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Bacterial Aggregating Activity in Human Saliva: Simultaneous Determination of Free and Bound Cells

E. E. GOLUB,* M. THALER, C. DAVIS, AND D. MALAMUD

Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Two new assays for saliva-mediated aggregation of oral bacteria have been developed, based on the use of [^{3}H]thymidine-labeled cells. One assay separates free cells from aggregated cells by centrifugation through sucrose, whereas the other utilizes membrane filters (8 μm, Nuclepore) to effect the separation. Comparison of these assays with the turbidity method reveals that they are faster (×20 to 40) and require 10 times less saliva and bacteria. The aggregation of *Streptococcus sanguis* M5, as determined with these assays, is complete in 5 min and is dose dependent on added cells and saliva. The reaction exhibits a temperature optimum of 42°C with no reaction at 0°C. If the pH is reduced to below 5, saliva-dependent aggregation is inhibited. The salivary factor(s) are heat labile, losing 100% of their activity after 100°C, 10 min or 70°C, 30 min.

Study of bacterial aggregation mediated by saliva has been prompted by the hypothesis that adsorption of bacteria to the tooth surface is an important process in the etiology of caries and periodontal disease. Specific salivary proteins have been shown to adsorb onto the surface of enamel to form the acquired pellicle (8). The specificity of bacterial adherence to the pellicle determines the observed specificity of bacterial colonization of the tooth surface (11). Saliva is also a primary defense mechanism against bacterial infection of the oral cavity (22) acting to (i) clear bacteria from the mouth by aggregation and flushing, (ii) directly attack bacteria, (iii) buffer and counteract bacterial acid production in plaque, and (iv) prevent demineralization of enamel and promote remineralization.

The role of saliva or adsorbed salivary components in modulating bacterial adherence to solid surfaces has been extensively investigated (6, 10, 14, 16, 20, 21, 24, 25). Quantitation of this phenomenon has been achieved through application of the Langmuir adsorption isotherm as a model for the binding of the cells to hydroxyapatite (7, 9). This analysis has been utilized to study the number of bacterial binding sites, the affinity of these sites for different bacterial strains, and the nature of interstrain interactions (1, 2). In addition, this procedure has been used to determine the role of extracellular polysaccharides and bacterial glucosyltransferase in the adherence of oral bacteria to hydroxyapatite (3, 4), to hydroxyapatite-coated glass beads (26), and to glass (12, 18). Three assay procedures have been reported for the study of the action of saliva on the aggregation of oral streptococci. Kashket and Donaldson (15) utilized a Coulter Counter assay to measure primary core aggregates of *Streptococcus sanguis* and *Streptococcus mitis*. Ericson and co-workers devised a turbidimetric approach which relies on the increased sedimentation of the aggregates leading to clearing of bacterial suspension (6). This assay has a "lag" period during which the clumped bacteria are settling out (5). Visual scoring of aggregation has also been used, both at the microscopic level (10, 13) and at the macroscopic level, by determination of the maximum dilution which will not cause visible clumping of the bacteria (17, 19, 23). This assay is technically simple but only semiquantitative.

In the present study we describe a sensitive, quantitative, and rapid assay for the aggregation of [^{3}H]thymidine-labeled oral bacteria. This assay has been used to characterize an *S. sanguis* aggregating activity in mixed human saliva.

MATERIALS AND METHODS

Saliva. Whole paraffin-stimulated saliva was collected into iced tubes and clarified by centrifugation at 20,000 × *g* for 20 min. Protein concentration was determined by the Lowry method with bovine serum albumin as a standard. The clear supernatant could be used fresh, frozen (−20°C), or lyophilized and resuspended in distilled water. Saliva stored at 4°C lost 25 to 50% of its aggregating activity over an 8-day period. Aggregating activity was stable in frozen or lyophilized saliva for at least 6 months.

