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Binding of *Streptococcus mutans* to extracellular matrix molecules and fibrinogen☆

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Abstract

We have determined the ability of *Streptococcus mutans* cells to bind to extracellular matrix (ECM) molecules and fibrinogen. *S. mutans* cells were found to bind fibronectin, laminin, collagen type I, and fibrinogen. An isogenic *S. mutans* strain with a defect in the expression of the major surface protein of *S. mutans*, antigen I/II, possessed a reduced ability to bind fibronectin, collagen, and fibrinogen but not laminin, suggesting that antigen I/II contributes during pathological processes to the interaction of *S. mutans* cells with fibronectin, collagen type I, and fibrinogen.

Keywords: *Streptococcus mutans*; Extracellular matrix molecules; Antigen I/II; Fibrinogen

The viridans streptococci are commonly associated with subacute infective endocarditis [1]. This heterogeneous group of microbes is generally found in the oral cavity and includes *Streptococcus mutans*, the etiologic agent of human dental caries [2]. Following injury to the endothelium, a sterile lesion consisting of platelet–fibrin deposits is formed, causing a condition called nonbacterial thrombotic endocarditis. Following trauma, such as a dental procedure, to the oral cavity a transient bacteremia can occur and members of the viridans streptococci, including *S. mutans*, can attach to the lesion and following growth a vegetation is formed resulting in infective endocarditis [3]. The mechanism as to how this interaction occurs between *S. mutans* cells and cellular components is not known. Since the bacteria colonize damaged heart tissue, it is believed that sub- endothelial matrix molecules, such as fibronectin, laminin, or collagen, function as cell receptors for the bacteria. A number of bacterial pathogens [4], including streptococci [5–8], have been shown to adhere to extracellular matrix (ECM) molecules or blood components, such as fibrinogen, via specific surface receptors. Interaction of *Streptococcus sanguis* cells with fibronectin appears to be an important virulence determinant for infective endocarditis caused by this viridans streptococci [9]. In this study, we characterized the binding of *S. mutans* cells to ECM molecules and the blood protein, fibrinogen. Additionally, we have examined the role of the predominant surface protein, antigen I/II (also designated antigen B, P1, and PAc) [10–13], in the binding of *S. mutans* cells to ECM molecules and fibrinogen.

Materials and methods

Bacterial strains. Streptococcus mutans M51 is the laboratory designation given to the bacterial strain used in this study to assess the ability of *S. mutans* cells to bind to extracellular matrix molecules and fibrinogen. Strain M51 is one of the twelve *S. mutans* strains obtained from Richard Facklam of the Centers for Disease Control and Prevention that were isolated from the peripheral blood of patients clinically diagnosed as suffering from infective endocarditis. *S. mutans* 834 is an isogenic mutant of *S. mutans* NG8 in which the *spaP* gene, which encodes the major surface antigen I/II (P1), has been insertionally inactivated with pVA981 which harbors a tetracycline resistance determinant [14]. Streptococcal strains were routinely grown in Todd–Hewitt (TH) media.

