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Immunological Relationship Between Anionic Antimicrobial Proteins from Caries-Free Individuals and Known Salivary Antimicrobial Factors

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We examined whether the anionic inhibitory proteins identified in mixed saliva from certain caries-free individuals are fragments or degradation products of recognized salivary antimicrobial factors. In the experiments reported here, the anionic inhibitory proteins did not produce precipitin reactions with antisera to any of the established salivary antimicrobial factors examined. Additionally, native, heat-treated, or urea-denatured known salivary antimicrobial factors did not react with the antiserum to the anionic inhibitory proteins. However, the antiserum to the anionic inhibitory proteins was found to be reactive with a protein concentrate from mixed saliva or from separate submandibular and parotid secretions from a number of different donors, as well as with a purified protein fraction containing the homologous anionic inhibitory proteins. These findings suggest that the anionic inhibitory proteins represent intact and unique salivary proteins and not the degradation fragments of salivary antimicrobial protein factors within the oral environment.

Previous studies have shown that mixed saliva from certain caries-free (CF) individuals inhibits the growth of *Streptococcus mutans* in a saliva protein-containing medium which normally supports the growth of this organism (7). The inhibitory nature of the mixed saliva was found to be associated with four anionic protein constituents having isoelectric points (pIs) of 4.70, 4.90, 4.98, and 5.05 and displaying antimicrobial properties. The electrophoretic mobilities of the anionic inhibitory proteins (AIP) differ from those of the principal antimicrobial factors of saliva, which include lysozyme, lactoferrin, salivary peroxidase, and secretory immunoglobulin A (s-IgA) (2–4, 13, 20, 21, 23). However, this difference alone does not exclude the possibility that the AIP might represent biologically active fragments of one or more of the cationic antimicrobial proteins. This study was undertaken as part of a broader investigation, the purpose of which was to elucidate the chemical and biological nature of the AIP. The potential of these proteins to immunologically cross-react with proteins known to contribute to the antimicrobial properties of saliva or with other proteins present in mixed saliva or in separate submandibular and parotid secretions was the subject of this investigation.

MATERIALS AND METHODS

Collection and treatment of saliva. Mixed saliva obtained from two previously studied CF donors (both age 21) was clarified by centrifugation (15,000 × *g*, 20 min) and concentrated fivefold by membrane ultrafiltration (7). The saliva protein concentrates were assayed for growth inhibitory activity and then separated by preparative isoelectric focusing as described previously (7) to obtain saliva protein fractions (SPF) having pIs in the following pH ranges: A-2, 4.30 to 4.70; B-1, 4.70 to 5.40; B-2, 5.50 to 6.60; and C-1, 6.65 to 6.80. SPF B-1 possessed all of the AIP of interest. For comparative purposes, mixed saliva was also collected from one other CF donor and from two caries-active (CA) donors (ages 21 to 33). This mixed saliva was clarified by centrifugation and concentrated fivefold by membrane ultrafiltration. Separate parotid and submandibular secretions were obtained from five additional donors by direct cannulation of the respective gland with 20G Teflon catheters inside diameter, 0.63 mm; outer diameter, 107 mm; length, 3.18 cm; Becton Dickinson and Co., Paramus, N.J.) to which an additional length of sterile medical tubing had been attached. Salivary flow was stimulated by the application of sterilized 2% citric acid (3 drops at 1-min intervals) to the back portion of the tongue; secretions were collected in chilled, sterilized, graduated cylinders. A total of 2 to 3 ml of each glandular secretion was obtained over a 5-min sampling period. The parotid secretions were divided into 0.5-ml aliquots and frozen at −20°C for later analysis. The submandibular secretions were immediately treated with 0.1% EDTA, transferred to sterilized, selective-membrane dialysis sacks (3,500-dalton cutoff; Spectrum Medical Industries, Inc., Los Angeles, Calif.), and dialyzed against 200 volumes of distilled water at 4°C overnight to remove calcium ions. The resultant protein-containing samples were divided into 0.5-ml aliquots and then frozen.