Bacteria. *S. sanguis* M5 (obtained from B. Rosan) was grown in brain heart infusion (Difco Laboratories) at 37°C. The cells were labeled with [^{3}H]thymidine

(0.5 μCi/ml, New England Nuclear Corp., 6.7 Ci/mmol) for 16 h. Labeled bacteria were washed three times in phosphate-buffered saline (PBS; 0.01 M KPO_4, 0.15 M NaCl, pH 7) and frozen in aliquots containing 200,000 cpm per 10^{10} cells. Since only about 2% of the label was incorporated into bacterial deoxyribonucleic acid, spent medium was treated with Norit activated charcoal (0.7 g/100 ml) and filtered to facilitate isotope disposal.

Aggregation assays. (i) Turbidity. The assay contained 1 ml of bacteria ($\simeq 10^{10}$ cells), 1 ml of PBS, and 1 ml of clarified saliva in a tube (13 by 100 mm) or a cuvette. The samples were blended gently in a Vortex mixer at zero time and thereafter kept stationary at 37°C. Optical density at 675 nm was monitored in a spectrophotometer against a blank containing 1 ml of bacteria and 2 ml of PBS.

(ii) Centrifugation. The reaction mixture contained 0.1 ml of labeled bacteria (≃20,000 cpm) suspended in PBS and 0.1 ml of clarified saliva. Tubes were incubated for 1 to 10 min at 37°C in a gyratory shaker (350 rpm). The reaction was terminated by adding 0.2 ml of 2.5% phosphate-buffered glutaraldehyde (0.01 M KPO_4, pH 7.2) and shaking for an additional 5 min. The entire reaction mixture was then layered over 1 ml of 25% sucrose in a glass tube (10 by 75 mm) with a Pasteur pipette. Tubes were centrifuged at 1,100 × *g* for 5 min. This produced a pellet of aggregated bacteria and a supernatant of free bacteria. The supernatant was counted directly in 4 ml of TX8 scintillation fluid (1 volume of Triton X-100, 2 volumes of xylene, 8 g of Omnifluor per liter; New England Nuclear Corp.). The pellet was resuspended in 1.4 ml of PBS and counted in 4 ml of TX8. The counting efficiency was 15% for both fractions. All assays were carried out in triplicate, and the results were expressed as percent aggregation after subtraction of a blank value obtained by incubation of bacteria with PBS and then treated with glutaraldehyde. The average blank aggregation was 10%.

(iii) Filtration. Samples were handled as described above for the centrifugation assay. After termination of the reaction with glutaraldehyde, aggregated and free bacteria were separated by filtration through 8-μm filters (Nuclepore Corp.) on a manifold (Millipore Corp.). Once again, both aggregated (filter) and free (filtrate) bacteria were analyzed and the data are presented as percent aggregation after subtraction of the blank value. All results reported here are the mean of triplicate determinations. The precision of these determinations was found to vary between 5 and 10%.

RESULTS

Our initial studies employed a turbidimetric assay of bacterial aggregation similar to the method of Ericson et al. (6). As shown in Fig. 1, the decrease in optical density and the duration of the lag period were related to the amount of added saliva. This reaction did not seem applicable to the assay of large numbers of samples. We felt that a more rapid assay of aggregation was necessary to study the initial reaction between bacteria and the salivary factor.

Development of new assays for bacterial aggregation. The analysis of bacterial aggregation can be resolved into two technical problems, (i) separation of free bacteria from aggregated bacteria, and (ii) detection and quantitation of the free and aggregated bacteria. The latter problem was solved by using radioactively labeled bacteria. In our assays we labeled bacteria with [^{3}H]thymidine (1, 4). Our initial efforts were therefore directed toward the development

