Co-aggregation as a virulent factor of *Streptococcus sanguis* isolated from infective endocarditis

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Abstract: The pathogenicity of strains of the *Streptococcus sanguis* group, isolated from infective endocarditis, was studied by measuring the development of subcutaneous abscesses in mice after infection with *S. sanguis* and *Actinomyces viscosus* either singly or as co-aggregated pairs. The pathogenicity of the co-aggregates was also examined in various viable combinations of the two bacterial species. More abscesses were formed by *A. viscosus* than the *S. sanguis* group including clinical isolates. Abscess formation by co-aggregates of combinations of each isolate and *A. viscosus* produced a higher percentage of abscess formation than those caused by infection with a pure suspension of *A. viscosus* or tested streptococci. Co-aggregated cells were more resistant to phagocytosis and killing by neutrophils *in vivo*. These results indicated that *S. sanguis* group streptococci isolated from infective endocarditis are able to co-aggregate and resist phagocytosis. The ability of co-aggregation of *S. sanguis* may serve as a survival mechanism in a host defense system and may be linked with virulence of this bacteria. (J. Oral Sci. 41, 117-122, 1999)

Key words: infective endocarditis; oral streptococci; *Streptococcus sanguis* group; co-aggregation.

Introduction

Infective endocarditis is frequently caused by oral alpha-hemolytic streptococci, such as *Streptococcus (S.) sanguis*, *S. gordonii*, *S. mutans* and *S. mitis* (1-3). The streptococci can enter the blood through breaks in the microcirculation of oral tissues induced by trauma, including dental manipulations, oral hygiene procedures, mastication, and oral infections (4).

Streptococci, circulating in blood, may then adhere to damaged endothelium (5, 6) or thrombi composed of platelets

and fibrin (5-7) on vessel walls, endocardium, and heart valves. Further, development of thrombi and verrucous vegetations on valves serve to embed the streptococci and provide protection from host defenses and antibiotic therapy (6, 7). Viridans group streptococci, the most common cause of bacterial endocarditis, also produce an exopolysaccharide (glycocalyx) which correlates with adherence to damaged heart valves (8). The amount of glycocalyx is correlated with the size of the infected cardiac vegetations and resistance to antimicrobial therapy (9).

Current evidence regarding the pathogenesis of bacterial endocarditis suggests that a crucial step in the development of infection is the initial adherence of bacteria to endocardium. Synthesis of glucan polymer has been correlated with the ability of *S. sanguis* to adhere to damaged heart valve leaflets *in vitro* and has been associated with increased infectivity *in vivo* (10). Dextranase-treated cells showed decreased adherence to fibrin-platelet matrix *in vivo* and decreased rate of infection in the animal model of endocarditis (10). Streptococcal virulence in endocarditis involves factors that promote infectivity and pathogenicity. Previous reports have shown that surface-localized glucosyltransferase mediates adhesion of *S. gordonii* to vein endothelial cells *in vitro* (11). Several species of oral streptococci have been shown to aggregate with other oral bacteria such as *Actinomyces spp.* and *Bacterionema spp.* in the maturation process of dental plaque. Unusual morphological arrangements have been observed when filamentous bacteria form specific aggregates with oral streptococci in the shape of corn cobs (12). It is well known that platelet aggregation and glycocalyx contribute the pathogenesis of oral streptococci in the initial step of bacterial endocarditis. However, there is little published information about the effect of co-aggregation of *S. sanguis* isolated infective endocarditis. In this study, therefore, we employed the clinical isolates to clarify the effect of coaggregation on the pathogenicity of *S. sanguis*. This study was performed to assess the pathogenicity of aggregates of *A. viscosus* and *S. sanguis* group streptococci.

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Materials and Methods

Bacterial strains and culture conditions

The following five viridans streptococci were used as the control strains in this study; *Streptococcus sanguis* ATCC10556, *S. oralis* strain 34, *S. gordonii* ATCC 10558, *S. salivarius* ATCC9222 and *S. mitis* (*S. oralis*) ATCC9811. Nine clinical isolates of viridans streptococci were isolated from confirmed cases of endocarditis in patients at the Department of Medicine, Tokyo Woman's Medical College. Bacterial classification was done using results from previous reports (13, 14). *Actinomyces viscosus* T14V was also employed in the co-aggregation experiment.

