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Inhibition of Enzymes by Human Salivary Immunoglobulin A

YOSHIO FUKUI, KAZUHIRO FUKUI, AND TAKAFUMI MORIYAMA

Department of Microbiology, Hiroshima University, School of Dentistry, Hiroshima 734, Japan

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Human whole saliva inhibited bacterial neuraminidases and the inhibition was found to reside in the salivary IgA fraction. Further, salivary immunoglobulin (Ig)A inhibited various bacterial enzymes and toxins: neuraminidases from *Streptococcus mitis*, *Streptococcus sanguis*, and *Clostridium perfringens*, hyaluronidase and chondroitin sulfatase from oral bacteria, diphtheria toxin, and streptolysin O. The inhibitory activity of salivary IgA did not correlate with that of serum on the basis of minimum inhibitory dose. A small amount of salivary IgA was required to inhibit oral bacterial neuraminidases, whereas a large amount was required to inhibit other bacterial neuraminidase. Therefore, it is concluded that the absence of neuraminidase activity of oral bacteria in whole saliva may be due to specific inhibition by salivary IgA.

Neuraminidase activity (EC 3.2.1.18; *N*-acetylneuraminate glycohydrolase) has been demonstrated in human whole saliva and submandibular-sublingual secretions (9, 13, 15, 16, 21). Moreover, it has been shown that some microorganisms from the oral flora produce the enzyme in culture media (4, 18, 19, 25). Therefore, it appeared likely that the oral bacteria may be a major source of salivary neuraminidase.

In our previous investigation (5) the salivary neuraminidases were characterized by polyacrylamide gel electrophoresis and density gradient centrifugation, and comparative studies of the enzymes from whole saliva and those of oral bacteria and human liver were also carried out in order to clarify the origin of the enzyme in whole saliva. However, neuraminidase activity originating from oral bacteria could not be found in fresh human whole saliva. Accordingly, the question has been raised whether the absence of bacterial neuraminidase is due to salivary immunoglobulin (Ig)A. The results of the study support this assumption. Further, evidence is presented to show that salivary IgA inhibits not only bacterial neuraminidase but also some other enzymes of oral bacteria and diphtheria toxin and streptolysin O as well.

MATERIALS AND METHODS

Collection and concentration of saliva. Twenty-five to 50 ml of unstimulated whole saliva were collected from healthy human adults in chilled beakers, concentrated with ammonium sulfate, and brought to 1 ml as described in a preceding paper (4).

Preparation of salivary neuraminidase. Salivary neuraminidases were isolated from concentrated whole saliva by polyacrylamide gel electrophoresis or sucrose density gradient centrifugation as described in the preceding paper (5).

Colostral IgA, salivary IgA, and antisera against serum and secretory IgA. Purified human colostral IgA was kindly provided by K. Inoue, Institute for Microbial Diseases, Osaka University, Osaka. Salivary IgA was purified by diethylaminoethyl (DEAE)-cellulose (Serva) chromatography and Sephadex G-200 (Pharmacia) filtration as described by Tomasi and Bienenstock (26). Secretory IgA, IgA, IgG, and IgM rabbit antisera were purchased from Behringwerke AG, Marburg-Lahn. Content of secretory IgA was determined by a single radial immunodiffusion method with purified colostral IgA as standard (12). The specificity of the antisera was checked by immunoelectrophoresis. Serum and salivary IgA were obtained from the same person for comparison of inhibitory activity.

Preparation of saliva antisera. Whole saliva was collected from 40 human adults and concentrated as described above. Rabbits were immunized with whole saliva by intramuscular injection at several sites (10 mg of protein of concentrated whole saliva in Freund complete adjuvant to a total of 4 ml). Each animal received three injections of the antigen at intervals of 2 weeks, and all were bled out 10 days after the third injection.

Immunoelectrophoresis. Immunoelectrophoresis was carried out on microscope slides covered with a layer of 1% agar in barbital buffer, pH 8.2, ionic strength, 0.05. Electrophoresis was performed in an apparatus at an applied potential of 4 mA per slide for 1 h in the cold by the method of Campbell et al. (1).

