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Short communication

Serotype-specific polysaccharide of *Streptococcus mutans* contributes to infectivity in endocarditis

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Streptococcus mutans and other viridans streptococci have been implicated as major etiological agents of infective endocarditis. The serotype-specific rhamnose-glucose polysaccharide (RGP) of *S. mutans* has several biological functions that appear to be essential for the induction of infective endocarditis. The aim of this study was to examine the contribution of RGP to the infectivity of *S. mutans* in infective endocarditis using a rat model. The RGP-defective mutant of *S. mutans* showed reduced ability to induce infective endocarditis compared to the parental strain. The ability of *S. mutans* to induce infective endocarditis was not consistent with the binding capacity of the organism to extracellular matrix proteins. The results suggest that *S. mutans* containing whole RGP is more virulent than the RGP-defective mutant, and the RGP has an important role for the induction of infective endocarditis by *S. mutans*.

Key words: endocarditis; serotype-specific polysaccharide; *Streptococcus mutans*

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The viridans streptococci constitute one of the principal groups of pathogenic organisms in infective endocarditis (9). The members of this group are inhabitants of the oral cavity, and *Streptococcus mutans*, the etiological agent of dental caries in humans, is also responsible for the induction of streptococcal infective endocarditis (4, 8). Once *S. mutans*, or other viridans streptococci, enters the bloodstream following oral surgery or other common dental procedures such as brushing or flossing, these pathogenic organisms are thought to adhere to damaged heart valves and develop infective endocarditis (9). The serotype-specific rhamnose-glucose polysaccharide (RGP) of *S. mutans* consists of a backbone structure of α1,2- and α1,3-linked rhamnosyl units with glucose side chains (14, 22). The RGP acts as a putative adhesin for the binding of *S. mutans* cells to human monocytic and fibroblastic cells (5) and to human and rabbit platelets (2). Other than the binding ability, several biological functions of *S. mutans* RGP have been reported that appear to be essential for the establishment of infective endocarditis: platelet aggregation (2), resistance to phagocytosis by human polymorphonuclear leukocytes (26), and triggering of cytokine (5, 25, 27) and nitric oxide (16) release.

Clinically, Fujiwara et al. (7) reported that *S. mutans* possessing RGP with very low glucose content was isolated from the peripheral blood of a patient with infective endocarditis. The molar ratio of rhamnose : glucose in RGP from their isolate was approximately 26, while that of reference strains of *S. mutans* was almost 2. However, the role of RGP in infective endocarditis has not been clarified *in vivo*. The purpose of this study was to examine the contribution of RGP to the infectivity of *S. mutans* in infective endocarditis using a rat model. We also examined the effect of RGP on the binding of *S. mutans* cells to extracellular matrix (ECM) proteins (laminin, collagen type I and fibronectin).

Induction of infective endocarditis

The laboratory strain *S. mutans* Xc (12) was grown in brain–heart infusion (BHI) (Becton Dickinson Co., Cockeysville, MD) broth. Strains Xc41 and Xc45 are isogenic mutants derived from the parental Xc strain; strain Xc41 is devoid of whole

RGP, and only the glucose content is reduced in strain Xc45 (28). Strains Xc41 and Xc45 were grown in BHI broth supplemented with erythromycin (10 μg/ml). Bacterial cells were collected by centrifugation and washed with phosphate-buffered saline (PBS; pH 7.4). Collected cells were then suspended in PBS and dispersed on ice by low-energy sonication.

Endocardiac infectivity was determined in catheterized rats as described previously (11, 18). The experiment to induce infective endocarditis in rats was performed with the approval of the animal care committee of Kagoshima University, Kagoshima, Japan. In brief, male Wistar rats (Kyudo, Kumamoto, Japan) with a body weight of 300–350 g were catheterized from the right carotid artery to the left ventricle through the aortic valve under anesthesia. Twenty-four hours after the operation, the rats were inoculated by injection of 0.5 ml of a suspension containing 5×10^7 colony-forming units (CFU) of bacteria into the tail vein. In preliminary experiments, we had found that a dose of 10^7 CFU per rat was necessary for the authentic induction of infective endocarditis using *S. mutans* Xc, and we also confirmed that a period of 3 days after the injection of the organism was sufficient for the development of vegetations according to the previous report (23). As controls, catheterized rats injected with PBS containing no bacterial samples, and non-catheterized rats injected with each bacterial sample (five in each group) were prepared. Three days after the injection, 1 ml blood was collected from the abdominal aorta of rats under anesthesia and was heparinized. The formed vegetation was excised from the heart, weighed and homogenized with 1 ml PBS. An aliquot from each of the serial dilutions of the homogenized vegetation was plated onto BHI agar with or without erythromycin (10 μg/ml). Collected blood was directly plated in a similar manner. The plates were incubated at 37°C under anaerobic conditions for 2 days, and the number of colonies formed was counted. When the bacterial colonies were present in the culture of the homogenized vegetation, it was judged that infective endocarditis had been induced in the rat.

