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ANTIMICROBIAL PROTEINS IN HUMAN UNSTIMULATED WHOLE SALIVA IN RELATION TO EACH OTHER, AND TO MEASURES OF HEALTH STATUS, DENTAL PLAQUE ACCUMULATION AND COMPOSITION

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Summary—Saliva antimicrobial proteins may interact in a common system to influence the oral ecology. Clinical studies of antimicrobial protein action thus may require a multiple-protein approach. Multivariate statistical methods have been used to describe possible patterns of interaction for lysozyme, lactoferrin, salivary peroxidase and secretory IgA in stimulated parotid saliva. However, oral microbes are most likely to encounter antimicrobial proteins in mixed resting saliva. Relationships among levels of lysozyme, lactoferrin, salivary peroxidase, and secretory IgA therefore were investigated in whole saliva from 216 subjects, and an attempt made to relate interperson variation in those proteins to differences in health and status, and dental plaque accumulation and composition. All proteins were significantly ($\alpha = 0.05$) correlated with each other ($r = 0.38$–0.52, $p < 0.001$). There was only one axis of common variation among proteins, and that axis was significantly correlated ($p < 0.001$) with total protein ($r = 0.84$) and flow rate ($r = -0.56$). That pattern deviated from the previous finding that proteins of acinar origin tended to vary independently from proteins of ductal origin in stimulated parotid saliva. The difference between parotid and whole saliva may reflect constitutive secretion of all proteins at low levels of stimulation. Common variation of unstimulated saliva proteins suggests that antimicrobial actions can be compared in subjects at population extremes. There were no significant associations between antimicrobial proteins in whole saliva and measures of health status or plaque accumulation. However, the proportions of *Streptococcus sanguis* were significantly correlated with lysozyme ($r = -0.26$), lactoferrin ($r = -0.34$), peroxidase ($r = -0.30$), total protein ($r = -0.37$), flow rate ($r = 0.24$) and principal-components scores ($r = -0.33$) in a subset of subjects ($n = 85$) where commercial biochemical tests were used to supplement species identification by colony morphology. Those findings may indicate that saliva antimicrobial proteins can affect the composition of dental plaque.

Key words: saliva, lysozyme, lactoferrin, peroxidase, sIgA, plaque, *Strep. sanguis*.

INTRODUCTION

Saliva contains a number of proteins with antimicrobial effects *in vitro*. Four of the best known are lysozyme, lactoferrin, salivary peroxidase and secretory IgA (Mandel, 1987). Each displays multiple actions in the laboratory. Effects include bacterial aggregation with inhibition of adherence to hydroxyapatite [lysozyme, sIgA (Liljemark, Bloomquist and Ofstenhage, 1979; Golub *et al.*, 1985; Tellefson and Germaine, 1986; Reinholdt and Kilian, 1987)], bacteriolysis or membrane damage [lysozyme, lactoferrin (Laible and Germaine, 1985; Pollock *et al.*, 1987; Ellison, Giehl and LaForce, 1988)], inhibition of metabolism [peroxidase, lactoferrin (Arnold *et al.*, 1982; Pruitt and Reiter, 1985)] and cell killing [lysozyme, lactoferrin, peroxidase (Arnold *et al.*, 1982; Laible and Germaine, 1985; Pruitt and Reiter, 1985)].

Synergistic or antagonistic interactions among antimicrobial proteins can modify their effects. Lactoferrin and sIgA both appear to increase production of bacteriostatic hypothiocyanite ion by peroxidase (Tenovuo *et al.*, 1982), and sIgA against bacterial iron-binding proteins may enhance bacteriostatic iron sequestration by lactoferrin (Ellison *et al.*, 1988). On the other hand, bactericidal actions of lactoferrin, which may involve the production of $OH^{\cdot}$ radical, can be blocked or enhanced by sIgA specific to particular species (Cole *et al.*, 1976; Lassiter *et al.*, 1987; Motley and Arnold, 1987). Polycationic antimembrane effects of lysozyme may be enhanced by peroxidase-generated hypothiocyanite (Pollock *et al.*, 1979; Pollock *et al.*, 1987), but peroxidase itself may non-specifically block bactericidal effects of lactoferrin (Lassiter *et al.*, 1987). Lysozyme and lactoferrin appear to bind sIgA, which could direct those proteins to specific bacterial targets (Germaine and Tellefson, 1979; Watanabe *et al.*, 1984). All three bind to bacteria-aggregating mucin glycoproteins (Tabak *et al.*, 1982), and sIgA may enhance the actions of the recently described parotid saliva agglutinins (Rundegren and Arnold, 1987). The histatin family of cationic peptides may enhance polycationic effects of lysozyme (MacKay *et al.*, 1984), and peroxidases also

might enhance those effects by catalysing formation of lysozyme polymers (Drozdz, Naskalski and Sznajd, 1988).

