Adsorption of Salivary Proteins to the Surface of Oral Streptococcal Cells

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Abstract

Oral tissues, especially tooth surfaces, are covered with a layer of salivary proteins. Oral bacterial cells that adsorb to salivary components accumulated on the tooth surface are, as a rule, covered with the same components, especially proteins. Thus, it is possible that the salivary proteins covering the bacterial cells are related to the adhesion of bacteria to oral tissues. The aim of this study was to clarify the mechanisms of adsorption of salivary proteins to the surface of *Streptococcus sanguis*, *S. mitis* and *S. salivarius* using an adsorption assay with salivary proteins labeled with tritiated formaldehyde. The results showed that salivary proteins adsorbed more to *S. salivarius* than to *S. mitis*, and least to *S. sanguis*. It was evident that hydrophobic bonding was involved in the adsorption of salivary proteins to the bacterial cells tested. The amount of salivary proteins adsorbed to *S. mitis* and *S. salivarius* was decreased by the presence of phosphate, that to *S. sanguis* was increased by the presence of a divalent cation such as Ca^{2+} , and that to all bacteria tested was inhibited in different ways by the presence of sugars. The amount of salivary proteins adsorbed to *S. sanguis* and *S. salivarius* was reduced effectively by pretreatment of the cells with trypsin, chymotrypsin and papain. In the case of *S. mitis*, the amount of adsorbed salivary proteins was decreased by pretreatment of the cells with chymotrypsin only, and was increased by pretreatment with lipase. These results indicate that there are different mechanisms of adsorption of salivary protein to the cell surfaces of oral streptococci.

Introduction

Dental caries and periodontal disease are caused by oral bacteria^[1-4], and therefore bacterial adhesion to the surface of oral tissues is observed in the early stage of these diseases. The mechanism of bacterial adhesion to oral tissues has been widely studied^[5-15], based mainly on just-harvested cultured bacterial cells. However, oral tissues such as teeth and the oral mucosa are quickly covered with a layer of salivary proteins, usually referred to as the acquired pellicle^[16].

At the same time, oral bacterial cells that adsorb to oral tissues are, as a rule, also covered with the same salivary pellicle. Thus it is possible that the salivary proteins covering the surface of oral bacteria are related to the adsorption of bacteria to oral tissues. In this respect, Tamura et al.^[17] pointed out nearly 6 years ago that differences in the number of bacterial cells adhering to salivary proteins on a membrane filter are observed between bacteria just-harvested from cultures and saliva-coated and that it was necessary for bacterial cells to be covered with body fluids when bacterial adhesion experiments were carried out^[18-24]. For this reason, as a first step toward clarifying the relationship between salivary proteins which adsorb to oral bacteria and the adhesion of oral bacteria to oral tissues, this study was performed to clarify the mechanisms of adsorption of salivary proteins to the surface of several species of oral streptococcal cells, *S. sanguis* (strain ATCC 10556) which is present in high numbers in dental plaque^[25,26], *S. mitis* (strain ATCC 9811) which is

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detected mainly on the surface of the oral mucosa^[25,26], and *S. salivarius* (strain HHT) which is detected in saliva and on the dorsal tongue^[25,26], using an adsorption assay employing salivary proteins labeled with tritiated formaldehyde.

Materials and Methods

Saliva collection and radiolabeling

Whole unstimulated saliva from a single donor, aged 31y, blood type A, was collected in a container chilled in ice, then heated at 56°C for 60 min to inactivate any degradative enzymes^[27] and clarified by centrifugation at 8000 × g for 10 min. Sodium azide was added at a final concentration of 0.04%. The saliva was filtered (Millipore, MA, U.S.A.), and dialyzed to remove inorganic substances. This preparation was finally lyophilized and stored at -70°C. Salivary proteins were dissolved in 0.067 M potassium phosphate buffer (pH 7.5), and radiolabeled with [³H]formaldehyde (Dupont NEN Research Products, MA, U.S.A.) by reductive methylation as described by Jentoft^[28].