Infected vegetations were induced in 81.8% of rats inoculated with *S. mutans* Xc, 36.4% of rats inoculated with RGP-defective mutant (Xc41), and 60.0% of rats inoculated with glucose-defective mutant (Xc45) (Table 1). Significant differences in the rate of induction of infected vegetations were observed between the Xc and Xc41 strains (Pearson's chi-square test, $P < 0.05$). The vegetations induced by the mutant strains of Xc41 and Xc45 were smaller than those induced by parental strain Xc, but the differences were not significant. The microbial density of the vegetations induced by the Xc41 strain was significantly lower than that of those induced by the Xc strain (Student's *t*-test, $P < 0.05$). Bacteraemia was detected in 27.3% of the rats inoculated with *S. mutans* Xc, but was not detected in the rats inoculated with either the Xc41 or Xc45 strains. No control catheterized or non-catheterized rats injected with bacteria developed infective endocarditis.

Binding of *S. mutans* cells to ECM proteins

An enzyme-linked immunosorbent assay (ELISA) was adapted to evaluate the binding ability of *S. mutans* cells to ECM proteins, as described previously (10). Mouse laminin (Becton Dickinson, Franklin Lakes, NJ), human collagen type I (CosmoBio, Tokyo, Japan) and human fibronectin (CosmoBio) were used. The bacterial cells were suspended in PBS (pH 8.0), and incubated with sulfosuccinimidyl 6-biotinamide-hexanoate (Pierce, Rockford, IL) (0.5 mg/ml final concentration) at 25°C for 30 min. By washing with PBS, the unbound biotin was removed from the bacterial cells. The wells of the ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 μl of either mouse laminin, human collagen type I, or human fibronectin (5 μg/ml) in PBS at 4°C overnight. The wells were filled with 2% skim milk in PBS containing 0.05% Tween 20 (PBST) at 25°C for 1 h. After blocking, suspensions containing 7.2×10^5 CFU of each kind of biotinylated bacterial cells in PBST with 2% skim milk were added and the plates were incubated at 25°C for a further hour. After washing, the bound bacterial cells were incubated with avidin-biotinylated alkaline phosphatase complex (Vector Laboratories, Burlingame, CA) diluted in PBST at 25°C for 1 h. The reaction products were visualized using *p*-nitrophenylphosphate (1 mg/ml) in 0.1 M diethanolamine buffer (pH 9.6) containing 2 mM $MgCl_2$, and the absorbance at 405 nm (A_{405}) was measured with a microplate reader (Bio-Rad Laboratories, Richmond, CA). The number of bacteria bound was determined using the standard curve between the number of bacterial cells and A_{405}, and the interpolation was performed exactly.

The Xc, Xc41 and Xc45 strains of *S. mutans* showed the ability to bind to laminin, collagen type I and fibronectin (Fig. 1). The ability of the Xc41 strain to bind both laminin and collagen type I was slightly lower than that of the Xc strain,

Table 1. Production of experimental infective endocarditis by inoculation with *Streptococcus mutans*

		Vegetation			Blood	
Strains	No. animals used	No. animals infected[1]	Weight (mg) (mean ± SD)	Log_{10}CFU/mg (mean ± SD)	No. animals infected[1]	Log_{10}CFU/ml (mean ± SD)
S. mutans						
Xc	11	9 (81.8)	5.47 ± 4.69	2.53 ± 1.59	3 (27.3)	0.34 ± 0.61
Xc41	11	4 (36.4) [2]	3.11 ± 2.49	1.14 ± 1.69[2]	0 (0)	0
Xc45	10	6 (60.0)	3.47 ± 2.76	1.89 ± 1.71	0 (0)	0
Control						
No bacteria[3]	5	0 (0)	1.12 ± 0.31	0	0 (0)	0
S. mutans						
Xc[4]	5	0 (0)	0	0	0 (0)	0
Xc41[4]	5	0 (0)	0	0	0 (0)	0
Xc45[4]	5	0 (0)	0	0	0 (0)	0

[1]Values in parentheses are the percentage of the total number of rats examined.
[2]$P < 0.05$ compared to *S. mutans* Xc.
[3]Catheterized rats were used.
[4]Non-catheterized rats were used.

