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Inhibition of Bacterial Aggregation by Serum- and Blood-Derived Proteins

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Human and animal sera contain potent inhibitors of saliva-mediated aggregation of oral streptococci. The inhibitors consist of a high-molecular-weight heat-labile factor and a lower-molecular-weight heat-activated factor. The latter appears to be serum albumin. Analyses of purified blood-derived proteins indicated that several high-molecular-weight proteins (fibrinogen, fibronectin, and ferritin) were able to inhibit aggregation at low concentrations. These data suggest that high-molecular-weight proteins may modulate the aggregation process.

Saliva-mediated bacterial aggregation involves a sequence of steps initiated by the binding of agglutinins to the cell surface and followed by a temperature-dependent agglutination of bacteria (9). Aggregation demonstrates bacterial species and strain specificity and calcium dependence (7–9). The salivary agglutinins appear to be high-molecular-weight glycoproteins secreted from both parotid and submandibular glands (4). To investigate the molecular properties of the aggregation process, we have been studying a variety of inhibitory molecules, most of which appear to interact with the salivary agglutinins rather than with the bacteria. For example, streptococcal aggregation mediated by salivary-derived agglutinins can be inhibited by extracts from human polymorphonuclear leukocytes and platelets (8a). In the present report, we demonstrate that serum contains at least two inhibitory activities—one is heat labile and has a high molecular weight (>160,000), whereas the other is heat activated and appears similar or identical to serum albumin. The inhibitory activities in serum are compared with a number of known serum and blood cell-derived proteins. Of these proteins, fibrinogen is the most potent inhibitor of saliva-mediated aggregation.

MATERIALS AND METHODS

Stimulated human parotid saliva was collected with a modified Carlson-Crittenden cup placed over the opening of the parotid duct. *Streptococcus sanguis* M5 and *Streptococcus mutans* KPSK2 were grown in brain heart infusion (Difco Laboratories), washed in phosphate-buffered saline (PBS; 0.01 M K_2HPO_4 + KH_2PO_4, 0.15 M NaCl [pH 7]), and frozen in portions containing 10^{10} bacteria per ml. Human fibronectin was obtained from Collaborative Research. Histones and serum proteins were obtained from Sigma Chemical Co.

Aggregation assay. Saliva-mediated bacterial aggregation can be monitored either by the centrifugation assay with ^3H-labeled bacteria (6) or by following the decrease in turbidity with unlabeled bacteria (2). We have previously reported that the turbidimetric assay is more sensitive for analysis of inhibitory molecules (8a). For this assay, cuvettes contained 0.1 to 0.2 ml of parotid saliva plus 0.2 ml of unlabeled bacteria with PBS or inhibitors added to a total volume of 1.0 ml. Samples were mixed and incubated at 37°C, and the decrease in optical density at 675 nm was monitored for several hours. The percent aggregation was determined from the linear portion of the dose-response curve when 30 to 50% of the bacteria in the saliva controls were aggregated. Results are expressed as percent of the total bacteria aggregated after subtraction of a blank containing bacteria and PBS only.

Serum was fractionated on Affi-Gel blue (Bio-Rad Laboratories) with a small column (bed volume ≈ 1 ml) prewashed with 0.02 M phosphate buffer (pH 7). Adsorbed proteins were eluted with 1.4 M NaCl. Gel exclusion chromatography was carried out on a column (1.5 by 54 cm) of Sephacryl 200 SF (Pharmacia Fine Chemicals) with PBS as eluent. Protein was monitored at an optical density of 220 nm or by the Lowry method with bovine serum albumin (BSA) as a standard. Serum proteins were also separated by isoelectric focusing in 4% polyacrylamide gels containing 2% Ampholines (3). Gels were focused to equilibrium, sectioned, and dialyzed overnight against PBS. After dialysis, the eluted fractions were tested for aggregation inhibitory activity. Replicate gels were used for determination of the pH gradient and for protein staining with Coomassie blue.