Bacterial enzymes and toxins. Neuraminidases were partially purified from *Streptococcus mitis* ATCC 9811, *Streptococcus sanguis* ATCC 10557 and strains of *S. mitis* and *S. sanguis* isolated from the human oral cavity, and the enzyme activity was determined as described in the preceding paper (5). Urinary mucoprotein was used as substrate for the enzyme assay. One unit of enzyme was defined as that amount of enzyme which catalyzed the release of 1 μmol of *N*-acetylneuraminic acid per h. Standardized streptolysin O and streptolysin O rabbit antisera were purchased from Nissui Seiyaku Co., Tokyo, and the assay procedure was carried out as described in the manual. Purified diphtheria toxin and standard toxin were kindly provided by M. Yoneda, Institute for Microbial Diseases, Osaka University, Osaka. The toxicity and the inhibitory effect on the toxin were measured by skin reaction in rabbits. One MRD toxin was added to 1 mg of salivary IgA, incubated at 4 C overnight, and then the toxicity was checked. Purified neuraminidase type V from *Clostridium perfringens* and hyaluronidase type I from bovine testes were purchased from Sigma Chemical Co. The activity of purified hyaluronidase was determined as the release of *N*-acetylglucosamine-reactive groups from hyaluronic acid by the method of Reissig, Strominger, and Leloir (20). Organisms producing hyaluronidase or chondroitin sulfatase, or both, were isolated from patients with periodontal disease, and direct localization and visualization of the enzymes in an agar plate were carried out as described by Hershon (7) and Smith, and Willet (24). Hyaluronic acid and chondroitin sulfate were purchased from Seikagaku Kogyo Co., Tokyo, and used as substrate.

Protein determination. The method of Lowry (11) was used with crystalline bovine serum albumin as a standard.

RESULTS

Inhibition of bacterial neuraminidase by whole saliva. During our investigation it was found that human whole saliva inhibited the activity of neuraminidase obtained from *S. mitis* ATCC 9811. To 0.1 ml of the concentrated whole saliva (8 mg of protein per ml), with or without heating at 100 C for 10 min, were added 0.02 ml of the partially purified neuraminidase containing 0.1 enzyme unit and 0.1 ml of 0.5 M phosphate buffer, pH 6.0. The reaction mixtures were incubated at 0 C overnight and, after the incubation, 0.2 ml of substrate containing 4 mg of urinary mucoprotein per ml were added to the mixtures and brought to 1 ml each with distilled water. Incubation was carried out at 37 C for 2 h, and released *N*-acetylneuraminic acid was determined. The results are summarized in Table 1, and indicate that whole saliva inhibits enzyme activity, but heated saliva does not. Further, the incubation before the addition of substrate was carried out also at 37 C, and the inhibitory activity was quite similar to that at 0 C. These results suggest that the inhibitory factor might be a protein, but not an enzyme.

TABLE 1. *Inhibition of streptococcal neuraminidase by heated and nonheated whole saliva*

Assay system[a]	Tube no.		
	1	2	3
Whole saliva	−	+	−
Heated whole saliva	−	−	+
Neuraminidase	+	+	+
Phosphate buffer	+	+	+
Substrate incubated at 0 C overnight	+	+	+
Percent inhibition incubated at 37 C for 2 h	0	92	0

[a] Assay system represents as follows: component present (+), component deleted (−).

The results in Table 1 were highly reproducible from preparation to preparation of concentrated whole saliva, and the inhibitory activity was also found in separately collected parotid or submandibular-sublingual secretions.

Isolation of the inhibitory factor. Separation of the inhibitory factor in whole saliva was attempted by using DEAE-cellulose. Concentrated whole saliva (8 mg of protein) was applied to a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer, pH 6.5, and eluted with a linear gradient of 0 to 0.5 M NaCl in 0.01 M phosphate buffer. A 0.5-ml amount of each eluate was sampled and checked for inhibitory activity as described above. There is a single peak with inhibitory activity in one of the four protein peaks (Fig. 1). The active fractions were concentrated with ammonium sulfate and further purified by gel filtration through a Sephadex G-200 column as described by Tomasi and Bienenstock (26). Figure 2 shows the immunoelectrophoretic pattern of the purified fraction with inhibitory activity. The results strongly suggest that the inhibitory factor found in whole saliva may be IgA.