A streptomycin-resistant strain of *A. viscosus* T14V was produced in our laboratory by a series of subcultures in Todd-Hewitt Broth (BBL, Cockeysville, MD, USA) containing 2 mg/ml streptomycin (Meiji Seika, Tokyo, Japan). Each culture was mixed with skimmed milk and stored frozen at -20°C . Cells were cultured anaerobically (CO_2 5%, N_2 95%) in Todd-Hewitt broth for 18 h at 37°C . Cells were harvested by centrifugation ($1200 \times g$ for 20 min) and washed three times with buffer: 1.0 mM Tris (hydromethyl)-aminomethane, adjusted to pH 8.0; 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , NaN_3 0.02% and 0.15 M NaCl. Heat-treated cells (85°C for 30 min) were also used in this study.

Co-aggregation assay

Co-aggregation was assayed by the method of Cisar et al. (15). Equal volumes (0.2 ml) of each cell suspension were mixed for 10 sec on a vibrator and allowed to stand at room temperature for 10 min.

Abscess development

The method for abscess development was described previously (16). Briefly, male mice (ddy) aged 4 weeks were obtained and observed for 7 days before the experiments. Bacteria were cultured and centrifuged as described above and washed with 25 mM potassium phosphate buffer (pH 8.0) containing 25 mM NaCl. Inocula were adjusted to a density of $1.0 \times 10^5 \sim 1.0 \times 10^{10}$ cfu/ml spectrophotometrically at 540 nm, then within 15 min, 0.1 ml was injected subcutaneously into the backs of the mice. The inocula for mixed cultures (0.1 ml) consisted of equal volumes (0.05 ml) of each species. Experiments were repeated four or five times with groups of five mice, and abscess formation was evaluated 2 days after inoculation. The mice were sacrificed with ether and abscess sizes were measured with calipers. Smears were also stained with Giemsa's solution and examined microscopically.

Phagocytosis of co-aggregates by neutrophil polymorphonuclear leukocytes (PMNs)

In vitro inhibition of phagocytosis was examined using the method described previously (16). In the experiments on the inhibition of phagocytosis and the reduction of the bactericidal activity of PMNs by actinomyces and streptococci, the ratio of test organisms that produced maximal co-aggregation with minimal free non-aggregated cells was calculated. After

spectrophotometric adjustment of each cell suspension to equal density, *A. viscosus* was mixed with the tested streptococcal strains. To separate non-aggregated and aggregated cells, each mixture was applied to the top of a glycerol column as described by Ochiai et al. (17). The mixture, consisting of discrete and aggregated cells, was applied to the step-gradient, glycerol column and 0.5-ml fractions were collected from the bottom. After 10 min, non-aggregates remained in the top layer of the column and any aggregated cells that settled in the glycerol 30% layer were determined spectrophotometrically at 540 nm. The effect of co-aggregates on phagocytosis was investigated with PMNs induced by injecting 2 ml of oyster glycogen (0.1% in 2mM phosphate buffered saline, pH 7.2; Sigma, St Louis, MO, USA) into the abdominal cavity of each mouse. The mice were sacrificed with ether 10-12 h later and peritoneal exudate cells were collected by the injection of RPMI-1640 Medium (Gibco, Rockville, MD, USA) supplemented with 2% fetal calf serum into the abdominal cavity. The RPMI-1640 was aspirated and the total number of cells was determined by haemocytometer (Kayagaki Irika Kogyo Co. Ltd, Tokyo, Japan) immediately after rubbing the abdomen. The number of PMNs present in peritoneal exudate cells was determined by Giemsa staining

Statistics

The significance of differences between groups was determined by Student's *t* test.

Results

Co-aggregation between *A. viscosus* and oral streptococci

When *A. viscosus* was mixed with the tested oral streptococci including nine strains of infectious endocarditis isolates, all the suspensions rapidly became granular and soon large clumps of bacteria formed, except for *S. gordonii* ATCC10558 and *S. salivarius* ATCC9222 (Table 1). Microscopic examination of these clumps showed that they were a mixture of the two organisms. However, non-aggregates were found when both strains were heated (Table 1).

Abscesses caused by non-aggregated cells

The results for pure bacterial suspensions injected subcutaneously into the backs of mice are shown in Fig. 1. Abscesses developed in all animals inoculated with $\geq 1.0 \times 10^7$ cfu of *A. viscosus* T14V, but this was reduced to 8.5% when an inoculum of 1.0×10^6 cfu was used. Abscesses developed in all mice inoculated with 1.0×10^9 cfu of all the tested *S. sanguis* including clinical isolates, in 31.3% with 1.0×10^8 cfu of *S. sanguis* ATCC10556, 55.4% with *S. sanguis* TW678 and 47.2% with *S. sanguis* TW644. However, no lesions were observed at concentrations of $\leq 1.0 \times 10^6$ cfu when the tested strains of streptococcus were used. These abscesses were smaller than those produced by *A. viscosus* T14V. Bacterial cells were present in all the stained smears of pus and the test species were isolated from the abscess samples initiated by viable bacterial suspension.

