germ-free rats [3]. Although it has been suggested that active de novo WIG synthesis from sucrose is essential for cell adherence [4–6], the exact relationship between WIG synthesis and cell adherence, as well as these mechanisms, are still not elucidated. The adhesive WIG synthesis of *S. mutans* strain B-13 (serotype *d*) is caused by a cooperative action of 2 GTase components, GT-I and GT-S, which catalyze mainly the formation of 1,3-α-D- and 1,6-α-D-glucosidic linkage, respectively [7,8].

Here, we describe evidence that the sucrose-dependent adherence of heat-killed B-13 cells to a glass surface is primarily mediated by GT-I, namely 1,3-α-D-glucan-forming GTase, and the coexistence of GT-S or primer dextran to allow the marked WIG synthesis is not necessary for the cell adherence.

2. MATERIALS AND METHODS

2.1. *Preparations of cells and enzymes*

Streptococcus mutans strain B-13 was used throughout. The organism was grown aerobically at 37°C for 20 h in defined medium M4 [9] with 0.5% glucose as a carbon source. The harvested cells were heated at 100°C for 20 min, washed twice with distilled water and lyophilized. GT-I and GT-S were purified from the culture fluids as in [6,7]. Homogeneity of both GTase components were confirmed by PAGE, SDS–PAGE and double immunodiffusion. In part, a 40% ethanol fraction [7] was used as a crude GTase preparation. Specific activities of the crude GTase, GT-I and GT-S, were 17.3, 25.5 and 56.4 IU/mg protein, respectively. The activity of GT-I was assayed turbidimetrically in the presence of 0.067% dextran T10.

2.2. *Adherence assay*

In vitro adherence of heat-killed cells to a glass surface was measured as in [4] with modification: 2 mg (dry wt) heat-killed B-13 cells, the appropriate amounts of GT-I, GT-S, and/or dextran T10 (Pharmacia) and 15 mg sucrose were allowed to react in 1.5 ml 50 mM acetate buffer (pH 5.5) containing 0.01% sodium azide. After incubation at 37°C for 16 h in a glass test tube (10 × 75 mm) held at an angle of 30°, a percentage of adhered cells to total cells (% adherence) was determined turbidimetrically as in [10].

2.3. *Glucan synthesis*

The reaction mixture (1.5 ml) consisting of the appropriate amounts of GT-I, GT-S, and/or dextran T10, 100 mM acetate buffer (pH 5.5), 0.01% sodium azide, and 50 mM sucrose was incubated under the same condition as the adherence assay.

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After the incubation for 16 h, the tube was rotated gently by hand at the angle of 30°, and the contents except adhered cells were decanted into a second tube. The first tube was washed with 1.5 ml 50 mM actetate buffer (pH 5.5) and released glucans were poured into the second tube. The adhesive WIG (ad-WIG) remaining in the first tube were washed 3 times with 50% ethanol in 50 mM acetate buffer (pH 5.5) by centrifugation. The non-adhesive WIG (non-ad WIG) in the second tube were homogenized ultrasonically at 100 W for 5 s, precipitated by centrifugation and washed in the same manner as above. Further, water-soluble glucans (WSG) in the supernatant liquid were precipitated by adding 3 vol. absolute alcohol at 4°C overnight. The precipitates were collected, and washed 3 times with 75% ethanol in 50 mM acetate buffer (pH 5.5). Then, the amounts of these washed polysaccharides were determined by phenol-sulphuric acid method [11]. The amount of cell-associated glucans produced by GT-I in the presence of the cells was estimated as follows: GT-I (1 μg) or heated GT-I (100°C, 3 min, 1 μg), sucrose (15 mg) and heat-killed B-13 cells (2 mg) were incubated in a similar manner as the adherence assay. After the incubation, the cells were collected by centrifugation, washed 3 times with 50 mM acetate buffer (pH 5.5), and subjected to the determination of neutral sugars by phenol–sulphuric acid method. The net amount of cell-associated glucans synthesized through incubation was derived by subtracting an amount of neutral sugars of the cells incubated with heated GT-I from that of the cells incubated with GT-I.

2.4. *Pretreatments of heat-killed cells*

Heat-killed B-13 cells (5 mg/ml) were pretreated at 37°C for 60 min with various reagents as follows: 0.1% Trypsin (Sigma) in 50 mM phosphate buffer (pH 7.2); 2.5 or 25 U dextranase/ml (Sigma, from *Penicillium* sp.) in 100 mM acetate buffer (pH 6.0); 1 N NaOH; and 1% SDS. After incubation, the cells were precipitated by centrifugation, washed 3 times with 50 mM acetate buffer (pH 5.5) and subjected to the adherence assay.

