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Differentiation and Interaction of Secretory Immunoglobulin A and a Calcium-Dependent Parotid Agglutinin for Several Bacterial Strains

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Previous studies have suggested that both secretory immunoglobulin A (sIgA) and various nonimmunoglobulin salivary glycoproteins are capable of agglutinating a variety of bacteria. The present study was designed to compare the nature of the agglutinins for *Streptococcus mutans* and *Salmonella typhimurium* in parotid saliva and colostrum. *S. mutans* was aggregated by saliva and colostrum, whereas *S. typhimurium* was aggregated only by saliva as detected by a spectrophotometric method. The principal salivary agglutinin for both *S. mutans* and *S. typhimurium* was calcium dependent and could be desorbed in phosphate-buffered saline (pH 6.8). In contrast, the colostral agglutinin was calcium independent and not readily desorbed. The agglutinin activities of saliva and colostrum for *S. mutans* were additive, suggesting independent target sites on the bacterial surface. The agglutinin activity of colostrum was totally associated with sIgA as was suggested by blocking of the agglutinating activity with anti-α-chain serum and the absence of blocking with an antibody specific for salivary agglutinin. Interestingly, anti-α-chain serum removed all agglutinating activity from saliva, but not from the phosphate-buffered saline-desorbed agglutinin. Dialysis of parotid saliva against 0.1 M disodium EDTA eliminated the agglutinin blocking activity of anti-α-chain serum but not that of the antiagglutinin antibody. The ability of anti-α-chain serum to block agglutination of the EDTA-dialyzed saliva could be restored by the addition of calcium chloride, suggesting that sIgA and salivary agglutinin are associated through a calcium-mediated interaction. These results indicate that bacterial agglutinating activity of colostrum, as detected spectrophotometrically, is mediated by sIgA, and that of saliva is mainly dependent upon a calcium-dependent nonimmunoglobulin agglutinin. The agglutinating activities of sIgA and parotid agglutinin seem to be additive, and their calcium-dependent association may favor the enhancement of their respective activities.

Substances in secretions mediating agglutination of bacteria may contribute to clearance of microorganisms by coating or agglutination of the cells. A variety of substances found in epithelial secretions have been suggested to be able to mediate bacterial agglutination. Among these are lysozyme (19), α-amylase (25), fibronectin (3), other high-molecular-weight glycoproteins (10, 12), and secretory IgA (sIgA) (1). Nonimmunoglobulin glycoproteins in saliva with bacterium-specific agglutinating properties have been described (2, 8, 15, 17). Some of the agglutinating glycoproteins in saliva also possess blood group reactivity (11, 14), which implies that these substances have different oligosaccharide configurations. Many of the glycoprotein agglutinins require calcium ions for their functioning (12, 14, 22), and a high-molecular-weight calcium-dependent agglutinin in parotid saliva has recently been shown to bind to or agglutinate a variety of oral bacterial species in a nonspecific manner (20). sIgA has been attributed a bacterium-agglutinating capacity in both colostrum and saliva (1, 26).

Complexing and joint action of glycoprotein agglutinins and sIgA in saliva have been implicated (6, 7, 18). The nature of the complexing has not yet been described, but treatment of saliva with antiserum to IgA has been shown to either inhibit or abolish agglutination (1, 7, 16), indicating a major role of IgA as an agglutinin in saliva.

The present study was undertaken to elucidate the mechanisms of interactions between sIgA and salivary glycoprotein agglutinins and also to determine their relative roles in mediating bacterial agglutination.

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MATERIALS AND METHODS

Microorganisms. Lyophilized samples of *Streptococcus mutans* TH16 (21) serotype c and *Salmonella typhimurium* HN202 (24) were inoculated on blood agar plates at 37°C in air containing 10% carbon dioxide for 48 h. Fresh colonies were transferred to tubes containing Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and grown to the early stationary phase by incubation at 37°C overnight. Cells were harvested by centrifugation and washed twice by suspending cells in a 10 mM potassium phosphate buffer (pH 6.8), containing 0.15 M NaCl (PBS). Stock suspensions in PBS had an optical density at 700 nm (OD_{700}) of 1.5 measured in a Cary 219 spectrophotometer (Varian, Palo Alto, Calif.).