☆ *Abbreviations:* ECM, extracellular matrix molecules; TH, Todd–Hewitt; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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Determination of binding of S. mutans to ECM protein and fibrinogen. The wells of microtiter plates (Costar EIA/RIA plate 3590; Costar Corporation, Cambridge, MA) were coated with protein by incubating 100 μl of a 10 μg ml^{-1} solution of either fibronectin, laminin, or collagen type I (all of human origin; Life Technologies, Gaithersburg, MD) or 100 μl of a 100 μg ml^{-1} solution of fibrinogen (Enzyme Research Laboratories, South Bend, IN), prepared in carbonate–bicarbonate buffer (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, pH 9.6), in assay wells overnight at room temperature. Collagen type I and laminin were obtained frozen in Tris buffer from the vendor and diluted before use in the carbonate–bicarbonate buffer. Wells were coated with 100 μl of a 100 μg ml^{-1} solution of bovine serum albumin (BSA) as blanks. Wells were then washed three times with water, blocked with PBS (0.01 M phosphate buffer [pH 7.4], 138 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween 20 and 0.25% BSA, and washed three times with water. Various concentrations of biotinylated *S. mutans* cells (sonicated 30 s at 50% power using a model 450 Branson digital sonifier to disrupt streptococcal chains into single and double cells) were added to wells and the plates were incubated 1 h at 37 °C. After 1 h, wells were washed twice with PBS containing Tween 20 to remove unbound cells. Bound *S. mutans* cells were detected with horseradish peroxidase-conjugated streptavidin. After incubation for 1 h at 37 °C with horseradish peroxidase-conjugated streptavidin and two washes with PBS containing Tween 20, color was developed using tetramethylbenzidine as substrate. Reactions were terminated by the addition of 1 M H_2SO_4 and the absorbance at 450 nm was read using a microplate reader (BioRad Model 3550-UV; BioRad Laboratories, Hercules, CA). For the experiments to determine inhibition by anti-fibronectin sera (Sigma Chemical, St. Louis, MO) of binding of *S. mutans* cells to fibronectin, various dilutions of antisera were added to fibronectin-coated wells, prior to the addition of biotinylated *S. mutans* cells. For the experiments that tested the ability of protease treated cells to bind fibronectin, biotinylated *S. mutans* M51 cells were incubated for 1 h at 37 °C with either proteinase K (50 μg ml^{-1}), trypsin (50 μg ml^{-1}), pronase E (25 μg ml^{-1}), or *Staphylococcus aureus* V8 endoproteinase Glu-C (25 μg ml^{-1}) (all from Sigma Chemical, St. Louis, MO) and washed three times with PBS to remove protease and then the cells were assayed for binding of fibronectin as described above. Biotinylated *S. mutans* cells were also used in experiments that measured the ability of *S. mutans* NG8 and its isogenic antigen I/II (*spaP*) mutant strain 834 to bind extracellular matrix protein and fibrinogen. The level of biotinylation of different preparations of the two *S. mutans* strains was determined by fixing with 0.25% (wt/vol) glutaraldehyde a known number of cells to the wells of microtiter plates followed by reaction with horseradish peroxidase-conjugated streptavidin. Only cell preparations with biotinylation levels within 10% of each other were used in these experiments and values obtained were corrected, if necessary, for differences in biotinylation levels.

Biotinylation of S. mutans cells. An overnight culture of *S. mutans* cells was diluted 1:20 and grown at 37 °C to an optical density of 0.6 at 600 nm. The cells were pelleted by centrifugation, washed twice in PBS, and suspended in PBS. *N*-hydroxysuccinimido-biotin was added to the cells at a concentration of 100 μg ml^{-1} and the cell suspension was incubated at room temperature for 1 h with gentle swirling. After three washes in PBS to remove unbound biotin, the cells were suspended in PBS and the optical density at 600 nm of the cell suspension was adjusted to 0.6 (approximately 5×10^8 cells ml^{-1}).

Results

Binding of S. mutans cells to extracellular matrix molecules and fibrinogen

Initially, we surveyed the ability of 12 *S. mutans* strains isolated from endocarditis patients to bind fibronectin. Eleven of the strains bound fibronectin at similar levels while one strain bound fibronectin at a level approximately 50% lower than the other strains. This study demonstrated the feasibility of examining the binding of *S. mutans* cells to extracellular matrix or blood constituents. Fig. 1 shows binding curves representative of four experiments of the binding of different concentrations of cells of one of the *S. mutans* strains, M51, to the wells of microtiter plates coated with either fibronectin, laminin, collagen type I, or fibrinogen. The binding of *S. mutans* cells to collagen type I and fibronectin was cell number dependent up to approximately 3×10^6 cells while the binding to laminin and fibrinogen peaked at approximately 1×10^7 cells. A decrease in binding to all of the molecules was observed at the highest cell concentrations which resulted in similar binding of cells to all of the molecules at the highest cell concentration (5×10^7 cells) used in the experiments. This observation may indicate that there is some dissociation of cells from the molecules at the higher cell concentrations. It should be noted that significant binding of *S. mutans* cells to fibrinogen could only be obtained with wells coated with a higher amount of fibrinogen than that used for the ECM molecules. Wells contained 10 μg fibrinogen versus 1 μg ECM molecule.