3. RESULTS

A crude GTase and mixture of GT-I (0.5 μg) and GT-S (0.25 μg) produced a large amount of ad-WIG from sucrose, and mediated the pronounced adherence of the heat-killed B-13 cells to a glass surface (table 1). GT-I alone exhibited no significant WIG synthesis; however, it mediated the remarkable adherence of the heated cells. Also, in the presence of dextran T10, GT-I caused a marked synthesis of non-ad-WIG from sucrose. The cell adherence by this system was inferior to that by GT-I alone. However, GT-S alone which produced only WSG from sucrose could not mediate the cell adherence.

The correlation between the cell adherence and GT-I and/or GT-S concentrations is shown in fig.

Table 1

Glucan synthesis and cell adherence by GT-I and/or GT-S

Enzyme system	Glucan synthesis (mg)			Adherence (%)
	ad-WIG	non-ad-WIG	WSG	
None (enzyme free)	–	–	–	16.9 ± 5.2[a]
Crude enyme (2 μg)	2.70	0.09	0.13	90.7 ± 0.2
GT-I (0.5 μg)	0.01	0.05	0.00	92.3 ± 0.3
GT-S (0.5 μg)	0.07	0.09	5.10	7.4 ± 0.7
GT-I (0.5 μg) + GT-S (0.25 μg)	6.00	0.02	0.17	86.1 ± 0.9
GT-I (0.5 μg) + dextran[b] (1 m)	0.31	2.20	0.03	78.0 ± 1.4

[a]Mean ± SD of triplicate determinations
[b]Dextran T10 (Pharmacia)

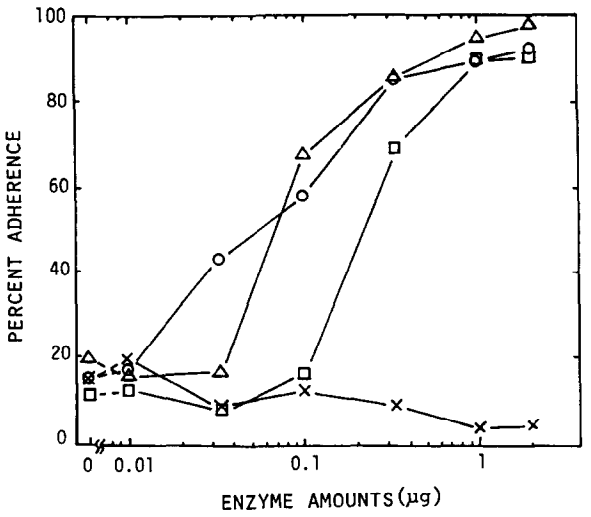

Fig. 1. Relationship between cell adherence and GT-I and/or GT-S concentrations. The cell adherence by GT-I alone (△), GT-S alone (×), GT-I + GT-S (S/I ratio; 0.5) (○), and GT-I + dextran T-10 (1.0 mg) (□) systems containing various concentrations of both components were measured as in section 2.

1. In all systems containing GT-I, the remarkable rises of the percentage in adherence accompanying an increased concentration of enzymes were observed. In particular, the system of GT-I alone was the most adequate for the cell adherence, and brought about the percentage in adherence to ~100% at 1 μg/reaction mixture.

Table 2 shows the effects of the protein ratio of GT-S to GT-I (S/I ratio) on glucan synthesis and cell adherence. The glucan synthesis was induced significantly with increasing concentrations of GT-S. Maximal WIG production occurred at a S/I ratio of 1.0, but the synthesis of ad-WIG proceeded strikingly only at S/I ratios of 0.25–0.5. In contrast, the cell adherence was inhibited apparently by the coexistence of GT-S. The inhibition by GT-S was very slight up to a S/I ratio of 0.75. When GT-S coexisted more than an equal amount of GT-I (S/I ratio ≧ 1.0), the majority of heat-killed cells failed to stick to glass surface.

Table 3 shows the glucan synthesis and the cell adherence mediated by GT-I in the presence of increasing concentrations of dextran T-10. Most WIG produced from sucrose by this system was non-adhesive glucans. Maximal production of non-ad-WIG occurred at 0.1% dextran T10, and

Table 2

Effects of S/I ratio on glucan synthesis and cell adherence by GT-I plus GT-S system

S/I ratio[a]	Glucan synthesis[b] (mg)			Adherence[c] (%)
	ad-WIG	non-ad WIG	WSG	
0.00	0.01	0.05	0.00	97.6 ± 0.5[d]
0.10	0.14	0.89	0.00	89.2 ± 8.8
0.25	2.96	0.02	0.01	94.9 ± 0.1
0.50	6.05	0.02	0.18	92.8 ± 0.7
0.75	0.24	6.65	1.23	90.3 ± 0.5
1.00	0.06	8.46	1.96	3.1 ± 2.8
1.50	0.09	6.74	4.29	3.6 ± 0.9
2.00	–	–	–	3.9 ± 3.3

[a] Protein ratio of GT-S (0–0.5 μg or 0–2 μg) to GT-I (0.5 or 1 μg)
[b] 0.5 μg GT-I was used; [c] 1.0 μg GT-I was used
[d] Mean ± SD of triplicate determinations