Antisera. Antiserum directed against SPF B-1 from one of the two CF donors was prepared by intracutaneous injection of a rabbit with 200 μg of SPF B-1 in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), followed 10 days later by 100 μg of SPF B-1 in incomplete Freund adjuvant. Antiserum to mixed human saliva protein concentrate (MSPC) was obtained after the administration of two injections, 2 weeks apart, of 2.5 mg of lyophilized, pooled saliva (in incomplete Freund adjuvant) from two CA donors. Myeloperoxidase (MPO) was isolated from human leukocytes as described by Bakkenhist et al. (5) and further purified by gel permeation and ion-exchange chromatography (19). The antiserum was prepared by the injection of 1 mg of MPO in complete Freund adjuvant, followed 2 weeks later by 1 mg of MPO in incomplete Freund adjuvant. Anti-bovine milk lactoperoxidase (LPO) was prepared by an

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initial injection of 1 mg of bovine milk LPO (Sigma Chemical Co., St. Louis, Mo.) in complete Freund adjuvant, followed by 2 more injections, at 2-week intervals, of 1 mg of LPO in incomplete Freund adjuvant and 1 mg of LPO in saline. For preparation of each of the antisera, rabbits were bled 2 weeks after the final antigen injections.

Human lysozyme, lactoferrin, IgG, s-IgA, α-amylase, and antisera to human IgG (heavy- and light-chain specific; gamma-chain specific) and α-amylase were obtained from Sigma Chemical Co. Anti-human lysozyme and anti-human lactoferrin were obtained from Accurate Scientific (DAKO), Westbury, N.Y. Anti-human immunoglobulin (light-chain specific; type kappa) and anti-free immunoglobulin (light-chain specific; type lambda) were obtained from Calbiochem-Behring, La Jolla, Calif. Antiserum to secretory component s-IgA and to human colostrum IgA (alpha-chain specific) were obtained from Bio-Rad Laboratories, Richmond, Calif. The specificity of the antigens and the antisera making up the test panels were determined by immunoelectrophoresis. The antiserum to MSPC produced multiple precipitin bands against MSPC, whereas the reaction between SPF B-1 and its antiserum produced multiple precipitin arcs by immunoelectrophoresis but only one precipitin band by immunodiffusion. This latter observation is consistent with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis findings, which indicated that SPF B-1 from both CF donors contained proteins having apparent molecular weights of 14,000 to 17,000. The antiserum to MSPC was also found to react with lactoferrin, s-IgA, and α-amylase. IgG and s-IgA exhibited cross-reactivity when tested against anti-IgG or anti-IgA. These proteins were distinguished on the basis of immunoelectrophoretic mobility or reaction with alpha- or gamma-chain-specific antisera. s-IgA also reacted with anti-lactoferrin, which was present as a known contaminant in the preparation. All of the other antisera used in this study produced precipitin arcs only with the homologous antigen. The specificity of the antiserum to free immunoglobulin (light-chain specific; type lambda) was verified by a negative reaction with a Bence-Jones protein (type kappa) isolated from the urine of a patient with multiple myeloma. Double immunodiffusion tests were also performed to determine the optimum antigen-antibody dilutions for precipitation.

Immunoelectrophoresis. Glass plates (84 by 94 mm) were coated with 1% agarose in 0.06 M barbital buffer, pH 8.6 (Sigma Chemical Co.), to produce 1-mm-thick gels. Wells (2.5-mm diameter) were filled with 5 μl of different test antigens. The antigens were tested at the following concentrations (milligrams of dry weight per milliliter): MSPC, 4.0 mg; SPF, 0.5 mg; and all other proteins, 1.0 mg. Electrophoresis was carried out at 300 V for 3 h at 10°C at a constant current with an LKB 2117 Multiphor system (LKB, Bromma, Sweden). Antiserum troughs were filled with 100 μl of the desired antiserum (diluted 1:4 to 1:8), after which the plates were incubated in a moist chamber for 48 h at 4°C. After incubation, the plates were soaked overnight in two changes of 0.9% saline and then for 1 h in each of two changes of distilled water. They were then dried and stained with 0.1% Coomassie brilliant blue R-250.

Dot blotting. A dot immunobinding assay (12) was used. Antigen concentrations were as described above. Each antigen solution (10 μl) was spotted onto nitrocellulose sheets in a microsample filtration manifold (Schleicher & Schuell Inc., Keene, N.H.). After being dried, the membranes were treated for 1 h with a blocking solution of 3% gelatin in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]). Filters were subsequently incubated for 2 h in 1% gelatin in TBS containing a 1:100 dilution of the test antiserum. After two 10-min washes in TBS with 0.05% Tween 20, the membranes were incubated for 1 h in 1% gelatin in TBS containing a 1:2,000 dilution of affinity-purified goat antirabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories). After two more washes in TBS with 0.05% Tween 20, the membranes were incubated in a solution of 0.015% H_2O_2 and 2.8 mM 4-chloro-1-napthol in TBS with 16% methanol. Development of the color reaction was terminated by immersion of the membranes in distilled water.