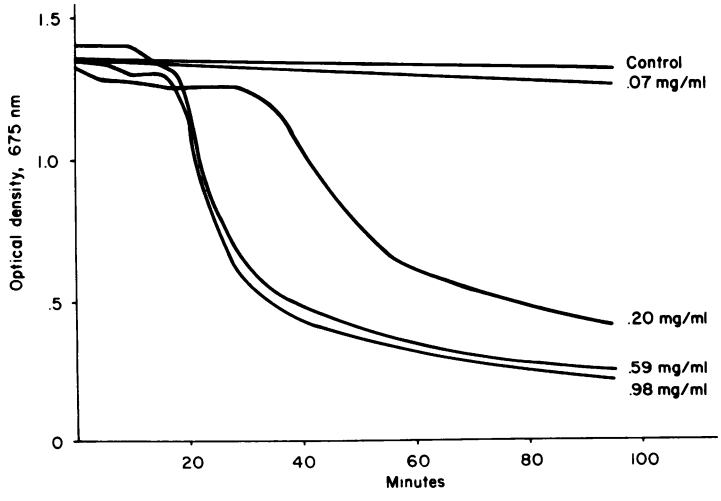

FIG. 1. *Analysis of bacterial aggregation by turbidity: effect of time and protein concentrations. Aliquots of S. sanguis (1.5 ml) were added to whole clarified saliva diluted with PBS to give the final protein concentration as indicated. The control contained bacteria plus PBS. Tubes were incubated at 37°C without shaking, and the optical density at 675 nm was read at 15-min intervals.*

and optimization of the separation techniques. Microscopic examination of *S. sanguis* after incubation with saliva revealed that the aggregates had a diameter of 10 to 35 μm. It therefore seemed likely that the passage of a mixture of free and aggregated bacteria through a Nuclepore membrane filter would result in quantitative retention of the aggregates with passage of the free cells through the filter. Trials with membrane filters of pore size 1 to 10 μm showed that an 8-μm pore size provided rapid filtration with retention of bacterial aggregates.

Centrifugation proved to be an alternative approach to the separation problem. Empirical trials led to the procedure of layering the reaction mixture onto 25% sucrose and centrifuging at 1,100 × *g* for 5 min. Some problems of reproducibility were initially encountered with this assay, resulting from disaggregation of the bacteria during the separation process. This problem was overcome by fixing the aggregated bacteria with 2.5% buffered glutaraldehyde (final concentration 1.25%), which has the dual advantage of rapidly terminating the reaction as well as stabilizing the aggregates. This procedure was used in both the filtration and the centrifugation assays. Addition of glutaraldehyde at zero time (i.e., at the time of saliva addition) resulted in total inhibition of the aggregation reaction, indicating that the factor(s) were glutaraldehyde sensitive. The low blank values demonstrated that fixation did not cause aggregation in the absence of saliva. The aggregation reaction was linear with time for the first two min and was complete within 5 min, well within the lag period for the turbidimetric assay (Fig. 1). To study the extent of the reaction, assays were routinely carried out for 5 min.

Dose dependency of the saliva-mediated aggregation reaction. Investigation of the effect of salivary protein concentration on aggregation showed that both the rate (1-min values) and the extent (5-min values) of the reaction were linearly dependent on the amount of added saliva over a wide range of protein concentrations. Figure 2 shows the effect of saliva concentration on the extent of aggregation (5 min) as measured both by the centrifugation and filtration assays. These data show the linearity of both assays and the agreement between them. When the same saliva sample and suspension of *S. sanguis* M5 were assayed by turbidity, a similar curve was generated (data not shown). The turbidity assays, however, required ten times as much of each reactant, and took 20 to 40 times as long to complete.

We also determined the effect of bacterial concentration on the aggregation reaction. A

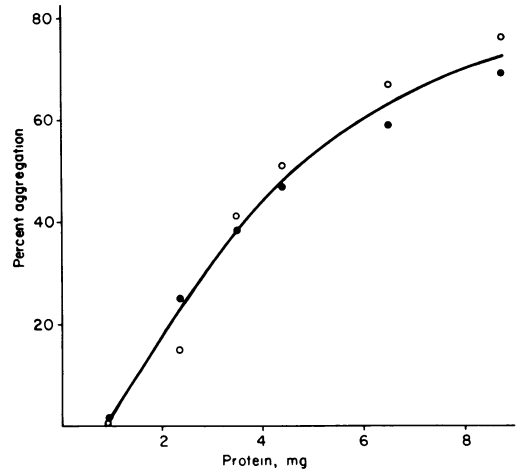

FIG. 2. *Analysis of bacterial aggregation by centrifugation or filtration. Aliquots of S. sanguis (0.1 ml) were incubated with dilutions of whole saliva in a final volume of 0.2 ml. Samples were incubated for 5 min at 37°C in a gyratory shaker. The reaction was terminated with 0.2 ml of 2.5% glutaraldehyde, and samples were assayed by the centrifugation (●) or filtration (○) technique. Values shown are the mean of triplicate percent aggregation values after subtraction of a blank obtained by incubating without saliva.*