The complexity of antimicrobial protein actions and interactions makes it difficult to understand the role of these proteins in oral health and ecology. Most studies have compared salivary levels of particular proteins in persons differing on some measure of oral health. However, persons with similar levels of one protein might be dissimilar for proteins that affect the action of the first. That would tend to limit detection of single-protein effects. Clinical studies of antimicrobial proteins thus may require that multiple proteins be considered as elements of a general defence system. That in turn requires a means for describing how antimicrobbial proteins vary together in a study population. We previously have used multivariate statistical methods to approach this problem, using stimulated parotid saliva from a pilot population of 44 dental students (Rudney and Smith, 1985), and a larger population of 198 dormitory residents (Rudney, 1989). Principal-components analysis was used to define patterns of common variation among lysozyme, lactoferrin, salivary peroxidase, and sIgA. The results suggested that antimicrobial proteins of acinar and ductal origin tended to vary independently of one another. A stronger stimulus was used with dormitory residents, and this appeared to increase acinar production of some proteins considered to be ductal (Rudney, 1989). Groups of subjects with distinct antimicrobial protein concentration profiles were identified by cluster analysis. There was some indication of an association between the health status of the subject and levels of antimicrobial proteins, as high-concentration clusters included higher proportions of subjects with symptoms of respiratory infection (Rudney, 1989).

Our working hypothesis has been that subjects with different profiles of antimicrobial protein concentrations may show different patterns of antimicrobial protein interaction, which may reflect distinct patterns of antimicrobial protein action. However, stimulated parotid saliva may not provide the best medium for *in vivo* tests of that hypothesis. Encounters between antimicrobial proteins and dental plaque bacteria are most likely to occur in whole saliva (Malamud, 1985). Moreover, unstimulated secretion predominates during sleep and most waking activities (Dawes, 1987). Relationships among antimicrobial proteins in mixed resting saliva thus may be more relevant to clinical and ecological studies. We have now addressed this issue by extending our studies to expectorated whole saliva from a large group of subjects. Our initial plan was to focus on three major questions. Firstly, what are the relationships among lysozyme, lactoferrin, salivary peroxidase and sIgA in expectorated whole saliva? Secondly, is the pattern of those relationships similar to that seen for stimulated parotid saliva? Finally, can differences in levels of antimicrobial proteins in expectorated whole saliva be related to differences in health status, plaque accumulation, and plaque composition? Two years were required to obtain the large number of samples required for statistical analysis, and some inadvertent changes in methods, instrumentation, and personnel occurred between year 1 and year 2. Those changes affected spectrophotometric assays (peroxidase, protein and $OSCN^-$), sIgA immunoassays and bacterial counts. This led us to add an unplanned question—would results for each year be affected by the methods used to obtain them?

MATERIALS AND METHODS

Study population

The population size was set at 264, to be comparable to the study with parotid saliva of Rudney (1989). A random sample of 128 students was drawn from a coeducational student dormitory in 1988. A second random sample of 136 students was drawn from a different dormitory in 1989. Procedures approved by the University of Minnesota Committee on the Use of Human Subjects in Research were used to obtain informed consent. An 87% response was achieved, and complete data were obtained from 216 subjects. That group included 106 women and 110 men aged 18–23 yr.

Saliva collection

Saliva was collected between 3:00 and 6:00 p.m. on Mondays. One sample was obtained for each subject over the 2-yr period. Subjects were asked not to eat, chew gum, use breath mints, sweets or tobacco, or drink anything but water for 1 h before their appointment. Subjects were instructed to swallow once when collection began, retain saliva until they would normally wish to swallow, and then spit into a volume-calibrated ice-cooled polypropylene tube. Retention and spitting was repeated without further swallowing until 5 ml were obtained or 30 min had elapsed. Collection time was measured from initial swallow to target volume, and actual sample volume was measured for determination of flow rate. Subjects were asked to refrain from talking or mechanical stimulation of salivary flow until collection was completed.