Bacterial strains and culture

S. sanguis ATCC 10556, *S. mitis* ATCC 9811 and *S. salivarius* HHT were used throughout the study. They were grown overnight at 37°C in Todd-Hewitt broth (Difco, MI, U.S.A.) after preincubation with Todd-Hewitt semi-solid agar medium. Organisms were harvested at the late logarithmic phase by centrifugation at 3000 × g for 10 min, washed twice in 0.05 M Tris-HCl buffer (pH 7.0), and suspended in the same buffer. The suspension was vigorously forced back and forth through a 25-gauge syringe needle to break up the streptococcal chains^[29] and then standardized to an optical density of 0.5 at 550 nm.

Adsorption assay

Bacterial pellets of 500 μl of several bacterial suspensions were harvested by centrifugation at 4500 × g for 3 min in a polypropylene microtube (Sarstedt, Rommelsdorf, Germany). After addition of radio-labeled salivary proteins (250 μg per 10⁹ cells for *S. sanguis*, 100 μg for *S. mitis* and 150 μg for *S. salivarius*^[22,24]) at -4°C for 60 min, the bacterial cells were washed three times in Tris-HCl buffer in the tubes to remove residual salivary proteins by centrifugation at 4500 × g for 3 min. The specific radioactivity of the labeled salivary proteins was counted. At the same time, the final cell number in each tube was counted to determine the volume of salivary proteins adsorbed per 10⁹ cells.

Effect of divalent cations, phosphate, and surface-active agent on adsorption of salivary proteins to bacterial surfaces

To test the effect of divalent cations on the adsorption of salivary proteins to bacterial cell surfaces, the amounts of adsorbed salivary proteins were measured using Tris-HCl buffer containing either Ca²⁺ or Mg²⁺ at concentrations of 10 or 100 mM. The effect of phosphate on the adsorption was compared when Tris-HCl buffer and potassium phosphate buffer at pH 7.0 were used. To test the effect of a surface-active agent on the adsorption, the amounts of adsorbed salivary proteins were measured using Tris-HCl buffer containing 0.1% Tween 80 for examining relationship between hydrophobic binding and salivary protein adsorption.

Effect of sugar on adsorption of salivary proteins to bacterial surfaces

To examine the lectin binding on the adsorption of salivary proteins to bacterial cells, the organisms were incubated in Tris-HCl buffer containing 1 mM CaCl₂^[30,31], and either 10 or 100 mM of sugars such as L-fucose, D-mannose, D-galactose, lactose, D-glucose, N-acetyl-D-galactosamine or N-acetyl-D-glucosamine.

Pretreatment of bacterial cells with enzymes

The influence of pretreatment of bacterial cells with several enzymes on the adsorption of salivary proteins to the cell surface was studied. The enzymes used were trypsin (Gibco, NY, U.S.A.; pH 8.0), chy-

motrypsin (Wako Pure Chemical, Osaka, Japan ; pH 8.0),papain (Wako ; pH 6.0) and lipase (Wako ; pH 9.0) at 37°C for 30 min (control consisted of non-treated bacterial cells suspended in potassium phosphate buffer)^[22,23]. After treatment with each enzyme, the bacterial cells were washed three times in Tris-HCl buffer by centrifugation at 3000×g for 10 min to remove residual enzymes, and were restandardized in the buffer, and the amount of adsorbed protein was assayed. The amounts of salivary proteins adsorbed to bacteria at pHs shown above were employed as controls^[22,24].

Results

Amounts of salivary proteins adsorbed to bacterial surfaces

The results are presented in Table 1. The amounts of salivary proteins adsorbed to the bacterial cell surfaces were 898.4 ng for *S. sanguis*, 1,000.7 ng for *S. mitis*, and 2,887.7 ng for *S. salivarius*. This table shows that differences in the affinity of salivary proteins for the bacterial surface are evident.

Table 1 Amounts of salivary proteins adsorbed to bacterial surfaces (ng/10⁹ cells)

<i>Streptococcus sanguis</i>	ATCC 10556	868.4 (74.7)*
<i>Streptococcus mitis</i>	ATCC 9811	1000.7 (106.1)*
<i>Streptococcus salivarius</i>	HHT	2887.7 (339.1)*

* : mean (S.D.)