Immunofixation. Analytical isoelectric focusing of test saliva preparations was performed with LKB thin-layer polyacrylamide gels containing ampholines in the pH range of 3.5 to 9.5. The samples (60 to 100 μg [dry weight]/20 μl) were applied in duplicate directly on the gel surface. An isoelectric focusing standard (Pharmacia Fine Chemicals, Piscataway, N.J.) consisting of 11 proteins with pIs between pH 3.5 and pH 9.3 was applied and run concurrently with the test samples. Electrophoresis was conducted at a constant power (8 W) for 90 min at 10°C. After electrofocusing was complete, the gel was divided into two sections. One section was removed, fixed in 12.5% trichloroacetic acid–30% methanol, and then stained with Coomassie blue for visualization of the electrophoretic protein patterns. The remaining gel section was left on the cooling plate and used for immunofixation. In this procedure, Whatman no. 1 filter paper strips (0.6 by 10 cm) which had been soaked in a 1:5 dilution of anti–SPF B-1 in normal saline containing 3% polyethylene glycol (molecular weight, 6,000) were placed on the gel surface in areas corresponding to each of the sample lanes. The gel sandwich was then removed and incubated in a moist atmosphere for 15 min, after which the strips were removed and soaked overnight in two changes of saline. They were subsequently washed for 1 h in each of two changes of distilled water, stained with Coomassie blue, and destained with 25% ethanol–7.5% acetic acid. Areas of staining on the strips were matched with the electrophoretic protein patterns to identify the pH region associated with protein reactivity to the antiserum. The pIs of the individual protein bands in the electrophoretic patterns were determined by comparison with a plot of pH versus distance of migration (to the nearest 0.1 mm) prepared with the isoelectric focusing calibration standard.

SDS-polyacrylamide gel electrophoresis. Discontinuous vertical gel slab SDS-polyacrylamide electrophoresis was performed with a 10% resolving gel (pH 8.8) containing 0.1% SDS and a 3% stacking gel (pH 6.8) containing 0.02% SDS. SPF B-1 fractions (50 to 100 μg [dry weight]) from each of the two CF donors were solubilized in 50 μl of SDS sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, 0.05 M Tris [pH 6.8]) and heated for 2 min at 100°C. Electrophoresis was conducted with 0.1% SDS–0.05 M Tris-glycine buffer (pH 8.3) at 20 mA per gel. Gels were fixed and stained with Coomassie blue. After being destained, the gels were soaked for 18 h in four changes of deionized water and then silver stained (Bio-Rad Laboratories silver staining kit). Migration distances of zones appearing after Coomassie blue or silver staining were measured, and apparent molecular weights were determined by comparison with molecular weight standards (Sigma Chemical Co.) run concurrently with the test samples.

RESULTS

We first examined the reactivity of the MSPCs from CA and CF donors or the electrophoretically separated SPF

TABLE 1. Reactivity of unfractionated MSPCs from CA and CF donors or SPF from CF donors with various antisera

Antiserum to:	Reactivity with[a]:								
	MSPCs					SPF			
	CF A[b]	CF B[b]	CF C	CA A	CA B	A-2	B-1	B-2	C-1
MSPC	+	+	+	+	+	+	+	+	+
SPF B-1	+	+	+	+	+	NR	+	NR	NR
Lysozyme	+	+	+	+	+	NR	NR	NR	NR
Lactoferrin	+	+	+	+	+	NR	NR	NR	NR
LPO (bovine milk)	+	+	+	+	+	NR	NR	NR	NR
MPO	+	+	+	+	+	NR	NR	NR	NR
s-IgA (α)	+	+	+	+	+	NR	NR	NR	NR
Secretory piece	+	+	+	+	+	NR	NR	NR	NR
IgG (heavy and light)	+	+	+	+	+	NR	NR	NR	NR
IgG (γ)	+	+	+	+	+	NR	NR	NR	NR
Light-chain immunoglobulin (kappa)	+	+	+	+	+	NR	NR	NR	NR
Light-chain immunoglobulin (lambda)	NR	NR	NR	NR	NR	NR	NR	NR	NR
α-Amylase	+	+	+	+	+	NR	NR	+	+

[a] MPSCs were from various donors (A, B, and C); SPF were from CF donors A and B; fraction B-1 (pI between pH 4.70 and pH 5.40) contained the AIP. +, Positive reaction; NR, no reaction.