Processing of saliva samples

Whole saliva is often centrifuged to remove exogenous material. However, centrifugation may lead to substantial underestimation of some proteins (Jenzano, Hogan and Lundblad, 1986). In preliminary studies, we compared the supernatant of samples centrifuged at 15,000 rev/min for 15 min to uncentrifuged portions of the same samples treated with EDTA to a final concentration of 10 mM/ml (centrifuged portions used for lysozyme assay were acid-treated to release bound lysozyme). Uncentrifuged portions were kept at 6°C or on ice at all times, and vortexed immediately before use. Samples for assay were taken with positive-displacement pipettes. Concentrations for centrifuged and uncentrifuged portions were correlated at $r \geqslant 0.90$ for all protein assays. However, lysozyme, lactoferrin and peroxidase concentrations were two-fold higher in uncentrifuged portions (sIgA was only slightly higher). Uncentrifuged samples treated in the manner described above therefore were used in all further studies.

Peroxidase assays

Total peroxidase was determined by the method of Mansson-Rahemtulla *et al.* (1986), as described

in Rudney (1989). Bovine lactoperoxidase (Sigma, St Louis, MO, U.S.A.) was used as a standard. Samples were screened for neutrophil myeloperoxidase by replacing KSCN with 150 mM KCl (Mansson-Rahemtulla *et al.*, 1986). Samples were run with buffer in place of KCl as a control for oxidation of endogenous SCN^- by myeloperoxidase and salivary peroxidase. Buffer activity was subtracted from KCl activity to estimate the portion attributable to myeloperoxidase. Adjusted KCl activity was then expressed in KSCN units by means of a regression equation obtained through studies of purified human neutrophil peroxidase (Calbiochem, San Diego, CA, U.S.A.). A Beckman model 44 spectrophotometer (Beckman, Fullerton, CA, U.S.A.) was used for peroxidase assays during the first year of study. Kinetic change was determined by measurement of slopes recorded on chart paper over 30 s. That instrument failed after the first year. It was replaced by a Beckman model DU-68, which processed kinetic information electronically. That allowed slopes to be determined for the first 10 s of change.

Other spectrophotometric assays

Endogenous hypothiocyanite ion was determined by the method of Thomas, Bates and Jefferson (1980) within 1 h of saliva collection. Total protein was determined by the method of Lowry *et al.* (1951) as described in Rudney and Smith (1985). The change in spectrophotometers described above also applied to these assays.