stock suspension of *S. sanguis* M5 was diluted with increasing amounts of PBS. The cell concentration in the undiluted suspension was determined by microscopic counting, and the dilution of the cells was monitored by measurement of the turbidity. Figure 3 shows a hyperbolic curve typical of a saturable process. Analysis of these data according to the Langmuir adsorption isotherm revealed an apparent dissociation constant of 5×10^8 cells per ml. If each cell was considered as a molecule, this would correspond to a molar dissociation constant of approximately 10^{-12} M.

Effect of temperature on the aggregation reaction. The effect of incubation temperature on the aggregation reaction is shown in Fig. 4. The reaction showed a temperature optimum near 42°C with no reaction at 0°C. Since this was quite different from the results of Kashket and Donaldson (15) who showed a temperature optimum of 10°C for the saliva-mediated aggregation of *S. sanguis*, we repeated the study with a different analytical method. Similar results were obtained with the turbidimetric assay (Fig. 5). Here, both the rate of the reaction and the length of the lag period were seen to be affected by the incubation temperature. Further investigation with the turbidimetric assay revealed that shifting from 37 to 0°C stopped the reaction

within 15 min. Shifting from 0 to 37°C immediately initiated the aggregation process.

The existence of an optimum at 42°C suggested that one or more components of the aggregation reaction might be temperature sensitive. Preincubation of saliva for 10 to 30 min at temperatures from 30 to 100°C, followed by assay at 37°C, revealed a progressive decrease in aggregating activity in samples heated in excess of 50°C, with a total inhibition after heating at 100°C for 10 min or 70°C for 30 min. In a similar experiment, bacteria were heated at 30°C to 100°C for 15 min and subsequently assayed at 37°C. There was a total loss of activity between 50°C and 60°C (data not shown). Taken together, these data show that the decreased aggregation at incubation temperatures in excess of 42°C resulted from the heat lability of both the salivary factor and the bacterial surface receptor.

pH dependence of aggregating activity. The aggregation assay was tested for pH dependence by titrating saliva with HCl. The results of this experiment are shown in Fig. 6A,

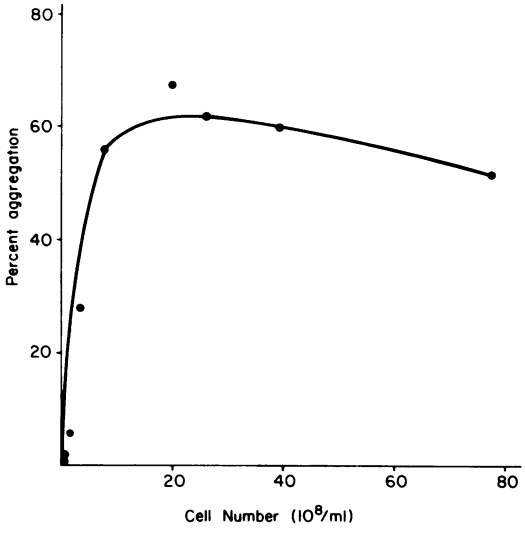

FIG. 3. *Effect of bacterial concentration on aggregation. A suspension of S. sanguis M5 containing 8 × 10^{10} cells per ml was diluted with PBS to give the indicated number of cells per 0.1-ml aliquot. Each bacterial concentration was incubated with 0.1 ml of clarified saliva for 5 min at 37°C. Samples were assayed for percent aggregation with the centrifugation assay. Each point represents the mean for triplicate determinations.*