Table 1 Coaggregation of infective endocarditis-isolated strains with *Actinomyces viscosus*

(A). Laboratory strain

<i>A. viscosus</i>	<i>S. oralis</i>	<i>S. sanguis</i>	<i>S. gordonii</i>	<i>S. salivarius</i>	<i>S. mitis</i> 1)
	34	10556	10558	9222	9811
----- Live-streptococcal cells -----					
T14V	4+	4+	±	—	4+
T14V(Heat T.) 2)	ND	3+	—	—	3+
----- Heat-treated cells -----					
T14V	2+	—	—	—	2+
T14V(Heat T.)	±	—	—	—	—

(B) Isolates from infective endocarditis

<i>A. viscosus</i>	<i>S. oralis</i>			<i>S. sanguis</i>					
	TW 50	TW 52	TW 644	TW 142	TW185	TW186	TW 675	TW 678	TW 681
----- Live-streptococcal cells -----									
T14V	3+	4+	4+	+	2+	2+	4+	3+	3+
T14V(Heat T.)	4+	2+	2+	±	+	+	—	4+	3+
----- Heat-treated cells -----									
T14V	+	+	—	±	±	±	2+	+	—
T14V(Heat T.)	—	—	—	—	—	—	—	—	—

1) *S. mitis* ATCC9811 is also classified in *S. oralis*.

2) Heat treated at 85 °C for 30 min.

Abscesses caused by co-aggregates

When co-aggregates of *A. viscosus* T14V (1.0×10^5 cfu) and various concentrations of *S. sanguis* TW678 were injected into mice, abscesses developed in 64.5% and 17.2% of animals for inocula of 1.0×10^7 cfu and 1.0×10^8 cfu, respectively (Fig. 2A). *S. sanguis* TW678 was at a higher percent of cells when compared with *S. sanguis* ATCC10556 at 1.0×10^8 cfu. A similar experiment was done with *A. viscosus* T14V (1.0×10^6 cfu) and *S. sanguis* TW678. Coaggregates were injected into mice, abscesses developed in 86.8%, 67.9% and 29.6% of animals for inocula of 1.0×10^8 , 1.0×10^7 and 1.0×10^6 cfu, respectively (Fig. 2B). This compares with a value of 8.5% when *A. viscosus* was tested in pure culture under the same conditions. Raised spherical abscesses, containing yellow and sticky pus, resulted from viable bacterial injections.

Anti-phagocytic action of cell aggregates

When *S. sanguis* TW678 was injected into mice, 66.3% of PMLs had phagocytosed bacteria 15 min after inoculation, and 76.4% after 60 min (Fig. 3). Similar results were obtained from *S. sanguis* ATCC10556. For *A. viscosus* T14V, the percentage of cells with phagocytosed bacteria was 59.6%, 65.2% and 74.2% after 15, 30 and 90 min, respectively.

When co-aggregates of *A. viscosus* and *S. sanguis* TW678 were inoculated into the abdominal cavity of mice after co-aggregation, the number of PMNs with phagocytosed bacteria was 39.4% after 15 min, 50.2% after 30 min and 48.2% after 90 min (Fig. 3). A significant difference ($p \leq 0.01$) was found between the co-aggregate and the pure culture groups of *A. viscosus* and *S. sanguis* tested at 60 and 90 min *in vivo*.

Fig. 1 Abscess formation in mice 2 days after injection of cell suspension of live strains of streptococcus and *A. viscosus*. Points represent the mean values from five experiments with five mice in each group; vertical bars represent the SD. Symbols represent *A. viscosus* (●), *S. sanguis* ATCC 10556 (○), *S. salivarius* ATCC 9222 (△) and strains isolated from infective endocarditis *S. sanguis* TW678 (■) and *S. oralis* TW 644 (□).