Immunoassays for salivary proteins

Sensitive, avidin–biotin sandwich, enzyme-linked immunoadsorbent assays (EIA) were used to quantify lysozyme and lactoferrin. sIgA was determined by single radial immunodiffusion in the first year (Mancini, Carbonara and Heremans, 1965; Rudney, 1989). A number of samples exceeded the range of the standard curve, so that assay was replaced by an avidin–biotin procedure in the second year. The lysozyme assay was first described by Francina *et al.* (1986). We adapted their procedure for use with lactoferrin and sIgA. The general protocol is outlined below; specifications for particular proteins are noted as they occur. Buffers were as described in Francina *et al.* (1986). except that 0.2 and 0.02% sodium azide were added to coating and diluent buffers, respectively. We accordingly used an avidin–alkaline phosphatase conjugate in place of avidin–horseradish peroxidase. EIA plates (Costar, Cambridge, MA, U.S.A.) were coated with 100 μl of capture antibody. Final dilutions were 1 μg/ml for rabbit anti-human lactoferrin IgG fraction (Cappel, Malvern, PA, U.S.A.) and rabbit anti-human alpha chain (Dako, Santa Barbara, CA, U.S.A.); 10 μg/ml for rabbit anti-human lysozyme (Dako). Coated plates were incubated overnight in a moist container at 37°C, and then washed three times at low intensity with a Microwash II plate washer (Skatron, Sterling, VA, U.S.A.). That wash cycle was used at all succeeding steps; reagents always were added in volumes of 100 μl. After washing, each plate received diluent buffer alone in columns 1 and 12, six dilutions of protein standard (one for each of columns 2–7), and two dilutions of two saliva samples (columns 8–11). Standard ranges were 2–10 ng/ml of human milk lysozyme (United States Biochemical, Cleveland, OH, U.S.A.), 0.25–7.5 ng/ml of human colostral lactoferrin (Cappel), or 0.5–10 ng/ml of human colostral sIgA (Sigma). Saliva dilutions for lysozyme and lactoferrin were 10,000- and 20,000-fold; dilutions for sIgA were 50,000- and 100,000-fold. Plates were incubated for 2 h at 37°C, and then received a 1000-fold dilution of capture antibody conjugated to biotin in a ratio of 0.2:1 by weight, according to the procedure of Francina *et al.* (1986). After a second 2-h, 37°C incubation, plates received a 2000-fold dilution of 1 mg/ml avidin–alkaline phosphatase conjugate (Cappel) for 1 h at 37°C. Plates then received 1 mg/ml Sigma 104 phosphatase substrate in 0.1 M sodium carbonate (pH 9.8) with 1 mM $MgCl_2$ for 1–2 h at room temperature. The reaction was stopped with 1N NaOH, and plates were read at 405 nm with a Model 2230 microplate reader (Bio-Rad, Richmond, CA, U.S.A.). Sample concentrations were determined by linear regression of log average column absorbance against log standard concentration. R-squared values for log–log standard curves typically were $\geqslant 0.98$; column coefficients of variation ranged between 10–20%. Correlations between sample dilutions were >0.90. Correlations with lysozyme, lactoferrin and sIgA (radial immunodiffusion) assays used in Rudney (1989) were 0.90, 0.77 and 0.93 in preliminary studies.

Questionnaire

Information on variables that might affect salivary function or concentrations of antimicrobial proteins was obtained with a questionnaire at the time of saliva collection. Items incuded age, sex, ethnic background, symptoms of respiratory/gastrointestinal infection, chronic disease, medications, tobacco, menstrual cycle and subject compliance with instructions for saliva and plaque collection (Mandel and Wotman, 1976; Tenovuo *et al.*, 1981; Rudney, 1989).

Intraoral procedures

Subjects were instructed to brush their teeth on arising without using dental floss or mouthwash, and then refrain from oral hygiene until after their appointment. Subjects completing a saliva collection were examined for clinical signs of respiratory infection. Supragingival plaque was then obtained as described below. Subjects then rinsed with disclosant dye (Butler, Chicago, IL, U.S.A.), and were scored for the PHP-M plaque index of Martens and Meskin (1972).

Microbiological procedures

Supragingival plaque was removed from buccal surfaces of right and left lower first permanent molars with a sterile curette. Plaque from both sides was pooled in pre-reduced VPI diluent (Holdeman and Moore, 1977), dispersed by sonication, and 10-fold serial dilutions plated on supplemented sheep blood and mitis salivarius agar (Di-Med, St Paul, MN, U.S.A.) with a spiral plater (Spiral Systems, Cincinnati, OH, U.S.A.). Blood plates were incubated anaerobically for 1 week at 37°C. Mitis salivarius plates were incubated anaerobically for 64 h, with an additional 24 h aerobically at room temperature. Simple counts made were made for total cultivable

flora and total streptococci. *Streptococcus sanguis*, *Strep. mutans*, and *Strep. salivarius* colonies were enumerated according to their morphology. The technician first assigned to make counts left the project at the end of the first year, so second-year counts were made by a different person (the first enumerator assisted in training the second). The Rapid Strep system (API, Plainview, NY, U.S.A.) was introduced in year 2 to facilitate training of the new enumerator. This system of biochemical tests was used to cross-check species identifications based on colony morphology (Coykendall, 1989).