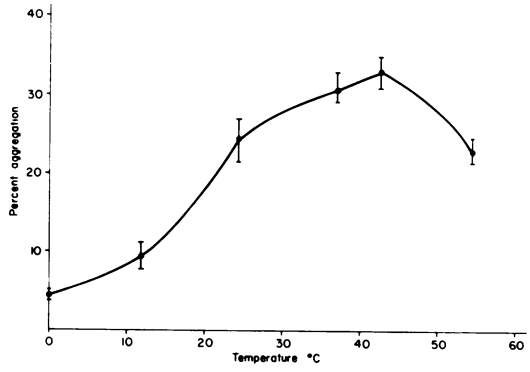

FIG. 4. *Effect of incubation temperature on bacterial aggregation. Samples containing S. sanguis M5 (0.1 ml) and whole saliva (0.1 ml) were incubated at the indicated temperature for a 5-min reaction. Samples were assayed by the centrifugation assay. Each point is the mean ± standard error for triplicate determinations.*

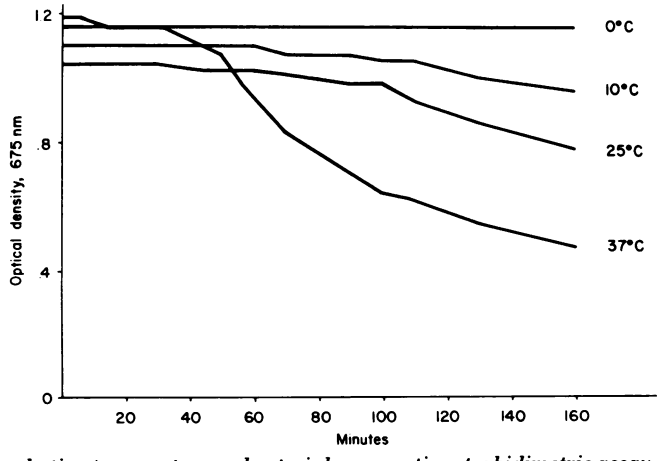

FIG. 5. *Effect of incubation temperature on bacterial aggregation: turbidimetric assay. Each tube contained 1 ml of S. sanguis, 1 ml of clarified saliva, and 1 ml of PBS. Tubes were incubated at the indicated temperatures for the entire reaction. Optical density at 675 nm was read at 15-min intervals.*

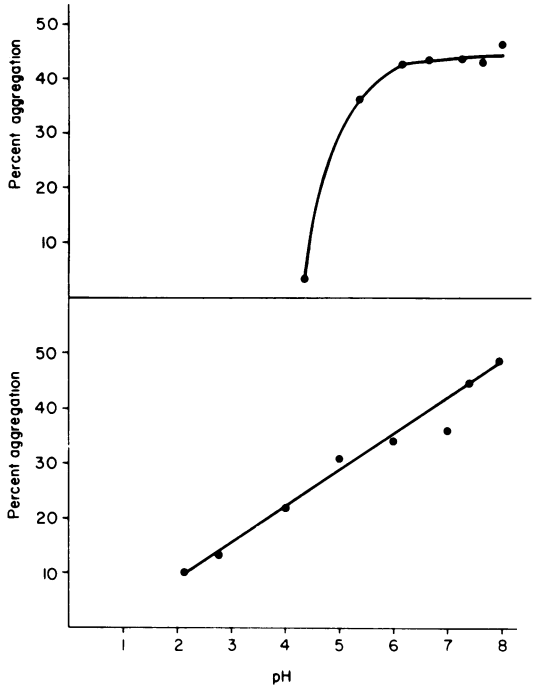

FIG. 6. *Effect of pH on bacterial aggregation. (A) Incubation. Mixtures of S. sanguis M5 (0.1 ml) and clarified saliva (0.1 ml) were adjusted to the indicated pH with HCl. The assay was carried out at 37°C for 5 min on a gyratory shaker. Triplicate samples were evaluated by the centrifugation assay. (B) Preincubation. Saliva samples were adjusted to the indicated pH values with HCl and allowed to stand at 0°C for 30 min. The pH was then adjusted to 7 with NaOH, and aggregation of S. sanguis was monitored by the centrifugation assay.*

the values on the abscissa indicating the pH of the reaction mixture at the end of the assay. When the pH was held above 5, there was little effect on the aggregation activity, whereas lowering of the pH below 5 resulted in a rapid decrease in activity. This inhibition could be (i) the result of denaturation of the salivary binding factor, (ii) an effect on the bacteria, or (iii) an effect on the assay. When saliva was titrated to low pH, then returned to pH 7 and assayed for aggregation activity, a progressive loss of activity was noted (Fig. 6B). Saliva exposed to pH 2 and then returned to pH 7 lost approximately 80% of its activity. These data suggest that the aggregating factor is acid labile.

