Agglutination of *Streptococcus mutans* by Low-Molecular-Weight Salivary Components: Effect of β₂-Microglobulin

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Radiolabeled monomeric human β₂-microglobulin (β₂m) was tested for binding to *Streptococcus mutans* strains in buffers containing 1 mM calcium (Ca²⁺). Binding was seen to strains with a previously established binding capacity of aggregated β₂m. Monomeric β₂m agglutinated β₂m-binding strains when Ca²⁺ was present. At Ca²⁺ concentrations of 1.4 mM, 0.032 μg of monomeric β₂m per ml caused bacterial agglutination. Parotid saliva was gel filtered on a Sephadex G-75 column, and low-molecular-weight fractions containing β₂m could agglutinate *S. mutans* cells. Five of six strains that could bind β₂m were agglutinated by these fractions, but only one of five nonbinding strains was. All strains tested were agglutinated by void volume fractions. A new method for the measurement of turbidity in bacterial agglutination inhibition experiments with parotid saliva was used. Suspensions containing parotid saliva, bacteria, and control serum were directly compared in a spectrophotometer with test suspensions containing goat anti-human β₂m, bacteria, and saliva. Thus, the spectrophotometer directly read the difference in agglutination of the two suspensions, and the result was presented as one curve by the recorder. Agglutination of five β₂m-binding strains of *S. mutans* was inhibited or decreased by the addition of goat anti-human β₂m as compared with control serum. The agglutination of two β₂m non-binding strains and one with variable binding was not inhibited. Thus, salivary β₂m may contribute to agglutination of *S. mutans* cells in parotid saliva.

Oral streptococci can be agglutinated by various salivary molecules including high-molecular-weight agglutins (13, 17–19, 27, 31), secretory immunoglobulin A (IgA) (20, 24, 33), blood group reactive substances (16, 34), and lysozyme (30). Generally, the agglutination phenomenon of bacteria in saliva has been considered to be a host defense system for the disposal of bacteria (32–34). However, the bacterial agglutinating factors can also serve as adherence-promoting factors in the pellicle (23–25), and the formation of small aggregates may increase the number of bacteria binding to saliva-coated hydroxyapatite (23). Even though bacterial binding of a particular molecule does not result in agglutination, the bacterial surface properties may be altered (26) and their colonization abilities may be changed.

It seems unlikely that one bacterial binding or agglutinating component is solely responsible for the clearance or attachment of a specific bacterium; several probably contribute to the resulting effect (15, 32). There have also been reports that indicate interaction between high-molecular-weight agglutins and secretory IgA, which could both have affinity for the same species (9, 27). This makes the functional picture more complex. Therefore, the importance of bacterial binding by a single isolated factor is not easy to demonstrate. Nevertheless, evaluation of the effect of single factors is necessary.

We have earlier demonstrated that aggregated β₂-microglobulin (β₂m) can interact with strains of oral streptococci (10, 12). β₂m is a low-molecular-weight protein occurring on surfaces of all nucleated cells as the light chain of the major histocompatibility antigens (for a review, see reference 3). β₂m is also present in saliva and other body fluids (2, 3, 11, 28). Previous studies have shown that aggregates, but not monomers, of β₂m bind to bacteria (12, 21). Aggregation of β₂m to high-molecular-weight complexes strongly increases the avidity of the interaction between bacteria and the β₂m complexes. The complexes then can bind to low-affinity receptors (10, 12). The need for a multipoint attachment has been considered significant in the binding of bacteria to cell surfaces (5), where β₂m seems to be present as repetitive units. In saliva, however, high-molecular-weight complexes containing β₂m have not been found, but β₂m is present as monomers (11). One aim of this study was to learn whether binding of β₂m monomers to *Streptococcus mutans* cells can be demonstrated. To do this, the influence of calcium ions (Ca²⁺) on the binding of the protein to bacteria was analyzed. Since some bacteria-binding salivary agglutins demand Ca²⁺ for interaction with bacteria (9, 17, 31) and others do not (22, 30, 33).

Another aim was to investigate whether β₂m monomers, the form present in saliva, could agglutinate strains of *S. mutans* and whether salivary fractions containing β₂m could agglutinate *S. mutans* cells. The effect of addition of anti-β₂m antibodies on the agglutination of *S. mutans* strains in parotid saliva was also studied.