Neutralization of the inhibitory factor with anti-IgA. To confirm the finding, blocking of the inhibitory factor with antisera was attempted. To 0.1 ml of concentrated whole saliva (8 mg of protein per ml) or the purified IgA fraction (1 mg of IgA per ml) were added 0.1 ml of IgA, IgG, IgM antisera, or normal rabbit serum. Merthiolate was added to prevent bacterial growth (1:1000). Each reaction mixture was incubated at 0 C overnight, and then a substrate was added. After the incubation, the enzyme activity of each tube was determined. The results showed that only anti-IgA (tube number 4) eliminated the inhibitory activity of whole saliva or of the purified IgA fraction (Table 2), indicating that the inhibitory factor of neura-

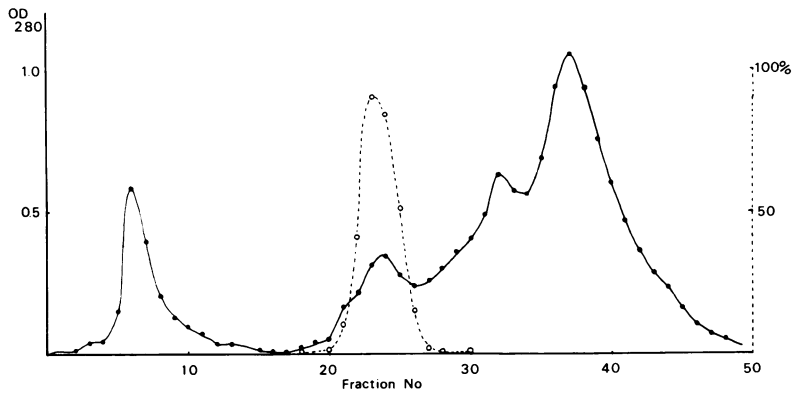

FIG. 1. *DEAE-cellulose column chromatography of the inhibitory factor. Column size was 1.0 by 20 cm, and fractions of 5 ml were collected. The solid line indicates protein and the dashed line the inhibitory activity.*

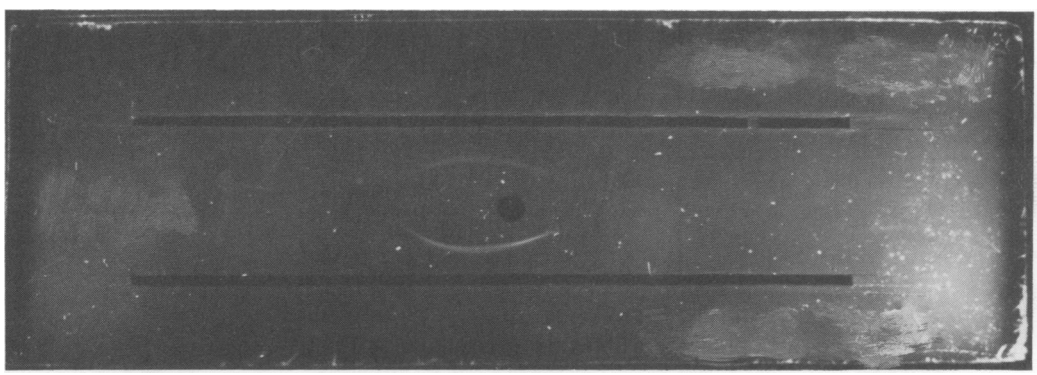

FIG. 2. *Immunoelectrophoretic pattern of the purified inhibitory factor. Center well contains 50 μg of the purified inhibitory factor, upper trough anti-whole-saliva serum and lower trough anti-IgA serum.*

minidase involved in whole saliva was salivary IgA.

Inhibition of other bacterial enzymes and toxins by salivary IgA. Neuraminidase prepared from *S. mitis* ATCC 9811 was inhibited by salivary IgA. Therefore, it was considered that salivary IgA may inhibit other bacterial enzymes and toxins. The results of such studies are summarized in Table 3. All preparations of bacterial neuraminidase were checked for activity by the same procedure as the enzyme of *S. mitis* ATCC 9811, and all were inhibited by salivary IgA.

The effect of salivary IgA on hyaluronidase and chondroitin sulfatase was studied next. The hyaluronidase-producing organisms leave a clear zone around the colony near a well containing saline, only a very small clear zone around a colony near the well containing human serum, but no such zone around a colony near the well containing salivary IgA (Fig. 3). The results indicate that salivary IgA and also human serum inhibit the hyaluronidase activity. Chondroitin sulfatase-producing organisms were checked for activity by the same method. The results showed that salivary IgA also inhibited the chondroitin sulfatase activity.

The effect of salivary IgA on diphtheria toxin and streptolysin O was tested as described in Materials and Methods. The results showed that salivary IgA inhibited both toxins.

On the other hand, neuraminidases from human saliva and hyaluronidase from bovine testes were not inhibited by salivary IgA.

Comparison of inhibitory activities of serum and salivary IgA. It appeared likely that serum and salivary IgA may differ in inhibitory activities of the enzymes of oral and non-oral bacteria. Comparative studies on the basis of minimum inhibitory doses were carried out accordingly.