DISCUSSION

Our study of saliva-dependent bacterial aggregation has been facilitated by the development of new assays to monitor the aggregation process. These assays are rapid, precise, and convenient. Each of the assays provides certain specific advantages. The turbidimetric assay is a progressive assay that permits alterations during the course of the reaction (e.g., temperature shifts or drug additions). However, the assay is cumbersome and monitors a late event in the aggregation sequence, whereas the centrifugation assay, for example, permits up to 100 assays in less than 2 h, with 5 to 10% variability among triplicates. The centrifugation and filtration assays gave similar results (Fig. 2), and the choice between them will be dictated by other factors in the experimental design (e.g., number of samples, available equipment, etc.).

One strength of the new assays is that both free and aggregated cells are determined simultaneously. This procedure makes the assays independent of cell specific activity and cell number and minimizes some experimental errors. Moreover, the recovery of labeled cells is determined for each analysis. The result of these advantages is seen in the high degree of precision and reliability achieved with these assays.

Glutaraldehyde has been used to rapidly terminate the aggregation reaction. Addition at zero time inhibits the process, indicating that the salivary factor is glutaraldehyde sensitive. Blank values are identical with or without addition of fixative, demonstrating that bacteria alone are not agglutinated by the fixative. Saliva-induced aggregates are stabilized by glutaraldehyde treatment to facilitate separation of free from bound cells. In experiments where it is desired to recover the free or aggregated bacteria or both for subsequent biological studies, the glutaraldehyde termination step may be eliminated with some loss in sensitivity.

The data in this paper were obtained exclusively with *S. sanguis* M5 since it gave high aggregation results in initial studies. Subsequent experiments indicate that other viridans streptococci (*Streptococcus mitis* and *Streptococcus salivarius*) are also aggregated by saliva, but to different extents. In preliminary experiments we have found that β-hemolytic streptococci are aggregated, but *Streptococcus faecalis* is not. We have not yet determined whether a single factor is responsible for the aggregation of all species.

The temperature dependence of the aggregation assay is similar to that of an enzymic reaction with an optimum near 40°C and no reaction at 0°C. The loss of activity at higher temperature could be easily explained by the heat lability of both the saliva and the bacteria.

The temperature dependence is strikingly different from that observed by Kashket and Donaldson (15) who showed a temperature optimum

of 10°C for bacterial aggregation with a sharp drop-off at higher temperatures. Clark et al. (2) also showed that adsorption of *S. mutans* to hydroxyapatite was the same at 37°C and at room temperature. A most surprising finding in this study is the lack of aggregation at low temperature. This behavior is usually associated with an enzymic reaction. Passive binding mechanisms such as antigen-antibody reactions usually continue at low temperature. Nonenzymic chemical reactions continue at low temperature, albeit at a lower rate. Whether an enzyme-catalyzed reaction is part of the mechanism of this process remains to be determined. In our hands most of the activity is heat labile and would be lost from saliva which had been heat treated to inactivate lytic enzymes (10, 13). The concentration dependence of activity shows saturation and fits the Langmuir isotherm. This behavior suggests that saliva contains a limited number of bacterial binding sites, and this conclusion would be consistent with either an enzymic or passive binding mechanism for aggregation. The affinity of the factor for *S. sanguis* M5 is high ($K_d \simeq 5 \times 10^8$ cells per ml). When this value is compared with the level of streptococci in saliva (10^6 to 10^8 cells per ml), it appears that aggregation mediated by this factor could occur in vivo.

The investigation of bacterial aggregation and adhesion phenomena in the pathobiology of oral disease is central to gaining an understanding of these disease processes. The assays described in this report will facilitate the gathering of such information. Moreover, they have already led to the partial characterization of a human salivary factor which mediates the aggregation of *S. sanguis* and which has properties different from previously reported aggregating factors.

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