**MATERIALS AND METHODS**

**Bacterial cultures and growth conditions.** Bacterial strains that were analyzed previously for binding aggregated β₂m were selected (10, 12). Binding of aggregated β₂m was scored as +, −, or (+) or (−) (see Table 1, footnote a) in the following strains: *S. mutans* AHT (+) and 3720 (+) (serotype a); BHT (−) (serotype b); P8 (−), KPSK 2 (+/−), 1449 (−) and NCTC 10449 (+) (serotype c); ME1 (+) (serotype d); LM7 (−) (serotype e); OMZ 175 (+) (serotype f); K1 (−); and OMZ65 (+) (serotype g). Bacterial cultures were maintained on blood agar plates and transferred to tubes containing 10 ml of dialyzed yeast extract medium (7). After incubation in anaerobic jars overnight in 95% N₂–5% CO₂, the bacteria were washed twice in phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl [pH 7.0]; PBS) and suspended to an optical density (OD) of 1.50 at 700 nm in a Beckman model 35 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

**Binding studies.** Binding of monomeric ¹²⁵I-labeled human β₂m to bacteria was tested as described earlier, with some exceptions (10, 12). Briefly, 200 μl of a suspension of 10⁹
bacteria per ml in KCl buffer was incubated with 25 µl (ca. 0.3 µg, giving a radioactivity of 10,000 cpm) of radiolabeled human β-m (these mixtures did not contain Tween 20 or human serum albumin [HSA] as in the earlier method). After incubation for 60 min, the suspension was washed with 2 ml of KCl buffer with 0.05% Tween and centrifuged. The radioactivity in the pellet was measured and compared with the radioactivity in 25 µl of labeled protein solution. In another experiment, 0.05% Tween 20 was added to the 25 µl of protein solution before incubation with the bacterial suspensions.

Saliva and gel filtration. Parotid saliva from two subjects was stimulated with citric acid to a flow rate of approximately 0.4 ml/min and collected in modified Lashley cups. Samples were used immediately for gel filtration. A 7-ml sample of saliva was fractionated on a Sephadex G-75 column (Pharmacia Fine Chemicals, Sweden) (2.5 by 42 cm) and eluted with 1 mM phosphate buffer (pH 6.95) (with 0.05 M KCl, 1 mM CaCl₂, and 0.1 mM MgCl₂; KCl buffer) containing 0.02% NaN₃. Fractions (4.9 ml each) were collected at an elution rate of 14.6 ml/h, and the absorbance at 280 nm was measured in the spectrophotometer. The elution positions of blue dextran 2000 (Pharmacia) and hen egg white lysozyme (HEWL) (E. Merck AG, Darmstadt, Federal Republic of Germany) were determined on the G-75 column.

IgA analysis. The concentration of IgA in every second fraction from the Sephadex G-75 column was measured by an immunobead enzyme-linked immunosorbent assay (6).

βₐm analysis. The concentration of βₐm in saliva fractions from the Sephadex G-75 column was determined by a radioimmunoassay, using 125I-labeled purified human βₐm and goat anti-human βₐm (GABₐm) (29). Preparation procedures and the properties of GABₐm are given extensively elsewhere (4). The elution position of βₐm on the Sephadex G-75 column was also determined by gel filtration of monomeric human 125I-labeled βₐm.

Amylase analysis. The presence of amylase in fractions was assayed by immunodiffusion in agarose gel (1% ME agarose [FMC Corp., Rockland, Me.] in 0.02 M barbital buffer [pH 8.6]) against rabbit anti-human secretory amylase (Nordic, London: batch 9-1177).

Agglutination assays. Fifty µl of gel filtration fractions was mixed with 25 µl of bacterial suspension in a microtiter plate (Dynatech M129A, Dynatech Deutschland, Federal Republic of Germany). Bacterial agglutination was determined after 90 min with a stereo microscope at 10× magnification by comparing βₐm-containing and βₐm-free suspensions. The degree of agglutination was rated + +, indicating heavy agglutination; +, indicating weak but definite agglutination; or −, indicating no agglutination.