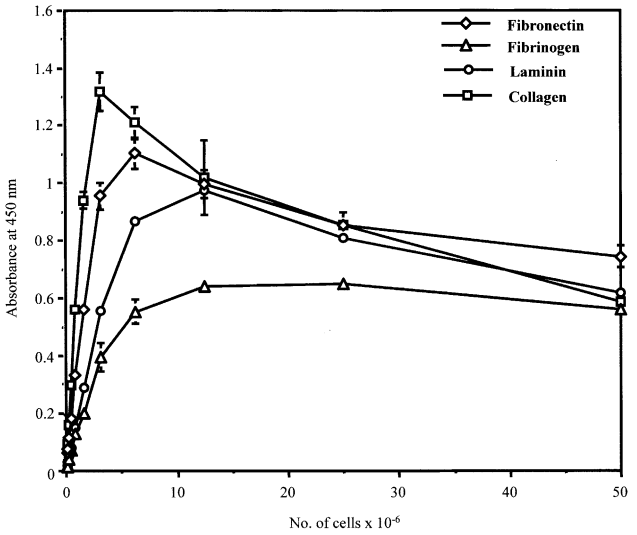

Fig. 1. Binding of *S. mutans* M51 cells to fibronectin (Fn), laminin (Ln), collagen type I (Cn), and fibrinogen (Fb). Values represent means and SEM of at least triplicate samples and are representative of four experiments.

Characterization of the binding of S. mutans cells to fibronectin

Since we were interested in the interaction of *S. mutans* with fibronectin, we characterized further the binding of *S. mutans* cells to this ECM molecule. To evaluate the specificity of binding of *S. mutans* cells to

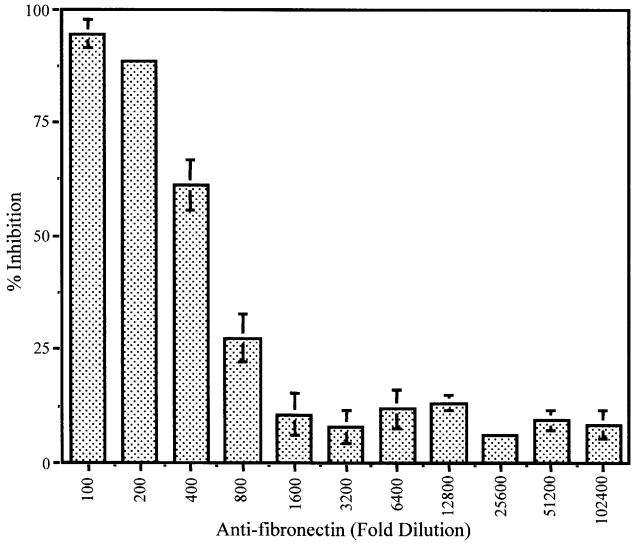

Fig. 2. Inhibition by anti-fibronectin sera of binding of *S. mutans* M51 cells (6×10^6 cells added to wells per dilution of antisera) to immobilized fibronectin. One hundred percent binding is the binding of cells to fibronectin-coated wells not treated with antisera. Values represent means and SEM of at least triplicate samples and reflect similar results of several experiments.

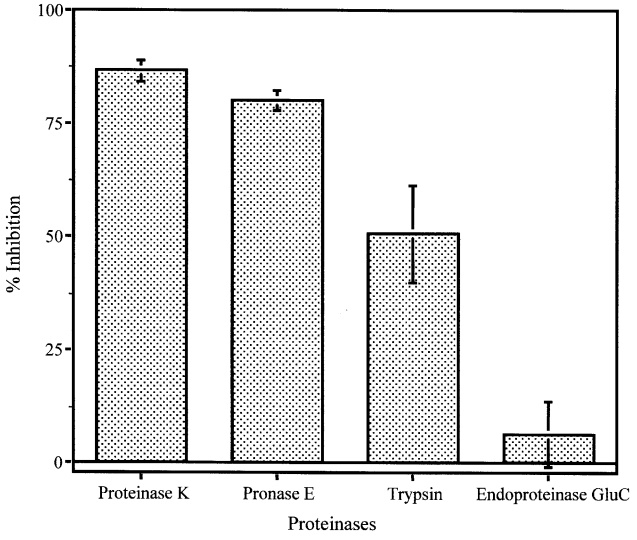

Fig. 3. Effect of protease treatment on binding of *S. mutans* M51 cells (6×10^6 protease-treated cells added per well) to immobilized fibronectin. Percent inhibition is the reduction in binding due to treatment of cells with the protease compared to binding of cells not treated with a protease. Values represent the mean and SEM of triplicate samples and reflect similar results of several experiments.

immobilized fibronectin, rabbit polyclonal antisera against human fibronectin were used to determine if the antisera could inhibit binding of *S. mutans* M51 cells to wells of microtiter plates coated with fibronectin. The results shown in Fig. 2 demonstrate that anti-fibronectin sera could inhibit at relatively high dilutions the binding of M51 cells to fibronectin.

When *S. mutans* M51 cells were treated with various proteases and assayed for their ability to bind fibronectin, the following results were obtained. Proteinase K and pronase E inhibited binding by approximately 80% while trypsin inhibited binding by about 50%. Treatment with endoproteinase GluC resulted in minimal or no inhibition (Fig. 3).