Data analysis

Statistical software and procedures were as described in Rudney (1989). Distributions first were tested for skewness. Power transformations were evaluated for skewed variables, and transformations effecting the greatest reduction in skewness were used in further analyses. As noted above, there were some inadvertent changes in methods, personnel, and instrumentation between the first and second year of study. *t*-Tests therefore were used to compare mean values between years. Some differences were found. Multivariate analyses accordingly were run separately for each year, and then for both years combined. In each case, relationships among saliva variables were first evaluated with Pearson correlation coefficients. Partial correlation then was used to estimate the effect of flow rate and total protein on antimicrobial protein correlations. Principal-components analysis were used to define major axes of common variation among antimicrobial proteins. Cluster analysis then was used to define groups of subjects with similar profiles of antimicrobial protein concentrations. Those groups were compared for questionnaire, plaque and microbiological variables by analysis of variance, nonparametric tests, or χ^2 as appropriate. Correlations of those variables with principal-components axes were also determined.

RESULTS

Summary statistics

Table 1 presents summary statistics for saliva variables, and indicates the transformations that yielded the closest approximation to a normal distribution (all variables were skewed). sIgA values were significantly higher in year 1. That seemed due to the change in methods, as radial immunodiffusion gave higher values when compared with the avidin–biotin assay in a subset of 24 samples (values were correlated at $r = 0.93$). The spectrophotometer used in year 1 gave much lower peroxidase activities for both samples and standards. Yearly differences were greatly reduced (although still significant) when total peroxidase was standardized to bovine lactoperoxidase. The new spectrophotometer yielded fewer out-of-range values for total protein, although yearly means were not significantly different. No yearly differences were

Table 1. Summary statistics for saliva variables in 216 subjects

Variable	Mean ± SD	Skewness[a]	Range[b]	Transform[c]
Flow rate (ml/min)	0.33 ± 0.16	1.07	0.04–0.94	Square root
Total protein (mg/ml)[d]				
Year 1	2.60 ± 1.19	2.38	0.71–8.78	log
Year 2	2.49 ± 0.81	1.02	1.16–5.88	log
Lysozyme (μg/ml)[e]	53.44 ± 28.36	1.33	8.02–188.60	log
Lactoferrin (mg/dl)[e]	1.09 ± 0.68	4.69	0.38–7.27	Reciprocal
Total peroxidase[f]				
Year 1				
Activity	1770 ± 673	1.25	206–3945	Square root
Standardized	5.10 ± 2.86	2.05	0.16–15.65	Square root
Year 2				
Activity	7842 ± 2667	0.62	2230–16,219	Square root
Standardized	7.53 ± 34.51	1.39	1.01–26.66	Square root
Estimated myeloperoxidase[g]				
Year 1 activity	569 ± 563	2.05	0–3363	Square root
Year 2 activity	1865 ± 1855	1.30	0–8441	Square root
sIgA[h]				
Year 1	24.26 ± 10.96	0.99	7.91–58.30	log
Year 2[e]	19.62 ± 8.41	2.27	8.38–58.07	log

[a]A measure of deviation from normality. Skewness equals zero in a normal distribution (Sokal and Rohlf, 1981).

[b]Minimum and maximum values obtained.

[c]Power transformation providing the closest approximation to a normal distribution. All further analyses done with transformed variables (Sokal and Rohlf, 1981).

[d]Different spectrophotometers in year 1 ($n = 107$) and year 2 ($n = 109$).

[e]Values reported are for the higher of two saliva dilutions used in avidin–biotin, enzyme-linked immunoadsorbent assay.

[f]Different spectrophotometers used in year 1 and year 2. Activities expressed as μM of reduced 5,5′-dithiobis (2-nitrobenzoic acid) ml^{-1} min^{-1} (Mansson-Rahemtulla *et al.*, 1986). Standardized values expressed as μg of bovine lactoperoxidase/ml (Rudney, 1989).

[g]Different spectrophotometers used in year 1 and year 2. Activities expressed as for total peroxidase; linear regression was used to estimate activity that would be obtained with KSCN as a substrate (Mansson-Rahemtulla *et al.*, 1986).

[h]Assayed by radial immunodiffusion in year 1; avidin–biotin, enzyme-linked immunoadsorbent assay in year 2.