Human βₐm was isolated from urine samples as previously described (2) and was aggregated with glutaraldehyde (1). Monomeric and aggregated βₐm were diluted in KCl buffer, and 50-µl samples were used for agglutination assays as described above, except that agglutination was determined after 2 to 3 h. Different volumes of 10 mM solutions of CaCl₂ in distilled water were added to incubation mixtures in some experiments to determine whether the divalent cation Ca²⁺ would influence agglutination. Lysozyme has a molecular weight somewhat higher than that of βₐm and has been reported to agglutinate oral streptococci (30). HEWL was therefore included as a control in agglutination assays.

Agglutination inhibition experiments. The five strains of S. mutans capable of binding aggregated βₐm were tested for agglutination by freshly collected parotid saliva mixed with GABₐm or preimmune control serum (CS) from the same animal. Two nonbinding strains and one with a variable binding were also tested. First, 10 µl of GABₐm or CS diluted 1:10 in PBS was added to 150 µl of parotid saliva diluted 1:2 in PBS, in Hellma Suprasil Quartz microcells (Hellma, Müllheim/Baden, Federal Republic of Germany) at 37°C. After 50 min, 250 µl of bacterial suspension was added. Agglutination was followed at 700 nm in a Beckman model 35 spectrophotometer with a four-position manual sample changer at 37°C. Positive controls contained 150 µl of parotid saliva diluted 1:2 in PBS and a 250-µl bacterial suspension. Negative controls contained PBS instead of saliva.

Cells containing positive control or CS were placed in reference positions, and cells containing negative control or GABₐm were placed in sample positions in the spectrophotometer (Fig. 1b). Agglutination in the positive control but not the negative control was read as an increase in absorbance. Similarly, when GABₐm-containing cells were read against CS-containing cells, a faster agglutination rate in the CS-containing cells was read as an increase of absorbance. If the agglutination rate was identical in the cells, no increase of absorbance was seen (Fig. 1c). For comparison, three of the strains were assayed according to Ericson et al. (13), using cells containing PBS in reference position (Fig. 1a, 2a, and 3a).

RESULTS

Binding experiments. Radiolabeled monomeric βₐm bound to three strains that had also bound aggregated βₐm and to one strain that had shown a variable affinity for aggregated βₐm (Table 1). The addition of 0.05% Tween 20 totally inhibited binding of all strains tested (Fig. 4).

Parotid saliva fractioned on a Sephadex G-75 column eluted as four peaks (Fig. 5). Salivary IgA eluted in the void volume and βₐm eluted in peak 3. The maximum concentration of βₐm was 4.7 µg/ml. In another control column run, radiolabeled human monomeric βₐm eluted at the same position. The molecular weight marker blue dextran 2000 eluted with void volume, and HEWL eluted in fractions 32 to 39. Amylase was detected in fractions 34 to 49 (peaks 2 and
3). All strains tested were agglutinated by fractions from peak 1. Of six strains that bound aggregated β₃m, five strains were agglutinated by fractions from peak 3, and of five negative strains, only S. mutans HBT was agglutinated in one of two assays (Table 1). Agglutination by void volume fractions was mostly seen after 30 min, but in low-molecular-weight fractions it was not seen until after 60 to 90 min. Preparations of aggregated human β₃m agglutinated all three strains that had bound aggregated human β₁m; bacteria were also agglutinated by aggregated β₃m in PBS free of Ca²⁺. For S. mutans ME1, showing the highest affinity for aggregated β₁m, the lowest agglutinating concentration was 0.02 μg/ml. Three nonbinding controls were also not agglutinated. Monomeric human β₁m agglutinated strains that bound the aggregated form of the protein. S. mutans ME1 was agglutinated at a concentration of 0.032 μg/ml.

HEWL agglutinated all six strains tested at concentrations from 39 to 200 μg/ml (Table 1). S. mutans HBT agglutinated at the lowest concentration. None of the bacteria tested were agglutinated by G-75 fractions at the elution position of HEWL.