Analysis of the involvement of antigen I/II protein in the binding of S. mutans cells to extracellular matrix molecules and fibrinogen

The predominant protein expressed on the surface of *S. mutans* cells is a protein of 190 kDa that has been designated by various laboratories as antigen I/II [10], antigen B [11], P1 [12], or Pac [13]. Here, we will refer to the protein as antigen I/II. Antigen I/II functions as an adhesin and has a role in the initial adherence of *S. mutans* cells to tooth pellicles in the etiology of dental caries [14]. Structurally, antigen I/II is associated with the fimbriae or "fuzzy coat" observed in electron micrographs of *S. mutans* cells [14]. To determine if *S. mutans* antigen I/II protein has a role in the binding of *S. mutans* cells to extracellular matrix molecules and fibrinogen, we performed a comparative analysis of the ability of wild-type *S. mutans* NG8 and its isogenic antigen I/II (*spaP*)-defective mutant strain 834 to bind to fibronectin, laminin, collagen type I, and fibrinogen. Results of our studies indicate that the mutant 834 strain had a reduced ability, especially at higher cell concentrations, to bind fibronectin and collagen (Figs. 4A and B) but bound laminin in a manner similar to that of the wild-type strain NG8 (Fig. 4C). Mutant strain 834 could only bind fibronectin and collagen at approximately 50% and 75%, respectively, of the level observed with the parental strain NG8. Mutant strain 834 bound fibrinogen poorly when compared to the parent strain NG8 (Fig. 4D).

Discussion

The important finding of this study is that cells of a *S. mutans* strain isolated from an endocarditis patient can bind to the extracellular matrix molecules fibronectin, laminin, and collagen type I and the blood component, fibrinogen. The interaction of *S. mutans* cells with ECM components and fibrinogen may be important in assisting in the adherence of bacteria to exposed matrix molecules during pathological situations in the oral cavity as well as adherence to fibrin–platelet matrices and damaged heart tissue during the colonization of lesions by this bacterium. The results presented here set the groundwork for studies aimed at the identification of the molecules on the surface

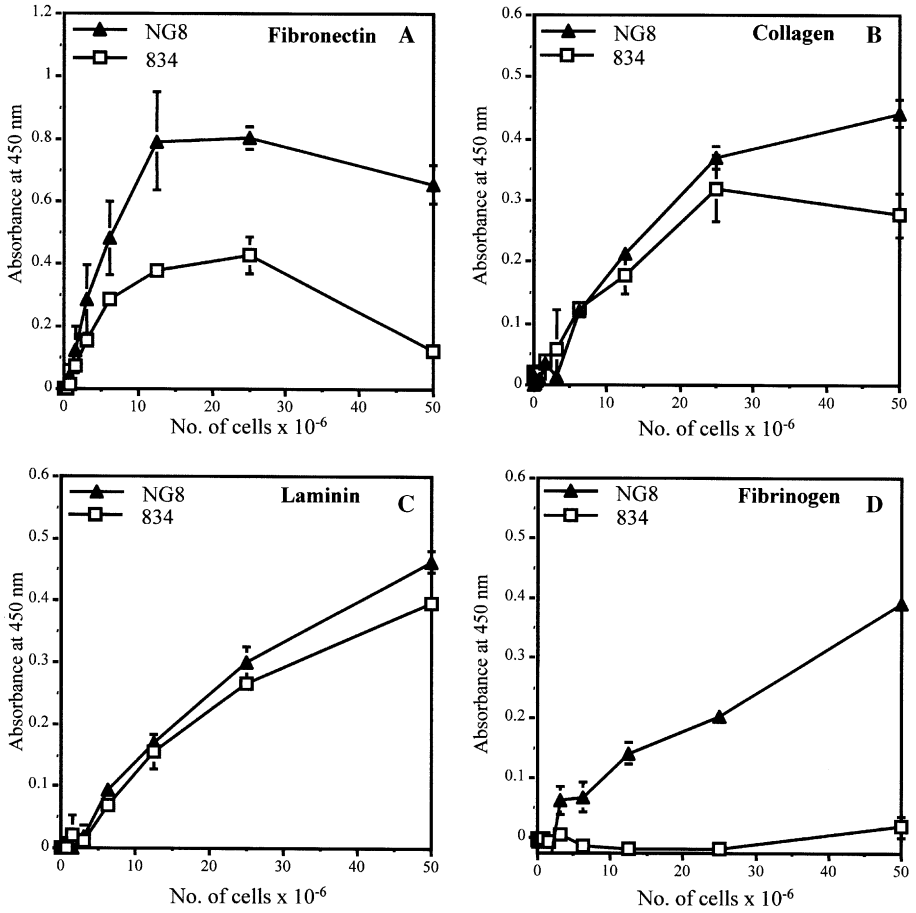

Fig. 4. Binding of *S. mutans* NG8 and *S. mutans* 834 to fibronectin (A), collagen (B), laminin (C), and fibrinogen (D). Values represent means and SEM of at least triplicate samples and are representative of several experiments.