RESULTS

Human serum was tested as an inhibitor of saliva-mediated bacterial aggregation by the turbidimetric assay. In a standard 1-ml assay, serum inhibited the aggregation of both *S. sanguis* M5 (50% inhibition [I_{50}] = 0.31 μl) and *S. mutans* KPSK2 (I_{50} = 3.8 μl) in a dose-dependent reaction (Fig. 1). Owing to the increased sensitivity of *S. sanguis*, this species was used for the remaining studies. As shown in Table 1, sera obtained from humans, rabbits, chickens, horses, and mice all inhibited bacterial aggregation mediated by human salivary agglutinins. Serum alone had no effect on bacterial aggregation in the absence of agglutinins. The serum inhibitory activity was stable after storage at 4°C, freezing, or overnight dialysis against PBS.

To determine whether inhibitory activity was heat labile, serum samples were heated for 30 min at 37 to 100°C and then tested for the ability to inhibit saliva-mediated bacterial aggregation. Preheating of serum above 80°C increased the inhibitory activity. A dose-response curve of heated (85°C) versus unheated serum indicated that the I_{50} was decreased from 0.3 to 0.08 μl; i.e., heated serum was approximately four times more potent as an aggregation inhibitor (Fig. 2). Heat activation was seen within 5 min at 85°C and reached a maximum value after 15 min (Fig. 3).

Serum (0.8 ml) was dialyzed against 0.02 M phosphate

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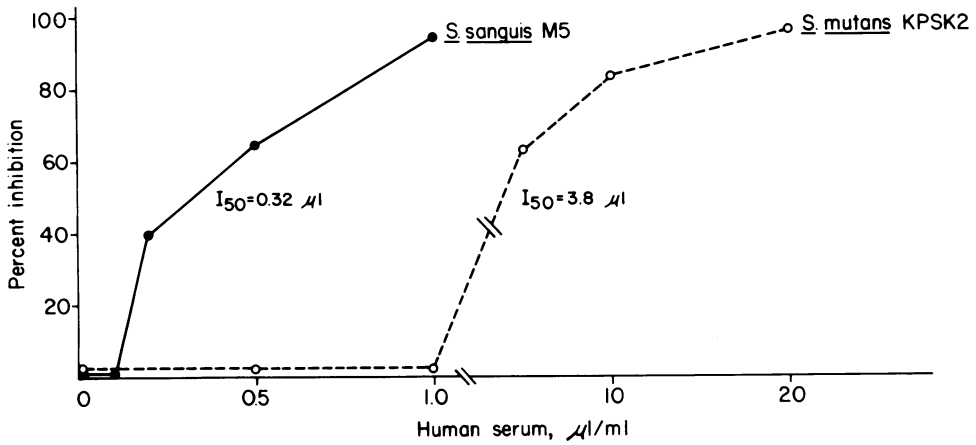

FIG. 1. Inhibition of saliva-mediated aggregation of *S. sanguis* M5 and *S. mutans* KPSK2 by human serum. Portions of *S. sanguis* M5 (●) or *S. mutans* KPSK2 (○) were incubated with parotid saliva and increasing concentrations of human serum in a total volume of 1.0 ml. Results are expressed as percent inhibition relative to a control sample with bacteria and saliva only. The I_{50} concentration of serum is indicated.

buffer and then fractionated on an Affi-Gel blue column. Unadsorbed proteins were eluted with 2 ml of phosphate buffer. The adsorbed proteins were eluted with 1.4 M NaCl. Aggregation inhibition activity was obtained in both the unadsorbed and adsorbed fractions but only the adsorbed activity demonstrated heat activation (data not shown).

Serum proteins were separated on polyacrylamide gels by isoelectric focusing. After focusing overnight, gels were sectioned into 0.5-mm pieces and dialyzed against PBS for 24 h to elute protein from the gels and remove Ampholines. Fractions were tested for inhibition of saliva-mediated aggregation. As shown in Fig. 4, aggregation inhibitory activity was found between pIs of 6.0 to 7.2 and at the top of the gel surface.