Saliva and colostrum. Unstimulated parotid saliva samples from three male donors were collected in ice-chilled tubes by using Lashley cups. Four mothers donated colostrum samples, which were defatted, decaseinated, and passed over a Heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) column to exclude lactoferrin and other heparin-binding components from the IgA-containing fraction (5).

Spectrophotometric agglutination assay. Largely the method described by Ericson et al. (9) was followed. Samples to be tested for mediating agglutination were diluted to a volume of 0.6 ml with PBS and then mixed with 1.2 ml of the bacterial stock suspension. The agglutination was measured at 20°C by recording change in OD_{700} during 1 h as a result of bacterial sedimentation.

Microtiter agglutination assay. The microtiter agglutination assay was only used in the experiments with antiserum

treatment of saliva. Twofold serial dilutions were made of 50-μl samples added to microtiter plates with V-bottom wells (Cooke Microtiter System; Dynatech Laboratories, Inc., Alexandria, Va.) containing 50 μl of 0.1% bovine serum albumin in PBS in each well. Fresh cells of *S. mutans* made up for the spectrophotometric assay were centrifuged and suspended in the PBS-bovine serum albumin buffer to give an OD_{660} of 0.1. A volume of 50 μl of the bacterial suspension was added to each well, and the plates were incubated at 37°C for 2 h and at 4°C overnight. The endpoint agglutination was read by using a magnifying mirror below the plate (1).

Desorption of agglutinins. A volume of 1.2 ml of bacterial suspension was mixed with 0.3 ml of parotid saliva or colostrum diluted to 0.6 ml with PBS. After incubation at 37°C for 30 min, the mixtures were centrifuged, and the pellets were suspended in volumes of 1.8 ml of PBS to desorb agglutinins. A new centrifugation was carried out to separate the bacteria from the agglutinin-containing supernatant (20). All agglutinin extracts were tested in the presence of 0.3 mM calcium chloride. The pellet was tested for aggregating ability in PBS. Colostrum-coated cells were also treated with 0.05 M glycine hydrochloride (pH 2.3) in the same way as with PBS.

Interaction between colostral and salivary agglutinins. Cells were treated with either colostrum or saliva by the procedure described above. After centrifugation, the colostrum-coated cells were suspended in 0.1 ml of saliva or 0.1 ml of colostrum diluted to 1.8 ml with PBS. Saliva-coated cells were treated in the same way as the colostrum-coated cells. Saliva- or colostrum-coated cells suspended in PBS only served as controls. The suspensions were tested for agglutination in the presence or absence of 0.3 mM calcium chloride.

Antisera treatment. Volumes of 0.3 ml of saliva or colostrum were incubated with 80 μl of rabbit anti-α-chain serum (Dako, Glostrup, Denmark) at 37°C for 2 h and at 4°C for 16 h. Precipitates were removed by centrifugation in an Eppendorf tube centrifuge. A volume of 1.8 ml of a salivary agglutinin-containing supernatant resulting from a centrifugation of saliva-coated *S. mutans* cells suspended in PBS was also treated with 80 μl of anti-α-chain serum. Saliva and colostrum samples were similarly treated with an antiserum raised to a purified parotid agglutinin derived from PBS washings of saliva-coated *S. mutans* cells (20). Antisera raised in rabbits to human milk lactoferrin, purified on a heparin affinity column (Pharmacia), and rabbit antisera to μ-chain (Dako) were also used for treatment of saliva and colostrum samples before testing for agglutination. Antisera that mediated agglutination were absorbed with *S. mutans* cells until no agglutinating activity was found.

In another set of experiments, the saliva and colostrum were dialyzed in the cold against three changes of 0.1 M sodium EDTA followed by four changes of PBS before treatment with the various antisera and recording of agglutination. A sample of the dialyzed saliva was also supplemented with a final concentration of 0.3 mM calcium chloride before a precipitation-agglutination experiment.

RESULTS

Saliva agglutinated both the *S. mutans* and *S. typhimurium* strains, whereas the colostrum only agglutinated the *S. mutans* strain. Salivary agglutinins could be desorbed from both strains by suspending the saliva-coated cells in Ca^{2+}-free PBS. Each supernatant had activity against both bacterial strains. The saliva-treated cells washed in Ca^{2+}-free PBS failed to agglutinate when suspended in PBS containing 0.3 mM $CaCl_2$. In contrast, agglutinins were not detected in the PBS wash of colostrum-treated cells of either of the strains, and colostrum-coated cells had the original agglutinating activity when suspended in PBS. When the colostrum-coated cells were treated with 0.05 M glycine hydrochloride (pH 2.3), agglutination was totally abolished. All saliva and colostral samples had IgA antibody activity for both *S. mutans* and *S. typhimurium* as detected by an enzyme-linked immunoassay with whole cells as the fixed antigen (unpublished observation).