Table 2 Effect of divalent cations, phosphate, and surface-active agent on the adsorption of salivary proteins to bacterial surfaces (% adsorption of salivary proteins compared to the control)

	<i>S. sanguis</i>	<i>S. mitis</i>	<i>S. salivarius</i>
Control	100	100	100
CaCl ₂ 1 mM	133.9	128.3	81.7
10 mM	134.7	81.5	66.6
MgCl ₂ 1 mM	98.6	86.2	83.7
10 mM	100.0	84.8	68.1
Phosphate	97.2	84.7	66.9
Tween 80	39.3	42.1	57.9

Effect of divalent cations, phosphate, and surface-active agent on adsorption of salivary proteins to bacterial surfaces

The results are shown in Table 2. The amount of salivary proteins adsorbed was increased by the presence of Ca²⁺ for *S. sanguis* and by the presence of 1 mM Ca²⁺ for *S. mitis*, but the amount was decreased by both Ca²⁺ and Mg²⁺ for *S. salivarius* and by Mg²⁺ and 10 mM Ca²⁺ for *S. mitis*. Phosphate inhibited the adsorption of salivary proteins to *S.mitis* and *S.salivarius* , but not to *S.sanguis*. For all bacterial cells, the amount of adsorbed salivary proteins was lowest in the presence of the surface-active agent Tween 80.

Effect of sugars on adsorption of salivary proteins to bacterial surfaces

The results are given in Table 3. The presence of lactose and N-acetyl-D-glucosamine inhibited the adsorption of salivary proteins to all bacterial cell surfaces, and the presence of L-fucose, D-mannose and D-galactose inhibited the adsorption to *S. sanguis* and *S. mitis*. The presence of D-glucose affected the adsorption to *S. sanguis* , but not to other strains. In the case of *S. mitis* and *S. salivarius* , the amounts of adsorbed salivary proteins were increased by the presence of N-acetyl-D-galactosamine.

Table 3 Effect of sugars on the adsorption of salivary proteins to bacterial surfaces
(% adsorption of salivary proteins compared to the control)

		<i>S. sanguis</i>	<i>S. mitis</i>	<i>S. salivarius</i>
Control		100	100	100
L-fucose	10m M	71.3	65.2	95.3
	100m M	65.3	71.2	93.2
D-mannose	10m M	95.4	92.7	101.2
	100m M	72.2	78.4	90.8
D-galactose	10m M	90.5	80.7	95.6
	100m M	79.0	80.0	90.6
Lactose	10m M	94.4	74.0	84.5
	100m M	67.3	64.3	86.4
D-glucose	10m M	85.0	116.8	95.1
	100m M	66.4	95.6	106.8
N-acetyl-D-galactosamine				
	10m M	92.0	132.5	158.8
	100m M	88.9	458.7	363.2
N-acetyl-D-glucosamine				
	10m M	79.8	80.4	95.1
	100m M	67.7	58.7	79.7

Table 4 Effect of pretreatment of bacterial cells with enzyme on the adsorption of salivary proteins to bacterial surfaces
(% adsorption of salivary proteins compared to the control)

	<i>S. sanguis</i>	<i>S. mitis</i>	<i>S. salivarius</i>
Control	100	100	100
Trypsin	40.4	98.9	29.1
Chymotrypsin	46.5	81.5	23.1
Control	100	100	100
Papain	40.9	95.2	18.4
Control	100	100	100
Lipase	89.0	218.7	97.1

Effect of enzyme pretreatment on adsorption of salivary proteins to bacterial surfaces

The results are given in Table 4. The amounts of adsorbed salivary proteins were decreased by pretreatment of the cells with trypsin, chymotrypsin, and papain for *S. sanguis* and *S. salivarius*. When *S. mitis* was used, the amount of proteins was decreased by chymotrypsin, and was increased by lipase.