To resolve the aggregation inhibitory activity by molecular size, serum was loaded onto a Sephacryl 200 SF column and eluted with PBS. Protein was monitored by following the optical density at 220 nm, and fractions were tested for inhibitory activity. As shown in Fig. 5, most of the inhibitory activity had molecular weights of 160,000 to 500,000. Samples from three regions (A, B, and C) were tested for inhibitory activity before and after heating at 85°C for 30 min. Fraction A was heat labile, fraction B was unaffected by heating, and only fraction C demonstrated heat activation of inhibitory activity.

Since results with Affi-Gel and Sephacryl fractionation suggested that the heat-activated inhibitor in serum might be albumin, we tested Cohn fraction V and purified bovine albumin (Sigma). Both of these preparations inhibited bacterial aggregation, and inhibitory activity was increased 8- to 10-fold by heating at 85°C. Analysis of albumin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that after heating to 85°C, there was a decrease in the 56,000 component and the generation of a series of higher-molecular-weight bands (data not shown).

To gain insight into the nature of the inhibitory molecules, we began to study purified compounds as inhibitors of bacterial aggregation. Initial studies were directed at highly charged molecules, since Ginsburg and Quie (5) have implicated charged molecules as modulators of inflammation. A series of acidic compounds including heparin and DNA were found not to influence saliva-mediated bacterial aggregation. A survey of the histones, however, revealed that some were inhibitors, whereas others actually induced bacterial aggregation in the absence of salivary agglutinins. As shown in Table 2, there appears to be a correlation of stimulatory or

TABLE 1. Inhibition of bacterial aggregation by human and animal sera[a]

Serum (µl/ml)	% Inhibition
Human (0.2)	61
Human (1.0)	100
Rabbit (0.2)	47
Rabbit (1.0)	100
Chicken (0.2)	53
Chicken (1.0)	100
Horse (0.2)	43
Horse (1.0)	100
Mouse (0.2)	11
Mouse (1.0)	51

[a] Each serum sample was tested at 0.2 and 1.0 µl in a 1-ml reaction volume as inhibitors of saliva-mediated aggregation of *S. sanguis* M5 by the turbidimetric assay. Results are expressed as percent inhibition compared with controls with saliva and bacteria only for triplicate determinations.

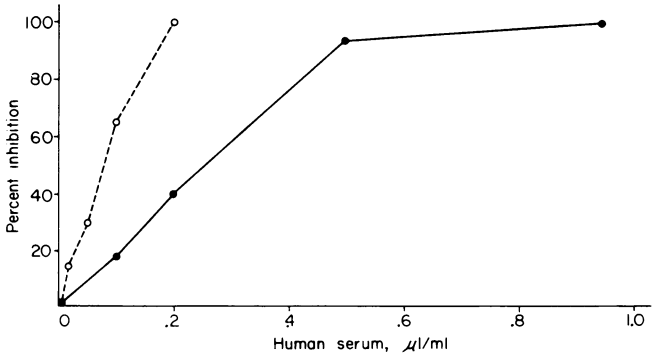

FIG. 2. Inhibition of bacterial aggregation by heated versus unheated serum. A titer of serum (●) or serum preheated to 85°C for 30 min (○) was determined by the turbidimetric assay with *S. sanguis* M5.

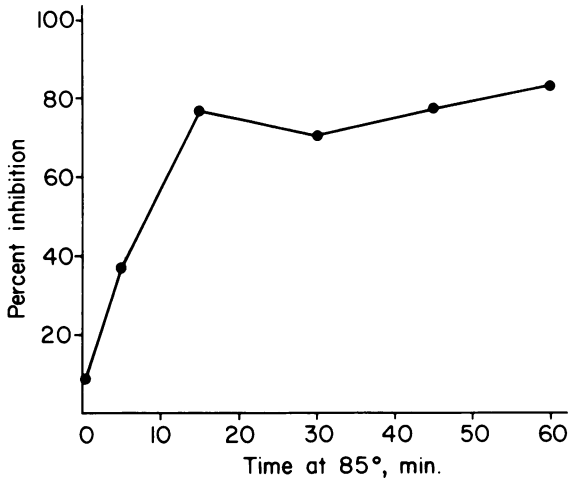

FIG. 3. Heat activation of serum inhibitory activity. Serum was heated to 85°C for 5 to 60 min and then tested for inhibitory activity for *S. sanguis* aggregation.