[b] MSPCs from these donors (A and B) possessed growth-inhibiting properties for *S. mutans* and *S. sanguis*.

derived from the MSPCs from the two CF donors with antisera to MSPC, the AIP-containing SPF B-1, or proteins representing the principal antimicrobial factors of saliva. Anti–α-amylase was included in the test panel as a positive control. The results of the immunoelectrophoretic reactions are shown in Table 1. MSPCs from each of the CF and CA donors reacted with antiserum to MSPC or SPF B-1. Each MSPC also reacted with antisera to human lysozyme, lactoferrin, secretory piece, s-IgA, IgG, and MPO and bovine milk LPO (detected only by the dot immunobinding assay), indicating that all of these factors were originally present in the donor saliva. Within the sensitivity limits of the immunodiffusion tests, the MSPCs of the two groups displayed no discernable differences in their reactivities with the different antisera.

The separate SPF that had pIs between pH 4.30 and pH 6.80 and that were derived from the two CF donors did not react with antisera to any of the known salivary antimicrobial factors. Although each of these SPF did react with anti-MSPC, only SPF B-1 (pI between pH 4.70 and pH 5.40) reacted with anti-SPF B-1. The reactions of the noninhibitory SPF B-2 and C-1 with anti–α-amylase were consistent with the known occurrence of α-amylase in these fractions. Although these results suggest that the AIP were not related to known salivary antimicrobial factors, additional possibilities had to be considered to explain the nonreactivity. One is that the AIP might represent fragments possessing altered conformational determinants of the original protein. Another is that they may possess antigenic determinants not normally exposed in the native protein structure. Therefore, we next examined the reactivity of native or denatured proteins representing the principal salivary antimicrobial factors as well as native or denatured MSPCs and SPF B-1 with antisera directed against the AIP (anti-SPF B-1). Additionally, the native or denatured proteins were tested for reactivity with antisera to the various known salivary antimicrobial factors. The proteins were denatured by being heated to 60°C for 30 min or by treatment with urea. The immunological reactivity of the various samples was evaluated by immunoelectrophoresis and dot blotting. The results revealed that within the denatured MSPC preparations there were components which still retained immunological reactivity with anti-SPF B-1 (Table 2). Heated samples of SPF B-1 also reacted with the antiserum, but samples denatured by 10.0 M urea were immunoelectrophoretically nonreactive. These preparations, however, reacted in the dot immunobinding assay. All of the known antimicrobial proteins tested maintained their reactivity with their homologous antisera after denaturation, although to a lesser extent. None of the known antimicrobial proteins tested were reactive with the antiserum to SPF B-1 under any of the study conditions.

In these studies it was observed that MSPCs from both CA and CF donors reacted with the antiserum to the AIP (anti-SPF B-1) from CF donors. Because the CA MSPC was known to be noninhibitory for oral streptococci and because the immunological reactivity of the CF MSPC was specifically limited to the SPF (B-1) containing the inhibitory proteins with pIs between pH 4.70 and pH 5.40, the specificity of the reaction with the CA MSPC was investigated. The CA MSPC was subjected to analytical isoelectric focusing followed by immunofixation with anti-SPF B-1. An examination of the immunofixed strips revealed a diffuse area of staining confined to a region corresponding to six proteins having pIs between pH 4.70 and pH 5.20. In

TABLE 2. Effect of denaturing conditions on the reactivity of various antigens with antiserum to the AIP

Antigen[a]	Reactivity with antiserum determined by[b]:			
	Immunoelectrophoresis		Dot blotting	
	AIP	Known proteins	AIP	Known proteins
MSPC	+	+	+	+
Heated MSPC	+	+	+	+
Urea-treated MSPC	+	+	+	+
SPF B-1	+	NR	+	NR
Heated SPF B-1	+	NR	+	NR
Urea-treated SPF B-1	NR	NR	+	NR
Known proteins	NR	+	NR	+
Heated known proteins	NR	+	NR	+
Urea-treated known proteins	NR	+	NR	+

[a] For heat treatments, samples (20 μl) were heated at 60°C for 30 min. For urea treatments, solid, recrystallized urea was added, to a final concentration of 10 M, to 100 μl of test sample containing 0.1% dithiothreitol.