Discussion

As dental caries and periodontal disease are caused by oral bacteria^[1-4], bacterial adhesion to the surface of oral tissues should be considered the first stage of these diseases. The surface of bacterial cells is covered with salivary proteins, like that of oral tissues, probably because the cells are suspended in saliva before bacterial adhesion to oral tissues, and thus it is possible that the cells' surface covered with salivary proteins is related to their adhesion to oral tissues.

As a first step toward clarifying the relationship between the salivary proteins adsorbed to bacterial

cells and their adhesion to oral tissues, the aim of this study was to clarify the mechanisms of adsorption of salivary proteins to several oral streptococci using an adsorption assay with salivary proteins labeled with tritiated formaldehyde. The results showed that salivary proteins had higher affinity for *S. salivarius* than for *S. mitis*, and for *S. mitis* than for *S. sanguis*. It may be assumed that there is some relationship between the high salivary protein affinity for *S. salivarius* and the isolation of *S. salivarius* in high number from saliva in the oral cavity.

The mechanism of oral bacterial adhesion to oral tissues is divisible into two types^[32], non-specific and specific binding. Thus, we had investigated the relationship between these types of binding and the mechanisms of adsorption of salivary proteins to bacterial cells.

Non-specific binding is known to occur through several mechanisms including bridging divalent cations^[32] and hydrophobic bonding^[32,33]. As divalent cations are related to bacterial adhesion, the effect of Ca^{2+} or Mg^{2+} on the adsorption of salivary proteins to bacterial cells was examined. The results show that the effects of divalent cations are found to be diverse and that Ca^{2+} promotes the adsorption of salivary proteins to *S. sanguis* only.

To test for hydrophobic bonding^[32,33], a surface-active agent, Tween 80, was added to the buffer. It is obvious that hydrophobic bonding plays a role in salivary protein adsorption, because the amount of adsorbed salivary proteins was greatly reduced by the addition of Tween 80 in all bacterial strains.

We also examined the effect of phosphate, which has a high affinity for Ca^{2+} and affects the formation of pellicle^[32,34], on the adsorption. The presence of phosphate was found to decrease the amount of salivary proteins adsorbed to *S. mitis* and *S. salivarius*, but not to *S. sanguis*. Thus the same mechanism may operate in both bacterial adhesion to oral tissues and adsorption of salivary protein to bacterial cells in the case of *S. mitis* and *S. salivarius*, but not in the case of *S. sanguis*.

With regard to lectin binding as a specific binding^[28,34], it has been reported that the latter is inhibited by addition of sugars^[35], and is dependent on the presence of divalent cations such as Ca^{2+} ^[28,29]. Accordingly, the effects of sugars on the adsorption of salivary proteins to bacterial cells were examined in the presence of 1 mM CaCl_2 . The presence of sugars, such as lactose and N-acetyl-D-glucosamine inhibited the adsorption of salivary proteins to all the bacterial cells examined, and the presence of L-fucose, D-mannose and D-galactose inhibited the adsorption of salivary proteins to *S. sanguis* and *S. mitis*. These results suggest that lectinbinding is involved in the adsorption of salivary protein to bacterial cells, especially *S. sanguis* and *S. mitis*. When *S. mitis* and *S. salivarius* were used, however, the amounts of adsorbed salivary proteins were increased by the presence of N-acetyl-D-galactosamine. This result differed from the previous reports^[34-36]. We are unable to conclude how this sugar is related to the mechanism of adsorption of salivary proteins, and further studies will be needed to clarify this.

We then focused on the surface component of cells involved in the adsorption of salivary proteins to the bacteria. Experiments involving pretreatment of bacterial cells with enzymes were reported previously by Liljemark et al.^[37] We used a modification of these methods in this study. The results indicate that the surface proteins of *S. sanguis* and *S. salivarius* as well as the cell surface lipid of *S. mitis* play a very important role in the mechanism of adsorption of salivary protein to their cells.

Conclusion

There are several mechanisms involved in the adsorption of salivary proteins to the surfaces of oral bacteria, and doubtlessly there is some relationship between bacterial adhesion to oral tissues and the adsorption of salivary proteins to bacterial cells. These differences in adsorption mechanisms may be responsible for the differences in distribution of the bacteria within the oral cavity.

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