The agglutination of eight S. mutans strains in parotid saliva with the addition of GAβ₂m or CS was followed spectrophotometrically. Figure 2a illustrates the agglutination of the β₁m-binding strain ME1. GAβ₂m totally inhibited agglutination (curve 2), and CS gave a minor inhibition (curve 3). Figure 2b shows the same events as in Fig. 2a, but the assay was performed by the method for Fig. 1b. In this assay, only the difference between the agglutinations in Fig. 2a was measured. The numbers (1 to 4) in Fig. 2a correspond to the same numbers in Fig. 2b. The curve 1 – 4 expresses the change of the absolute value of the difference in OD between curve 1 and 4 in Fig. 2a. Thus, the suspension that contained saliva and bacteria agglutinated faster than did the suspension with PBS and bacteria. Similarly, the curve 2 – 3 increases, showing that GAβ₂m inhibited agglutination as compared with CS.

The β₁m-nonbinding LM7 was tested (Fig. 3a and b). GAβ₂m did not inhibit agglutination as compared with CS (Fig. 3a). In Fig. 3b, the curve 2 – 3 does not increase, which shows that bacterial suspensions with parotid saliva and CS or GAβ₂m agglutinated almost identically. The curve 1 – 4 expresses the change of the absolute value of the difference in OD between curve 1 and 4 in Fig. 3a. Thus, the suspension that contained saliva and bacteria agglutinated faster than did the suspension with PBS and bacteria. Similarly, the curve 2 – 3 increases, showing that GAβ₂m inhibited agglutination as compared with CS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rate of binding of aggregated radiolabeled β₁m in previous studies*</th>
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<tr>
<td>Agglutination by peak</td>
<td>+</td>
</tr>
<tr>
<td>3 from Sephadex G-75</td>
<td>5/6</td>
</tr>
<tr>
<td>Uptake of radiolabeled monomeric β₁m</td>
<td>3/3</td>
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<td>Agglutination by aggregated β₁m</td>
<td>3/3</td>
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<tr>
<td>Agglutination by lysozyme</td>
<td>3/3</td>
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</tbody>
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* Expressed as number of reactive strains/total number of strains tested. +, Binding of aggregated β₁m; −, no binding of aggregated β₁m; +/−, variable binding of aggregated β₁m.

* Strain HBT.

FIG. 2. Agglutination of S. mutans ME1 (a β₁m-binding strain) in parotid saliva and buffer. (a) Curves: 1, bacteria and buffer (negative control); 2, bacteria, saliva, and GAβ₂m; 3, bacteria, saliva, and CS; 4, bacteria and saliva (positive control). (b) According to Fig. 1b, the increasing OD value shows that agglutination is taking place in cells in control position (as compared with in the cell in the sample position). Curves: 1 – 4, the sample-positioned cell contains ME1 and buffer, and the reference-positioned cell contains ME1 and saliva; 2 – 3, the sample-positioned cell contains bacteria, saliva, and GAβ₂m; the reference-positioned cell contains bacteria, saliva, and CS. The increase in the OD value indicates that GAβ₂m inhibits or decreases agglutination as compared with CS.

FIG. 3. Agglutination of S. mutans LM7 (a β₁m-nonbinding strain). Symbols are the same as in Fig. 2.
been ascribed to interaction between the Sephadex gel and amylase (14), and might explain the lower absorption at 280 nm by higher-molecular-weight fractions.

Among the Sephadex G-75 fractions, all bacteria were agglutinated by void volume fractions. The lower-molecular-weight fractions not containing IgA or high-molecular-weight agglutinins, but containing βm, could agglutinate five of six strains that readily bound βm. The low concentration of βm in the fractions may explain the failure of one strain to agglutinate. The slower agglutination seen in the lower-molecular-weight fractions indicates that agglutinating factors are either not as strong or not as concentrated as those seen in the void volume.

Strain BHT was the only βm-nonbinding strain that was variably agglutinated by fractions containing βm. This strain has been reported to show a high affinity for lysozyme (30). However, since HEWL eluted in fractions just before peak 2 on the G-75 column, and these fractions never agglutinated bacteria, lysozyme here is probably not responsible for the agglutination of strain BHT. Also, binding and agglutination by βm did not correlate with agglutination by lysozyme for the strains tested.