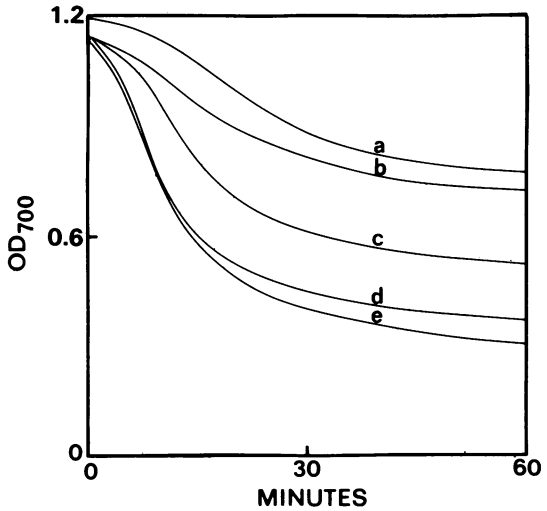

FIG. 1. Agglutination of saliva- or colostrum-coated *S. mutans* cells suspended in additional saliva or colostrum. Curves: a, saliva-coated cells plus saliva; b, colostrum-coated cells plus colostrum; c, saliva- or colostrum-coated cells alone; d, saliva-coated cells plus colostrum; e, colostrum-coated cells plus saliva.

Suspension of colostrum-coated *S. mutans* cells in saliva increased the rate of agglutination substantially when compared with a similar treatment with additional colostrum (Fig. 1). Likewise, saliva-coated cells demonstrated an increased rate of agglutination when suspended in colostrum relative to when they were suspended in additional saliva. The kinetics of the saliva- and colostrum-mediated agglutination were similar in that both produced the same kind of sigmoidal absorbance curves resulting from sedimentation of bacterial aggregates (Fig. 1). Saliva- or colostrum-coated cells suspended in the homologous secretion showed a lower rate of agglutination than when the cells were suspended in PBS only.

Colostrum and saliva precipitated with anti-α-chain serum showed no agglutinating activity in the spectrophotometer, whereas a similar treatment of the salivary agglutinin desorbed from the bacterial cell surfaces gave no inhibition of its agglutination (Fig. 2). The antiserum to the purified parotid saliva agglutinin preparation did not inhibit colostrum-mediated agglutination, but it totally abolished saliva-mediated agglutination (Fig. 3). Although the antiagglutinin treatment abolished saliva-mediated agglutination, such treatment did not reduce the IgA concentration in the saliva as measured by rocket immunoelectrophoresis.

Colostral agglutination was not affected by treatment with either antilactoferrin or anti-μ-chain, whereas agglutination mediated by saliva was partially inhibited by both of these antisera (Fig. 4). When the saliva, dialyzed against EDTA followed by PBS, was treated with antiserum before the

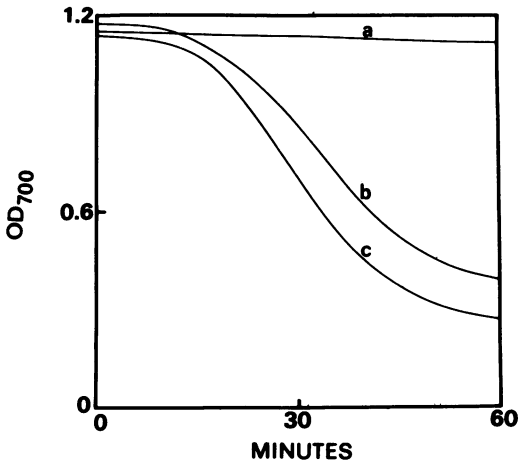

FIG. 2. Agglutination of *S. mutans* mediated by saliva or colostrum samples treated with anti-α-chain serum. Curves: a, saliva, colostrum, or EDTA-dialyzed saliva in 0.3 mM Ca; b, EDTA-dialyzed saliva; c, EDTA-dialyzed saliva or native saliva with PBS instead of antiserum and agglutinin plus anti-α-chain serum.

agglutination test, only antiagglutinin could totally block agglutination. The anti-α-chain serum resulted in only minimal inhibition of agglutination, whereas the antilactoferrin and anti-μ-chain sera gave no inhibition (Fig. 2 through 4). When calcium was added back to the EDTA-dialyzed saliva at a final concentration of 0.3 mM before a repeated precipitation-agglutination experiment, the anti-α-chain serum again totally blocked agglutination (Fig. 3).