Fig. 2 The effect of co-aggregation of *A. viscosus*, A, 1.0×10^5 ; B, 1.0×10^6 and various concentrations of either *S. sanguis* ATCC10556 or TW678 on abscess formation in mice. Strains were injected after co-aggregates had formed. Points represent the mean values from five different experiments with five mice in each group; vertical bars represent the SD. Symbols represent *A. viscosus* + *S. sanguis* ATCC10556 (○) and *A. viscosus* + *S. sanguis* TW678 (●). Values for co-aggregates were significantly higher than with *A. viscosus* or *S. sanguis* alone (* $p < 0.01$).

Fig. 3 The effect of bacterial co-aggregates on the ability of polymorphonuclear leukocytes to act as phagocytes in vivo. Points represent the mean values from five different experiments with three mice in each group; vertical bars represent the SD. Symbols represent *S. sanguis* ATCC 10556 (○), *S. sanguis* TW678 (□), *A. viscosus* (△), *A. viscosus* + *S. sanguis* ATCC10556 (●), *A. viscosus* + *S. sanguis* TW678 (■). Values for co-aggregates were significantly lower than with *A. viscosus* or *S. sanguis* alone (* $p < 0.01$).

Discussion

Streptococcal infection of heart valves and endocardium accounts for as many as half of the cases of native valve endocarditis (18). These infections may occur when oral bacteria enter the circulatory system and adhere and colonize damaged heart tissues (19). The virulence of viridans streptococci in the pathogenesis of infective endocarditis remains an enigma. *S. sanguis* is a normal inhabitant of the human oropharyngeal mucosa and dental plaque. In this environment, it is considered indigenous and relatively avirulent. However, this organism may escape the oral cavity following dental or specialized procedures and enter the blood stream. The resulting bacteremia may cause infective endocarditis. Certain strains of *S. sanguis* group streptococci induce human platelets to aggregate *in vitro* (20). Streptococcal glycocalyx are important virulence factors in the pathogenesis of endocarditis. These surface polymers may facilitate the binding of bloodborne bacteria to valve surfaces, thereby initiating endocardial infection (21).

Bacterial surface polysaccharide and proteins mediate aggregation and co-aggregation, therefore, many of streptococci found in dental plaque also have the ability to coaggregate with *A. viscosus*. Co-aggregation is highly specific, where the partners of one cell type may not be partners of a closely related cell type (22). It has been reported that saliva and dextran induce aggregation in oral streptococci (23). These species exhibit both homotypic and heterotypic aggregation with the latter being related to cell-to-cell interactions between the adhesin-receptor complementary pairs on the two cell types (15, 24). Ochiai et al. (16) reported that *S. mitis* could evade phagocytosis by forming co-aggregate with *A. viscosus*. Aggregation of platelets by bacteria is well studied as a causative agent of oral streptococci for infective endocarditis, however, coaggregation is not well elucidated. Actinomyces

are pathogenic bacteria that cause chronic inflammatory lesions in man and animals (25). Engel et al. (26) have reported that *A. viscosus* releases various extracellular inflammatory substances that cause increased migration of PMNs and accelerate tissue destruction. They also reported that inoculation of *A. viscosus* cells caused a high rate of abscess development. Co-aggregates between *A. viscosus* and *S. sanguis* (Fig. 2A and B) exhibited greater pathogenicity than that shown by single bacterial cells (Fig. 1). These results suggest that the pathogenicities of *A. viscosus* and *S. sanguis* are increased by co-aggregation. It was found that *A. viscosus* was more resistant to phagocytosis than *S. sanguis* in pure culture, and when actinomyces formed part of the co-aggregates, their uptake by PMNs was decreased further *in vivo* (Fig. 3). Since the ability to co-aggregate with *A. viscosus* induce *S. sanguis* to survive at a higher rate, co-aggregation of oral bacteria may inhibit phagocytosis.

Some polysaccharides from *S. sanguis* act as a receptor for a Gram-negative bacterium (27). Previous studies have shown that these Gram-negative bacteria produce immunosuppressive factors intra- and extra-cellularly (28-30). The strains of *S. sanguis* and *S. oralis* co-aggregate with *Capnocytophaga* (*C. ochracea*) with these polysaccharides. Recently, several reports have described periodontopathic bacteria, such as *C. ochracea* and *Actinobacillus* spp. as the cause of septicemia and endocarditis in compromised and compromised hosts (31, 32, 33).

The present study reveals that *S. sanguis* could evade phagocytosis by forming co-aggregates with other oral bacteria. Coaggregation of oral bacteria would be an important virulent factor to cause systemic infection. Further investigations are required to elucidate this mechanism.

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