The method presented in this paper had some advantages as compared with previously described methods (13) for turbidimetric measurements of bacterial agglutination inhibition experiments. In measuring the effect of added antisera against a specific salivary molecule, a CS often also interferes with agglutination. This effect is often not interesting to study per se, but it has to be subtracted from the phenomenon one wishes to study. In measuring only the differences between agglutination in saliva treated with antisera and with CS, the effect of CS was subtracted in the spectrophotometer. Thus, small differences in agglutination could be detected, and specific inhibition by the antisera was directly revealed. Also, as the agglutinations in control and test cuvettes were measured against each other, and not against a buffer control, only half the number of cuvettes was used as compared with the method described for Fig. 1a, 2a, and 3a. The results obtained with this method correlate with those obtained by other methods (13) (Fig. 2a and b, Fig. 3a and b).

For the highest affinity strain, S. mutans ME1, agglutination was inhibited almost completely when Gβm was added (Fig. 2a and b). Agglutination of other βm-binding strains tested was not so strongly affected by Gβm. Since agglutination of bacteria can be caused by several factors in saliva, one would expect that interfering with one factor with antibodies would not totally inhibit agglutination. However, this was seen with strain ME1. As ME1 showed the highest affinity of the strains tested, it might have been possible that complexes between antibody and βm were absorbed onto the bacteria without causing agglutination. Receptors for other bacterial agglutinating factors may then have been blocked sterically.

The fact that several factors in saliva interact with the indigenous flora is perhaps an important feature of the bacteria. Maybe it is important for the indigenous flora to have several ways to interact with host substances, instead of one strong interaction that might be more vulnerable. Microheterogeneities among salivary molecules and thus many interaction sites have been suggested to provide "zip codes" for disposal or attachment of microorganisms (32). A favorable combination of surface receptors on the bacteria would be the key to attachment. It is therefore important to clarify all types of interactions between bacteria and salivary factors to be able to understand better the oral

**FIG. 5.** Elution profile of 7 ml of parotid saliva separated on a Sephadex G-75 column. Symbols: ---, absorbance at 280 mm; ----, radioactivity corresponding to 125I-labeled human monomeric βm; ····, IgA concentrations as measured by immunobead assay.

In increases, which shows that the strain LM7 was agglutinated by parotid saliva but not by PBS.

The agglutination of the five strains tested that could bind aggregated βm was inhibited by Gβm. ME1 was inhibited the most. The nonbinding and the variably binding strains were not affected differently by Gβm or CS.

**DISCUSSION**

It was demonstrated that βm monomers also can bind to S. mutans strains, and not only in the aggregated form as earlier assumed (10, 12, 21). As with some other salivary molecules (9, 17, 31), binding of βm to bacteria seemed to require the presence of Ca++. Previous βm-binding studies were performed with Ca++-free buffers containing Tween 20 or HSA. When a KCl buffer with 1 mM Ca++ and without Tween 20 or HSA was used, monomeric βm was bound to the four strains that also bound aggregated βm, but not to the negative strains. HSA or Tween is often used to prevent binding to reaction vessels and might interfere with low-affinity interactions. Even though a higher adsorption of βm to tubes and negative control bacteria was found in this study, there was a clear difference between controls and βm-binding strains. The presence of Tween or HSA might mask the presence of low-affinity receptors, but it does not seem to interfere with the higher avidity reactions between βm aggregates and bacteria (10, 12, 21).

Purified preparations of human monomeric and aggregated βm agglutinated the strains that bound the protein but not the negative strains. As little as 0.032 μg of monomeric βm per ml induced agglutination. However, in gel-filtered saliva fractions causing agglutination, the maximum concentration of βm was 0.0047 μg/ml. Purification procedures and storage might have decreased the affinity of the purified βm to bacteria as compared with freshly tested salivary βm. The concentration of βm in unfractioned saliva is 0.2 to 0.9 μg/ml (11), which is several times the concentration needed for agglutination.

Amylase, having a molecular weight of ca. 5.5 × 10⁶, eluted in fractions with lower molecular weights. This has
ecology. $\beta$-m interacts with hydroxypapitate (8) and with some strains of oral bacteria (10, 12). $\beta$-m also participates in the saliva-induced agglutination of these strains. Even though S. mutans can agglutinate in salivary fractions without $\beta$-m, binding of $\beta$-m seems to be of significance for agglutination of these strains.

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LITERATURE CITED