A comparison of the two agglutination assays showed that saliva treated with antiserum to either salivary agglutinin or α-chain to eliminate activity in the spectrophotometric assay still retained some activity in the microtiter assay (Table 1), suggesting the increased sensitivity of the latter. The anti-α-chain serum reduced the salivary microtiter more than did the antiserum to agglutinin. In contrast, the microtiter for the PBS-desorbed agglutinin was only reduced one $\log_2$ step by treatment with anti-α-chain serum, whereas the antiagglutinin serum reduced the titer 5 $\log_2$ steps.

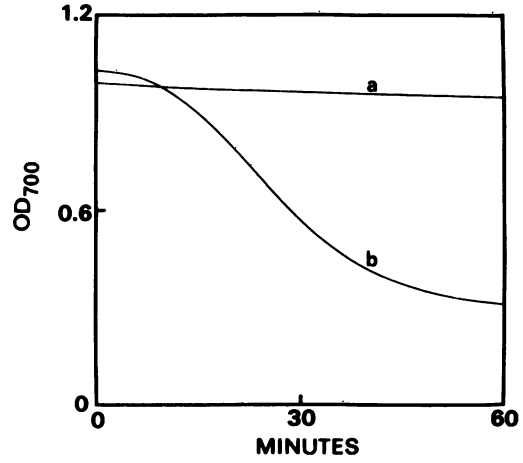

FIG. 3. Agglutination of *S. mutans* mediated by saliva or colostrum samples treated with antiagglutinin. Curves: a, saliva or EDTA-dialyzed saliva; b, colostrum or saliva with antiagglutinin substituted for PBS.

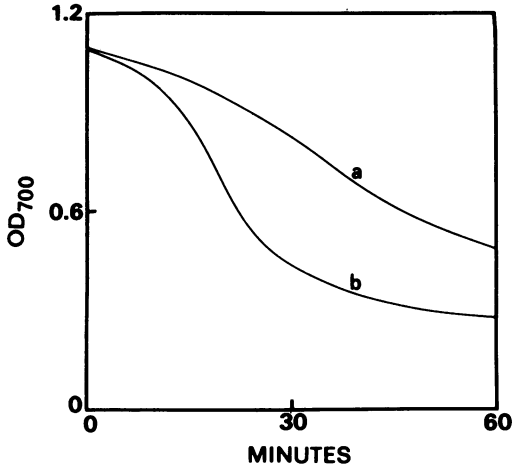

FIG. 4. Agglutination of *S. mutans* mediated by (a) saliva treated with antilactoferrin or anti-μ-chain or (b) EDTA-dialyzed saliva with and without treatment with antilactoferrin or anti-μ-chain.

DISCUSSION

The saliva- and colostrum-mediated agglutination of *S. mutans* and *S. typhimurium* indicated the presence of two different kinds of agglutinins. The salivary agglutinin, which was easily desorbed in a calcium-free buffer, showed no species specificity, since it mediated agglutination of both species, in agreement with other recent findings (20). The colostral agglutinin on the other hand, could not be desorbed in PBS. Moreover, the colostrum sample obviously contained a species-specific agglutinin, since it only reacted with the *S. mutans*.