[b] The known proteins tested included human lysozyme, lactoferrin, s-IgA, IgG, and MPO and bovine milk LPO. +, Positive reaction; NR, no reaction.

comparison, electrofocused CF and CA MSPCs immunofixed with antiserum to MSPC each showed defined bands of staining corresponding to pIs of pH 6.50 and pH 5.80, respectively. There was also a continuous area of staining between pH 4.70 and pH 5.90 which was consistent with 12 to 17 protein bands identified in the corresponding gels.

To further establish that the AIP were of glandular origin, the immunological reactivity of cannulated parotid and submandibular secretions with anti-SPF B-1 was examined. In these tests, four of the five submandibular secretions and all of the parotid secretions reacted with the antiserum. Double immunodiffusion tests with submandibular and parotid secretions and SPF B-1 against anti-SPF B-1 produced a reaction of identity between the test samples.

DISCUSSION

The major aim of this study was to obtain information as to whether the AIP identified in the saliva of certain CF individuals (7) might represent fragments or degradation products of one or more of the proteins normally considered to contribute to the antimicrobial properties of saliva. Based on their apparent molecular weights (14,000 to 17,000), as determined by SDS-polyacrylamide gel electrophoresis, it is conceivable that the AIP could represent degradation fragments of any of the established salivary antimicrobial proteins, except for lysozyme. In the experiments reported here, we were not able to demonstrate any reactions either between the SPF containing the AIP and antisera to any of the established inhibitory proteins of saliva or between the latter proteins and an antiserum to the AIP (anti-SPF B-1). In contrast, the finding that the AIP antiserum was reactive with proteins in the separate glandular secretions from a number of different donors lends strong support to the argument that the AIP represent intact salivary proteins and not the degradation fragments of a glandular secretion within the oral environment.

It can be argued that the likelihood of a reaction between the AIP and antisera to the recognized salivary antimicrobial factors is not great if the AIP represent fragments, as alteration of conformational determinants frequently results in a loss of antibody reactivity directed against the native protein (6, 14, 15). There is a greater likelihood, however, that the antiserum to the AIP would recognize antigenic sites on the native protein, although with diminished antibody-binding affinity (14, 15). Our experiments designed to test these possibilities yielded negative results in precipitin reactions and in a dot immunobinding assay.

Because urea is known to destabilize protein conformation, resulting in protein unfolding (11), it was anticipated that these experiments would reveal whether the AIP possessed antigenic determinants for sites which normally are not exposed in the native conformations of the salivary antimicrobial factors. The antiserum to AIP did not react with any of the nontreated or denatured known inhibitory proteins. The AIP–anti-AIP experiments revealed that the AIP were sensitive to urea denaturation, as evidenced by the loss of immunoelectrophoretic reactivity at antiserum dilutions which yielded precipitin arcs with the nondenatured proteins. These findings further support the premise that the AIP likely represent intact salivary proteins and not fragments or degradation products of known salivary antimicrobial factors.

The urea-denatured AIP, however, did react with the antiserum to the native AIP in the dot immunobinding assay. It is possible that during the assay the concentration of urea was reduced to a level which permitted sufficient protein refolding to account for the reactivity with the antiserum. Additionally, as the antiserum used in these studies was not monospecific, the possibility that it contained antibody subpopulations recognizing determinants other than those for the native conformations of the proteins cannot be ruled out.

The immunological reactivity of mixed saliva or separate glandular secretions from CA as well as CF individuals with the antiserum to the AIP is consistent with the known occurrence of protein constituents in these secretions having pIs identical to those determined for the AIP (7–10). Although the electrophoretic protein patterns of the salivary secretions of CA and CF individuals may appear similar (1, 16–18, 22), previous studies (7, 8, 10) have shown that proteins in these secretions having identical pIs may, nonetheless, exhibit quite different biological properties. Confirmation that the salivary secretions of CA and CF individuals contain closely related proteins with only minor structural differences which confer different biological properties could provide important new insights into the relevance of the salivary proteins as determinant factors of oral microbial ecological relationships and ultimately of an individual's inherent susceptibility to dental caries.

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