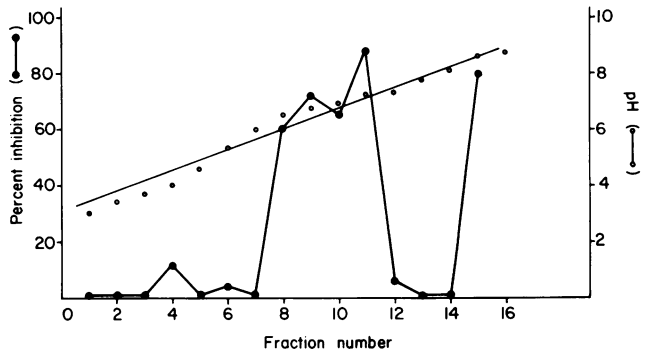

FIG. 4. Fractionation of aggregation inhibitory activity by isoelectric focusing. Serum was applied to the top (basic) end of gels containing 4% polyacrylamide and 2% Ampholines. Gels were run overnight at 250 V at 4°C, sectioned into 0.5-mm pieces, and dialyzed overnight against PBS. Portions of the eluted fractions were tested for aggregation inhibition activity with *S. sanguis* M5. The pH was determined on a replicate gel. This experiment was carried out three times with similar results.

inhibitory activity with the relative content of lysine and arginine. Histones containing high amounts of arginine-induced bacterial aggregation, whereas lysine-rich histones inhibited saliva-mediated aggregation.

Complete dose-response studies for the inhibition of bacterial aggregation were carried out with a series of blood-derived proteins. The data are expressed as the I_{50} for the inhibition of saliva-mediated bacterial aggregation in Table 3. There is a wide range of inhibitory potential, with fibrinogen being the most potent substance tested as an inhibitor of