Doses of salivary IgA and serum inhibiting by 50% 0.1 U of neuraminidases obtained from *S. mitis* or *S. sanguis* were determined. It is evident that the 50% inhibitory dose was 80 μg for salivary IgA (83 μg for salivary IgA collected from 40 adults) (Fig. 4). It was 90 μg for colostral IgA and more than 0.4 ml for serum (6 mg of IgG and 1 mg of IgA) (Table 4). On the other hand, the respective doses for neuramini-

TABLE 2. *Elimination of the inhibitory factor with antisera*

Assay system[a]	Tube no.						
	1	2	3	4	5	6	7
Whole saliva or purified salivary IgA	−	−	+	+	+	+	+
Anti IgA	−	+	−	+	−	−	−
Anti IgG	−	−	−	−	+	−	−
Anti IgM	−	−	−	−	−	+	−
Normal rabbit serum	+	−	−	−	−	−	+
Phosphate buffer	+	+	+	+	+	+	+
Neuraminidase incubated at 0 C overnight	+	+	+	+	+	+	+
Substrate incubated at 0 C overnight	+	+	+	+	+	+	+
Percent inhibition incubated at 37 C for 2 h	0	0	87[b]	0	85[b]	85[b]	85[b]

[a] Assay system represents as follows: component present (+), component deleted (−).

[b] The values of percent inhibition of other four experiments were more than 85.

dase of *C. perfringens* were 1.3 mg for salivary IgA and less than 0.01 ml for serum (0.15 mg of IgG and 0.025 mg of IgA). Further, 1 U of streptolysin O was inhibited by 1.16 mg of salivary IgA, and by 1/50 ml of serum (0.25 mg of IgG and 0.042 mg of IgA), as shown in Table 4.

TABLE 3. *Inhibition of bacterial enzymes and toxins with salivary IgA*

Enzyme or toxin	Inhibition with IgA[a]
Neuraminidase	
From *Strep. mitis* ATCC 9811	+
From *Strep. sanguis* ATCC 10557	+
From an isolated strain of *Strep. mitis*	+
From an isolated strain of *Strep. sanguis*	+
From *Cl. perfringens*	+
Hyaluronidase from oral bacteria	+
Chondroitin sulfatase from oral bacteria	+
Streptolysin 0	+
Diphtheria toxin	+
Neuraminidase from human saliva	−
Hyaluronidase from bovine	−

[a] Inhibition represented as follows: inhibited (+), not inhibited (−).