of *S. mutans* cells that mediate the binding to extracellular matrix molecules and fibrinogen.

During the characterization of the binding of *S. mutans* cells to fibronectin, we observed that incubation of cells with soluble fibronectin failed to inhibit binding of cells to fibronectin-coated wells which suggests that *S. mutans* cells do not bind well to soluble fibronectin. This result, which was also reported recently by Chia et al. [16], may explain the finding of an earlier study [17] which concluded that *S. mutans* cells bind poorly to fibronectin since in that study the binding assay utilized soluble fibronectin to measure the binding of *S. mutans* cells to fibronectin. Additionally, we have observed that monoclonal antibodies against the cell attachment domain or the carboxyl terminal region of fibronectin do not inhibit binding of cells which suggest that these domains may not participate in the interaction of fibronectin with *S. mutans* cells. The results that digestion of *S. mutans* cells with several proteases indicate that the molecules on the surface of *S. mutans* M51 cells that interacts with fibronectin is substantially proteinaceous in nature.

The studies presented here suggest that the major surface protein of *S. mutans*, antigen I/II, may be involved in the interaction of *S. mutans* cells with fibronectin, collagen, and fibrinogen. Several reports in the literature have suggested that the antigen I/II or a homolog expressed by other oral streptococci may in addition to involvement in the adhesion of cells to salivary pellicles also interact with specific extracellular matrix molecules in pathogenic processes such as in the establishment of endodontic infections [18,19]. In one of these studies, Love et al. [18] found that strain NG8 could bind collagen and that the antigen I/II (*spaP*) mutant strain 834, the same strain used in the present study, exhibited a reduced ability to bind collagen when compared with NG8. Our studies are in agreement with the studies of Love et al. [18] that the mutant 834 strain has impaired ability to bind collagen. In another study, Sciotti et al. [19] reported that purified antigen I/II protein of *S. mutans* OMZ175 had the capacity to bind to a number of matrix molecules, including fibronectin, collagen, and laminin. In the present study, we observed that cells of the endocarditis strain, M51, bound collagen type I, fibronectin, laminin, and fibrinogen in descending order, while the primarily laboratory strain NG8 bound fibronectin better and binding to collagen type I, laminin, and fibrinogen was similar. We do

not know the reason for the difference in the binding of two *S. mutans* strains to the same molecules, but may indicate an adaptation of the endocarditis strain to become a better pathogen in its role as a causative agent of infective endocarditis. The difference in binding properties of the two strains is not because of the amount of surface localized antigen I/II because strain NG8 has about 40% more antigen I/II on its surface as strain M51 when determined using reactivity of cells of the strains to anti-antigen I/II sera. Binding of *S. mutans* cells using antigen I/II as a ligand to tissue via exposed fibronectin or collagen may only be one of the several mechanisms that permits these cells to adhere since only partial reduction of binding was observed when the antigen I/II mutant strain was evaluated in the binding assay. This may be specifically true for the binding of cells to fibronectin since recently the identification of a 130 kDa fibronectin-binding protein of *S. mutans* has been reported [16]. On the other hand, antigen I/II may play a more significant role in the adherence of *S. mutans* cells to fibrinous clots since the antigen I/II mutant strain was found to possess little binding activity for fibrinogen. To our knowledge, this is the first study that demonstrates an interaction of *S. mutans* cells with fibrinogen and that the molecule on the surface of *S. mutans* cells that mediates the interaction is antigen I/II protein. The identification of the domain within the structure of the antigen I/II protein that binds fibrinogen will allow for a greater understanding of the role of antigen I/II in pathogenic process of infective endocarditis. In conclusion, the present study supports the conclusion that *S. mutans* cells bind extracellular matrix molecules and fibrinogen and that antigen I/II of *S. mutans* plays a role in the interaction of *S. mutans* cells with fibronectin, collagen, and fibrinogen.

Acknowledgments

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