Table 3

Effects of dextran T10 concentrations on glucan synthesis and cell adherence by GT-I plus dextran T10 system

Dextran T10 added (%)	Glucan synthesis[a] (mg)			Adherence[b] (%)
	ad-WIG	non-ad-WIG	WSG	
0.00	0.01	0.05	0.00	97.0 ± 0.2[c]
0.01	0.06	0.91	0.00	83.1 ± 0.2
0.03	0.11	1.43	0.03	83.0 ± 3.2
0.1	0.16	1.85	0.30	81.9 ± 0.2
0.3	0.13	1.62	2.60	78.5 ± 0.1
1.0	0.02	0.17	14.4	73.0 ± 2.1
3.0	0.00	0.00	42.7	69.0 ± 3.8

[a] 0.5 μg GT-I was used; [b] 1.0 μg GT-I was used
[c] Mean ± SD of triplicate determinations

no production at 3%. The presence of dextran was inhibitory for the cell adherence, however the inhibition was not so remarkable. Even when dextran was 3% at which WIG synthesis was completely inhibited, ~70% of the cells could stick to a glass surface.

The adherence of B-13 cells by GT-I was entirely dependent on the prolonged incubation with

>2.0 mM sucrose and sucrose could not be replaced by either glucose nor fructose. Needless to say, GT-I heated at 100°C for 3 min lost an ability completely to mediate the cell adherence. The presence of dextranase (50 mU/ml) in the incubation mixture did not affect mostly for the adherence by GT-I. Pretreatment of the heated cells with dextranase (2.5 U/ml) also did not show any effect. However, pretreatment with a high concentration of dextranase (25 U/ml) resulted in a partial (50%) inhibition. Pretreatments of the cells with trypsin, SDS, and NaOH caused a nearly complete loss of the cell adherence by GT-I.

However, GT-I (1 μg) produced the cell-associated glucans of 0.64 mg from sucrose under the adherence assay condition using 2.0 mg heat-killed B-13 cells. The amount was ~5-times as much as that of glucans produced by cell-free system. The cell-associated glucans were highly resistant against dextranase treatment (2.5 U/ml, 37°C, 2 h).

4. DISCUSSION

We demonstrated in [6,7] that a cooperative action of the 2 GTase components was essensial for the synthesis of adhesive WIG. The result the adhesive WIG synthesis proceeds strikingly only when GT-I and GT-S coexisted at S/I ratios of 0.25–0.5, confirms our conclusion. Similar results have been reported using 2 GTase components from *S. mutans* strain OMZ-176 (serotype *d*) [12].

This study shows that in vitro adherence of heat-killed B-13 cells to a glass surface is mediated primarily by GT-I, and the coexistence of GT-S or primer dextran to allow the marked WIG synthesis is not only unnecessary but also inhibitory for the cell adherence. These findings suggest strongly that sucrose-dependent adherence of *S. mutans* may be independent of WIG synthesis. Thus far, a concept that WIG synthesis is responsible for cell adherence has been generalized using crude GTase preparations. However, we can find in the reports several data that did not agree with this concept. For example, there are different inhibitory effects by dextranase [13] and a discrepancy of optimum pH ranges [14] for cell adherence and WIG synthesis, and a specific inhibition for cell adherence by egg albumin [14]. We have also recognized that bovine serum albumin (1 mg) or serum (50 μl) inhibits remarkably the adherence of B-13 cells without affecting the WIG synthesis (not shown).

This study shows that a simultaneous incubation of the cells, native GT-I and sucrose is necessary for the cell adherence. Furthermore, the amounts of cell-associated glucans produced by GT-I in the presence of cells are slight, but apparently superior to the cell-free system. These results suggest that a catalytic action of GT-I undoubtedly relates to a process for adherence. Therefore the process for adherence of the B-13 cells to a glass surface may involve the de novo synthesis of a specific cell-associated glucan by the primer-dependent GT-I reaction which requires a certain polysaccharide on cell surface as a glucose acceptor. The exact nature of the cell-associated glucan is still unknown, although it is presumed that it consists mainly of a 1,3-α-D-glucosidic linkage. The acceptor substance is also unknown, but appears to be other than a glucose polymer consisted of 1,6-α-D-glucosidic linkage, because neither dextranase treatment nor addition of dextran (table 3) causes the vigorous inhibition to the cell adherence.

However the fact that the cells pretreated with trypsin, SDS, and NaOH lose the ability to stick to a glass surface suggests that a heat-stable protein-like component on the cell surface could function as an important factor in the adherence of *S. mutans* cells. Similar findings have been reported using cells and crude GTase preparations of *S. mutans* strain 6715 [14,15].

Thus, we claim in this report that sucrose-dependent adherence of *S. mutans* cells to smooth surfaces may be performed primarily by the mechanism containing actions of GT-I (1,3-α-D-glucan forming GTase) and a cell-surface protein-like component. Experiments to clarify the detailed mechanism of cell adherence are in progress.

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