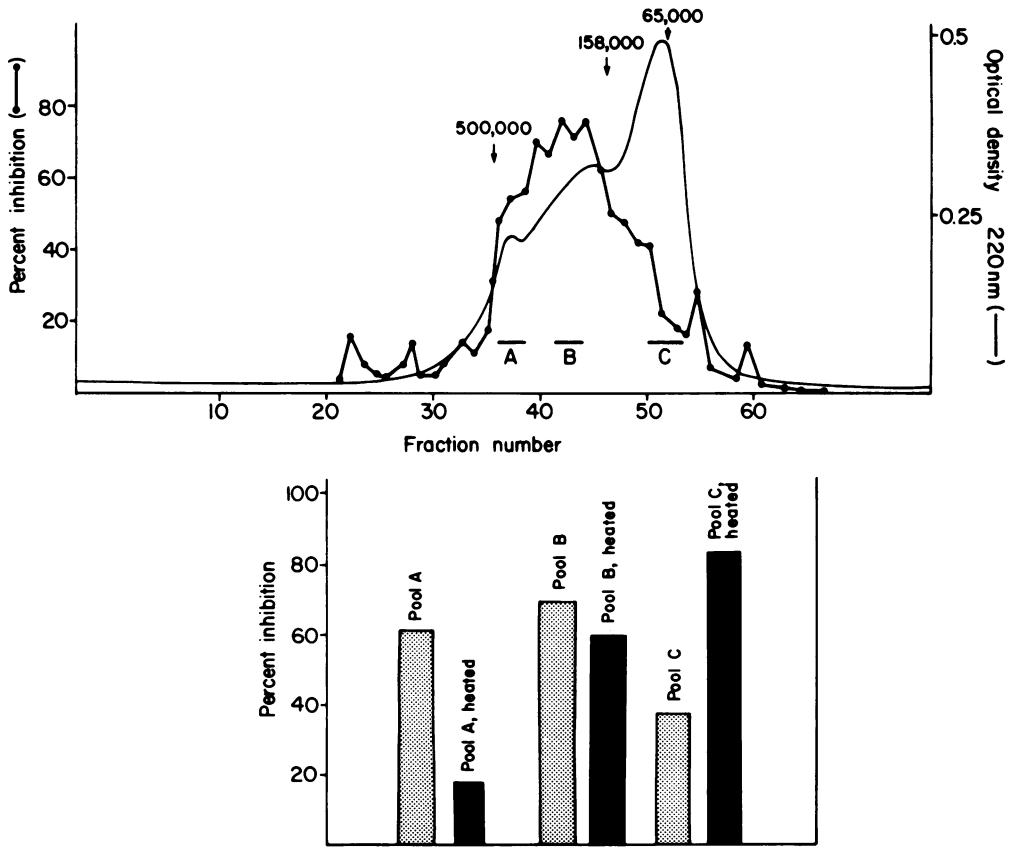

FIG. 5. Fractionation of inhibitory activity on Sephacryl 200 SF. Serum was loaded onto a Sephacryl 200 SF column, and protein (———) and aggregation inhibitory activity (●) was monitored. Three regions, as indicated, were tested, dialyzed against 1:10 PBS, and lyophilized. Each of these fractions was tested for inhibitory activity before and after heating to 85°C for 30 min. The results shown are from one of three column runs, each giving consistent inhibitory patterns.

saliva-mediated aggregation. As can be seen in Table 3, egg white albumin, like BSA, shows heat-stimulated inhibitory activity. Analysis of egg white albumin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the appearance of higher-molecular-weight forms as a result of heating to 85°C as had been seen with BSA. The inhibitory activity of Cohn fraction II was heat labile.

DISCUSSION

Serum contains potent inhibitors of saliva-mediated strepnuclear leukocytes and platelet extracts (8a), it appears that the inhibition involves an interaction of the serum factors with salivary agglutinins, rather than a binding of the inhibitor to the bacterial surface. For example, pretreatment of bacteria with serum followed by washing does not lead to inhibition of aggregation (data not shown). Dose-response studies with sera from different animal species showed similar I_{50} values, with the exception of mouse sera which was a less potent inhibitor. The presence of inhibitory activity in sera from diverse animal species against human saliva suggests that the inhibitor is a ubiquitous serum component. Ericson et al. (1) reported that anti-immunoglobulin antisera and high concentrations of human serum albumin more effectively inhibited saliva-mediated aggregation of *S. sanguis* than of *S. mutans*. As in the present study, the inhibition appeared to be on the salivary agglutinin rather than on the bacterial component.

A surprising finding was that the preheating of serum led to a marked increase in inhibitory activity. Fractionation studies of human serum revealed at least two inhibitory fractions—a high-molecular-weight (>160,000) heat-labile component and a low-molecular-weight (≈60,000) component that was heat activated within 5 min at temperatures above 80°C. This heat-activated component appears identical to serum albumin, and indeed, purified BSA inhibits bacterial aggregation. Heat activation appears to involve the formation of high-molecular-weight aggregates of albumin—a phenomenon observed with serum, purified BSA, and egg white albumin.

The second inhibitory fraction in serum has a molecular weight of >160,000 and a pI of 6.0 to 7.2. These molecular characteristics resemble immunoglobulin G (IgG), however, we were unable to deplete inhibitory activity from human serum with protein A or anti-human IgG. There is no obvious correlation between protein pI and inhibition of bacterial aggregation. However, most of the potent inhibitors are high-molecular-weight proteins (fibrinogen, 341,000; fibronectin, 440,000; ferritin, 450,000). Coupled with the observation that heating bovine and egg white albumin generates high-molecular-weight aggregates with increased inhibitory potential, this suggests the possibility that high-molecular-weight compounds inhibit aggregation by physically blocking the binding of agglutinins to the bacterial surface.

The biological role of serum inhibitors of bacterial aggregation is not clear. Although serum albumin is present in saliva, the requirement for heat activation suggests that this molecule does not play an important role in situ. If bacterial aggregation is viewed as a means of clearing microorganisms from the oral cavity (10), then inhibition of this aggregation could serve to promote bacterial colonization. It is possible that in the course of gingival inflammation and periodontal disease, the increased serum components released into the gingival crevice could serve to impede salivary antibacterial activities and exacerbate the disease process. Since the colonization of bacteria within the oral cavity represents an interplay of clearance and adherence mechanisms (10), selective inhibitors can facilitate the identification of the salivary molecules involved in these two distinct processes (11).