The additive agglutinating effect shown by saliva- or colostrum-coated *S. mutans* cells suspended in the heterologous secretion sample compared with that of cells exposed to the homologous secretion indicates that the salivary and colostral agglutinins engage different cell receptor sites or physical mechanisms for agglutination to occur. The addition of homologous agglutinin gave a slower agglutination rate than when the cells were coated with saliva or colostrum and suspended in PBS only, suggesting a prozone effect. Both saliva and colostrum mediated similar sigmoidal absorbance curves for agglutination. Thus, when cross-linking of cells is obtained to form small, nonsedimenting aggregates, the same type of physical mechanism, e.g., colloidal stability

TABLE 1. Comparison of agglutinating activity for *S. mutans* in the microtiter and spectrophotometric assays with saliva or salivary agglutinin before and after treatment with antisera

Prepn	Agglutination assay results	
	Microtiter[a] ($\log_2$)	Spectrophotometer[b]
Saliva plus PBS	10, 10	Yes
Saliva antiagglutinin	5, 5	No
Saliva plus anti-α-chain	3, 4	No
Agglutinin plus PBS	8, 8	Yes
Agglutinin plus antiagglutinin	3, 3	No
Agglutinin plus anti-α-chain	7, 7	Yes

[a] Microtiter results are given in duplicate as the $\log_2$ reciprocal of the last dilution to give a detectable difference in button pattern from the negative control.

[b] Agglutination was recorded as clear agglutination or no agglutination after 1 h.

(23), may determine the kinetics for the sedimentation of larger aggregates.

Parotid saliva dialyzed against EDTA and then precipitated with anti-α-chain serum showed almost the original agglutinating activity. When calcium was added back to the dialyzed saliva before precipitation, agglutination was abolished. Consequently, it seems as if the salivary agglutinin is complexing with salivary IgA in a calcium-dependent fashion and that the saliva-mediated spectrophotometric agglutination is mainly due to nonimmunoglobulin agglutinin. The colostrum-mediated agglutination was not inhibited by agglutinin-specific antiserum, but was abolished by the anti-α-chain serum. Affinity-purified colostral sIgA retained *S. mutans* agglutinating activity (Rundegren and Arnold, manuscript submitted), indicating that colostrum-mediated agglutination is mainly dependent on IgA. The ionic calcium found in parotid saliva is known to allow calcium-dependent protein interactions in the saliva for acidic proteins such as statherin (13) and the acidic proline-rich proteins (4). The calcium-dependent parotid agglutinins (20) also seem to have a pI in the acidic range (unpublished observation). Although sIgA has a pI in the neutral range, acidic domains of its molecular subunits may account for the cation-dependent interaction with parotid agglutinins. Repeated treatment of complexed salivary mucin-IgA with 6 M urea (J. Reinholdt and M. Kilian, J. Dent. Res. **63**[Special issue], abstr. no. 513, 1984) most likely would interfere with this protein-protein interaction as also shown by the resulting depletion of IgA activity in the mucin preparation.

Previous studies indicate that saliva-mediated agglutination could be due mainly to IgA antibodies (1) or to nonimmunoglobulin agglutinins (2, 8, 20). The present results indicated that different agglutination assays may be measuring the effect of different agglutinins, since there was a considerable titer in the microtiter assay in the absence of detectable agglutination in the spectrophotometric assay. The microtiter results also showed that both IgA and salivary agglutinin are responsible for the saliva-mediated activity detected in this assay. The anti-α-chain serum had little effect on the activity of the desorbed agglutinin, whereas the antiagglutinin had a substantial effect. In contrast, the anti-α-chain serum lowered the saliva-mediated titer more than the agglutinin-specific serum, suggesting that IgA is the major contribution to the microtiter activity. There are purified sIgA preparations that contain significant activity in the absence of detectable spectrophotometric activity and samples with relative low microtiters with significant spectrophotometric agglutination (Rundegren and Arnold, manuscript submitted). Such data suggest that the two assays may be detecting qualitative differences in interactions of agglutinins with bacterial cells.

The small inhibition in spectrophotometric agglutinating capacity of α-chain-precipitated, EDTA-dialyzed saliva compared with nontreated saliva (Fig. 2) indicates that IgA could contribute to some of the agglutination observed. The abrogation of agglutinating activity in saliva with antiagglutinin serum (Fig. 3) implies that only a negligible portion of the total IgA is complexing with the agglutinin, because the total IgA in agglutinin-precipitated and untreated control salivas was the same. It is also possible that the EDTA dialysis may not completely remove all of the calcium needed for complexing; thus, some agglutinin in saliva may still be precipitated by the anti-α-chain serum. The treatment of saliva with either antilactoferrin or anti-μ-chain gave a partial inhibition of agglutination, whereas the same treatment of EDTA-dialyzed saliva gave no inhibition. This inhibition represents further evidence for a calcium-dependent complexing of proteins in saliva.

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