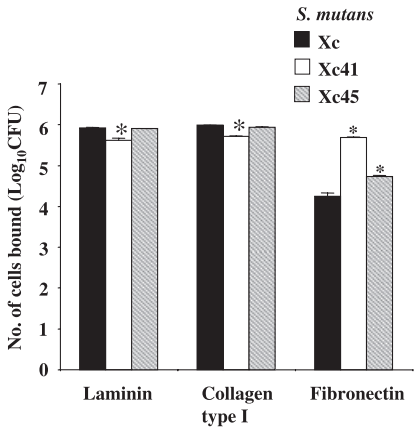

Fig. 1. Binding of *Streptococcus mutans* Xc, Xc41, and Xc45 strains to laminin, collagen type I and fibronectin. The values represent means and standard deviations of triplicate assays. The experiments were performed three times independently, and similar results were obtained in each experiment. * $P < 0.05$ compared to *S. mutans* Xc.

although the differences were significant (Student's *t*-test, $P < 0.05$). In contrast, the Xc41 and Xc45 strains showed higher binding abilities for fibronectin than the Xc strain ($P < 0.05$).

Several virulence factors of *S. mutans* for infective endocarditis have been examined using animal models. Loss of either synthesized glucan and fructan, which are sucrose-derived exopolysaccharides, or SloA, which is an ATP-binding protein responsible for the metal transport of *S. mutans*, reduced the induction rate of infective endocarditis (17, 20). In this study, compared to the parental strain *S. mutans* Xc, the induction rate of infected vegetations decreased significantly and bacteraemia was not observed in catheterized rats after inoculation with the RGP-defective mutant strain (Xc41) (Table 1). These findings support the possibility that the strain containing RGP is more virulent than the mutant that is deficient in whole RGP.

The adherence of microorganisms to damaged heart tissue can be crucial for the development of infective endocarditis (9). In our study, *S. mutans* Xc bound to immobilized collagen type I, laminin and fibronectin. Compared to Xc strain, the RGP-defective mutant Xc41 exhibited reduced binding to collagen type I and laminin. There have been reports that antigen I/II (also designated PAc) (1, 21), fibronectin binding protein (3) and the collagen-binding adhesin family (24) were involved in the adherence of *S. mutans* cells to ECM proteins. Sato et al. (24) demonstrated that the *S. mutans* strain harboring collagen-binding adhesin (Cnm protein), encoded by the *cnm* gene, exhibited binding to immobilized collagen and laminin, while the Cnm-deficient mutant showed reduced binding ability. Since *S. mutans* Xc also has the *cnm* gene (Y. Sato, personal communication), the binding of the Xc strain to collagen and laminin may be attributed to the function of the Cnm protein. Further work is needed to define the binding mechanism of *S. mutans* to collagen and laminin through RGP in connection with the Cnm protein and/or other cell surface molecules. On the other hand, the binding ability of *S. mutans* strains to fibronectin was not consistent with the ability of the strains to induce experimental infective endocarditis. Fibronectin binding has been thought to be a major process in the induction of infective endocarditis, and this hypothesis was supported by findings in which several strains of *Staphylococcus aureus* (13) and *Streptococcus sanguis* (15) with reduced fibronectin-binding capacity showed reduced adherence to valvular vegetations. While, Flock et al. (6) showed no differences in the induction of infective endocarditis between the parental *S. aureus* strain and its mutant with reduced fibronectin-binding capacity in a rat model, and strongly pointed out that fibronectin binding alone is not enough to promote the adhesion of *S. aureus* cells to heart valve vegetations. In our study, the virulence of *S. mutans* containing whole RGP for infective endocarditis was not explained by its ECM-binding ability. There have been reports that *S. mutans* RGP has several functions that seem to be associated with the establishment of infective endocarditis other than its binding ability (2, 5, 16, 25–27), and it is possible that the RGP contributes to the infectivity of *S. mutans* through the integration of these functions in infective endocarditis.

The glucose side chains of the RGP-defective mutant (Xc45) did not show a reduced ability to cause experimental infective endocarditis in this study. Fujiwara et al. (7) isolated an *S. mutans* strain possessing very low glucose contents of RGP from the peripheral blood of a patient with infective endocarditis. Nakano et al. (19) demonstrated that *S. mutans* strains defective in glucose side chains of RGP were less susceptible to phagocytosis by human polymorphonuclear leukocytes. These results suggest that glucose side chain-defective strains of *S. mutans* are able to survive longer in blood, but the direct evidence indicating that glucose side chains have important roles in the induction of infective endocarditis has not been presented. From the results obtained here, we think that the glucose side chains of RGP might have no major roles in the induction of experimental infective endocarditis. Pritchard et al. reported that a common structure composed of a polyrhamnose backbone exists in streptococcal polysaccharides (22) and this structure might be related to the infectivity of the viridans streptococci in infective endocarditis. Further studies are necessary to clarify the role of serotype-specific polysaccharide in the induction of infective endocarditis by viridans streptococci including *S. mutans*.

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