TABLE 2. Histones as aggregators and inhibitors of bacterial aggregation[a]

Histone	Lys-Arg	% Aggregation without saliva (10 μg of histone/ml)	I_{50} for saliva-mediated aggregation (μg/ml)
H3	0.7	50.5	
H4	0.8	45.6	
H2A	1.2	45.3	
H2B	2.5	16.8	20
H1	19.0	0	150

[a] Each histone was tested at a series of concentrations as a stimulator (without saliva) or inhibitor (with saliva) of *S. sanguis* M5 aggregation. The percent aggregation shows results obtained with 10 μg of each histone per ml. The I_{50} indicates the concentration of histone giving 50% inhibition of saliva-mediated aggregation. Histones H3, H4, and H2A were not tested as inhibitors since they demonstrated high endogenous aggregation activity.

TABLE 3. Inhibition of saliva-mediated aggregation of *S. sanguis* M5 by purified proteins[a]

Substance	I_{50}	
	μg/ml	nM
Fibrinogen	1	2.9
Fibronectin	16	36.4
Ferritin	40	80
Lactoferrin	9	120
Cohn fraction II	40	270
Cohn fraction II, heated to 85°C	—	—
Egg white albumin	—	—
Egg white albumin, heated to 85°C	25	560
BSA	900	14,500
BSA heated to 85°C	10	150

[a] Each substance was tested as an inhibitor of saliva-mediated aggregation at six concentrations. The I_{50} results are given in micrograms of inhibitor per milliliter of reaction mixture and as nanomolar concentration. Cohn fraction II, egg white albumin, and BSA were tested before and after heating (85°C for 30 min). —, No inhibitory activity.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Ericson, T., D. Bratthall, and J. Rundegren.** 1979. Bacterial agglutination induced by saliva. Inhibition studies with anti-Ig antisera and serum components, p. 243–254. *In* I. Kleinberg, S. A. Ellison, and I. D. Mandel (ed.), Saliva and dental caries (a special supplement to Microbiology Abstracts) Information Retrieval, Inc., Washington, D.C.
2. **Ericson, T., K. Pruitt, and H. Wedel.** 1975. The reaction of salivary substances with bacteria. J. Oral Pathol. **4**:307–323.
3. **Franks, D. J., and D. Malamud.** 1976. Isoelectric focusing of brain adenylate cyclase. Anal. Biochem. **73**:486–492.
4. **Gibbons, R. J., and J. van Houte.** 1975. Bacterial adherence in oral microbial ecology, Annu. Rev. Microbiol. **29**:19–44.
5. **Ginsburg, I., and P. G. Quie.** 1980. Modulation of human

polymorphonuclear leucocyte chemotaxis by leucocyte extracts, bacterial products, inflammatory exudates, and polyelectrolytes. Inflammation **4**:301–311.

6. **Golub, E. E., M. Thaler, C. Davis, and D. Malamud.** 1979. Bacterial aggregating activity in human saliva: simultaneous determination of free and bound cells. Infect. Immun. **26**:1028–1034.
7. **Kashket, S., and C. G. Donaldson.** 1972. Saliva-induced aggregation of oral streptococci. J. Bacteriol. **112**:1127–1133.
8. **Malamud, D., B. Appelbaum, R. Kline, and E. E. Golub.** 1981. Bacterial aggregating activity in human saliva: comparisons of bacterial species and strains. Infect. Immun. **31**:1003–1006.

8a. **Malamud, D., R. Goldman, and N. S. Taichman.** 1983. Modulation of bacterial aggregation by PMN and platelet extracts. Inflammation **7**:133–144.

9. **Malamud, D., and E. E. Golub.** 1981. A model for saliva mediated bacterial aggregation. Adv. Physiol. Sci. **28**:277–282.
10. **Mandel, I. D.** 1976. Immunologic aspects of caries resistance. J. Dent. Res. **55**:C22–C31.
11. **Rosan, B., D. Malamud, B. Appelbaum, and E. Golub.** 1982. Characteristic differences between saliva-dependent aggregation and adhesion of streptococci. Infect. Immun. **35**:86–90.