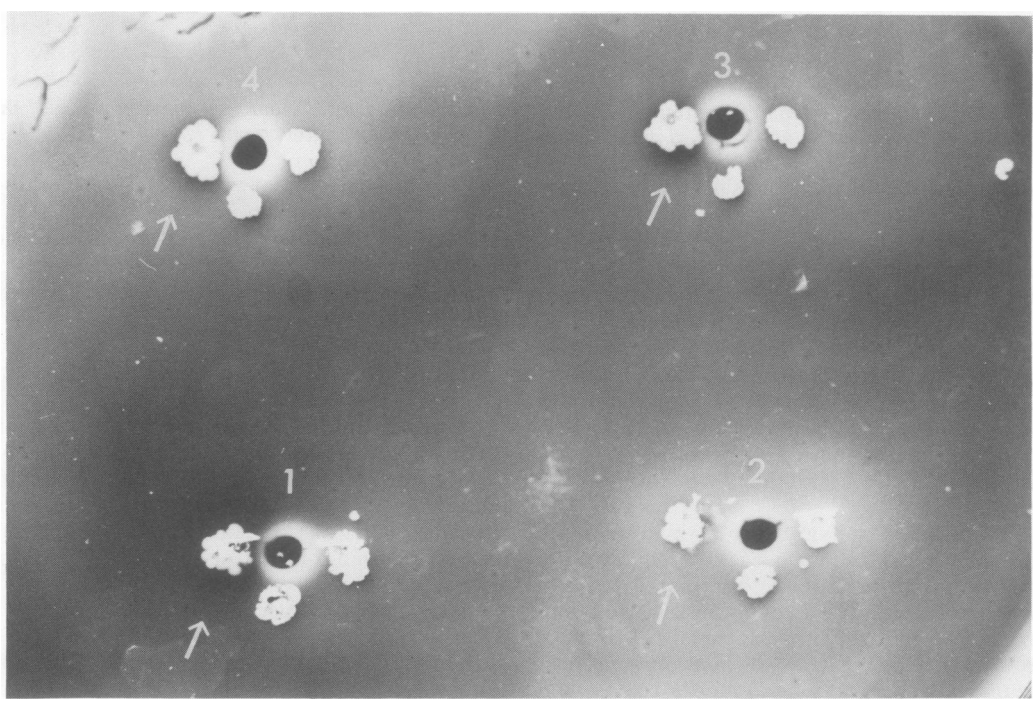

FIG. 3. *Inhibition of hyaluronidase of oral bacteria by salivary IgA and serum. The well (1) contains saline; the well (2) 50 μg of salivary IgA; the well (3) 10 μg of salivary IgA; the well (4) 10 μliters of serum. The arrows indicate hyaluronidase-producing organisms which inoculated from the same colony, and the others were hyaluronidase-nonproducing organisms.*

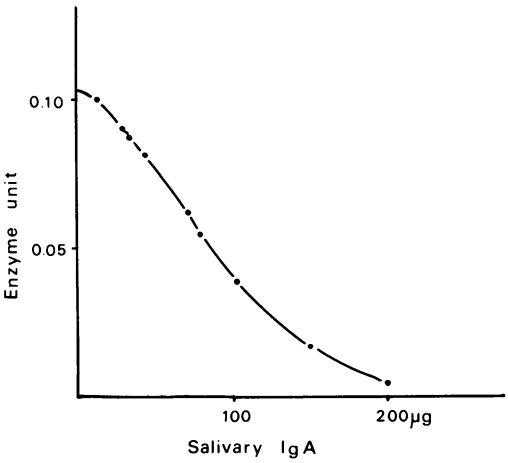

FIG. 4. *Dose curve of salivary IgA to inhibit 0.1 U of streptococcal neuraminidase. Each tube contains 0.1 U of streptococcal neuraminidase. After addition of appropriate amount of salivary IgA, enzyme activity of each tube was determined as described in Table 1.*

TABLE 4. *Comparison of inhibitory doses of salivary IgA and serum*

Sample	Inhibitory dose	
	Salivary IgA (μg)	Serum (ml)[a]
Neuraminidase from oral bacteria	80[b]	4/10[b] (6 mg IgG and 1 mg IgA)
Neuraminidase from *C. perfringens*	1,300[b]	1/100[b]
Streptolysin O	1,160[c]	1/60[c]

[a] The salivary IgA and serum were obtained from the same individual. These inhibitory doses represent mean values of three experiments.

[b] These values represent the doses to inhibit 50% activity of 0.1 unit of bacterial neuraminidase.

[c] These values represent minimum doses to inhibit 1 U of streptolysin O.

It is clear that there are considerable differences between the inhibitory activities of salivary IgA and serum, and between the activities of salivary IgA to inhibit oral and non-oral bacterial neuraminidases.

DISCUSSION

In the previous study (5) we have reported that neuraminidase activity originating from oral bacteria was not found in human whole saliva. The present study clarifies the reason for this finding, since human whole saliva inhibited completely oral streptococcal neuraminidases but not salivary neuraminidase. The inhibitory factor involved in saliva has been characterized as salivary IgA. Further, we have shown that salivary IgA inhibited the activities of hyaluronidase and chondroitin sulfatase produced by oral bacteria. Therefore, when failing to demonstrate bacterial enzymes and toxins in saliva the possibility of their inhibition by salivary IgA must be borne in mind. An enzyme may be demonstrated even under these conditions when it utilizes substrates of small size. We found that salivary IgA inhibited only 50% activity of streptococcal neuraminidase when *N*-acetylneuramine lactose was used as substrate instead of urinary mucoprotein.

Patton and Pigman (14) demonstrated the presence of an 11*S* component in parotid, submaxillary and sublingual secretions by analytical ultracentrifugation. It has also been established by Simons et al. (22) and Tomasi et al. (27) that the IgA immunoglobulin class predominates in human saliva. Moreover, a large number of reports have appeared concerning the possible relationship of the antibodies present in saliva to dental caries and periodontal disease (2, 6, 8, 10), and concerning antibacterial activity in whole saliva (3, 23, 28). This problem is extremely complex both because of limited information available concerning the character and function of salivary components and their influences on the production of caries and periodontal disease. Further studies are needed to elucidate the nature of differences in inhibitory activity of salivary IgA and to determine whether this inhibitory activity is due to local or systemic antigen stimulation. In any case, our results strongly suggest that salivary IgA may play an important role in the physiology and pathology of the oral cavity.

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