Table 2. Correlations (Pearson's r) for saliva variables in 216 subjects

Variable	sIgA	Lysozyme	Lactoferrin	Salivary peroxidase	$OSCN^-$	Flow rate
Lysozyme	0.47*					
Lactoferrin	0.48*	0.39*				
Salivary peroxidase	0.42*	0.42*	0.52*			
$OSCN^-$	−0.01	0.05	−0.07	0.04		
Flow rate	−0.50*	−0.50*	−0.32*	−0.40*	0.01	
Total protein	0.57*	0.62*	0.61*	0.77*	0.13**	−0.50*

Data pooled over years 1 and 2.
*Statistically significant ($\alpha = 0.05$) at $p < 0.001$, **$p = 0.05$.

found for lysozyme, lactoferrin, flow rate or $OSCN^-$ (myeloperoxidase is discussed below). Values for sIgA, peroxidase and total protein were equalized for analyses of the entire data set by calculating z-scores for each year (Sokol and Rohlf, 1981). Yearly analyses were based on yearly values.

Correlation and principal-components analysis

Correlation and principal-components analysis gave consistent results in pooled and yearly data. Salivary antimicrobial proteins showed strong significant correlations with each other, and with total protein (Table 2). Individual proteins and total protein likewise showed significant negative correlations with flow rate. Correlations of hypothiocyanite ion with individual proteins, total protein or flow rate were low, and generally non-significant. Large reductions in correlations between antimicrobial proteins occurred when partial correlation was used to remove common variation attributable to total protein and flow rate (Table 3). The greater part of the decrease was seen when total protein alone was held constant; there was little additional change when flow rate also was controlled.

The results of principal-components analysis were quite different from our previous findings for stimulated parotid saliva. We identified only one major axis of common variation within the correlation matrix for antimicrobial proteins in Table 2 (there was essentially no common variation within the partial correlation matrix). That axis accounted for 60% of total variation among antimicrobial proteins. It was strongly and positively correlated with lysozyme, lactoferrin, peroxidase and sIgA (Table 4). It likewise showed a strong significant positive correlation with total protein, and a more moderate significant negative correlation with flow rate (Table 4 and Fig. 1).

Cluster analysis

Total protein and flow rate were included with individual antimicrobial protein concentrations as variables in cluster analysis. Seven groups with distinct concentration and flow-rate profiles were found in pooled data. Forty-eight per cent of subjects fell within one group with variable means essentially equal to population means. An additional 18 and 13% fell into two clusters moderately higher or lower than population means. The remaining subjects could be split into four small clusters. Two showed low flow-rate and high antimicrobial (and total) protein means; the other two showed high flow-rate and low protein means (clusters within each set showed minor differences in lysozyme, lactoferrin, or sIgA).

Cluster profiles are not shown, because they were not consistent when separate analyses were run for each year. That prompted use of the 'bootstrap method' to evaluate the results of principal-components and cluster analysis (Efron, 1982). The sample population is first replicated many times, and the replicates pooled to form one very large population. Multiple random samples of the same size as the original population are then drawn and analysed separately. 'Bootstrapping' gives an estimate of the effect of sample composition on results of statistical procedures. Principal-components results were very consistent in 10 'bootstrapped' samples of 100 and 200 (five of each). The results obtained agreed with those for the original sample. Cluster analyses of the same 'bootstrapped' samples showed great variability. The only consistent features were high flow-rate/low-protein and low flow-rate/high-protein clusters defining sample extremes. 'Bootstrap' findings suggested that principal-components analysis was relatively insensitive to minor differences in population structure. Cluster analysis was sensitive to such differences, and thus suitable only for identification of extreme subjects.

Table 3. Partial correlation coefficients for antimicrobial proteins in 216 subjects with total protein and flow rate held constant

Variable	sIgA	Lysozyme	Lactoferrin
Lysozyme	0.10		
Lactoferrin	0.21*	0.01	
Salivary peroxidase	−0.06	−0.12	0.10

Data pooled over years 1 and 2.
*$p = 0.002$; all other coefficients not statistically significant ($\alpha = 0.05$).

Table 4. Principal-components analysis of saliva variables in 216 subjects

Variable	Correlation with principal component axis
sIgA	0.77*
Lysozyme	0.73*
Lactoferrin	0.79*
Salivary peroxidase	0.77*
Flow rate	−0.56*
Total protein	0.84*
Total variance (%)†	60%

Data pooled over years 1 and 2.
*Statistically significant ($\alpha = 0.05$) at $p < 0.001$.
†The proportion of total variation in the multivariate data set attributable to the principal-component axis.

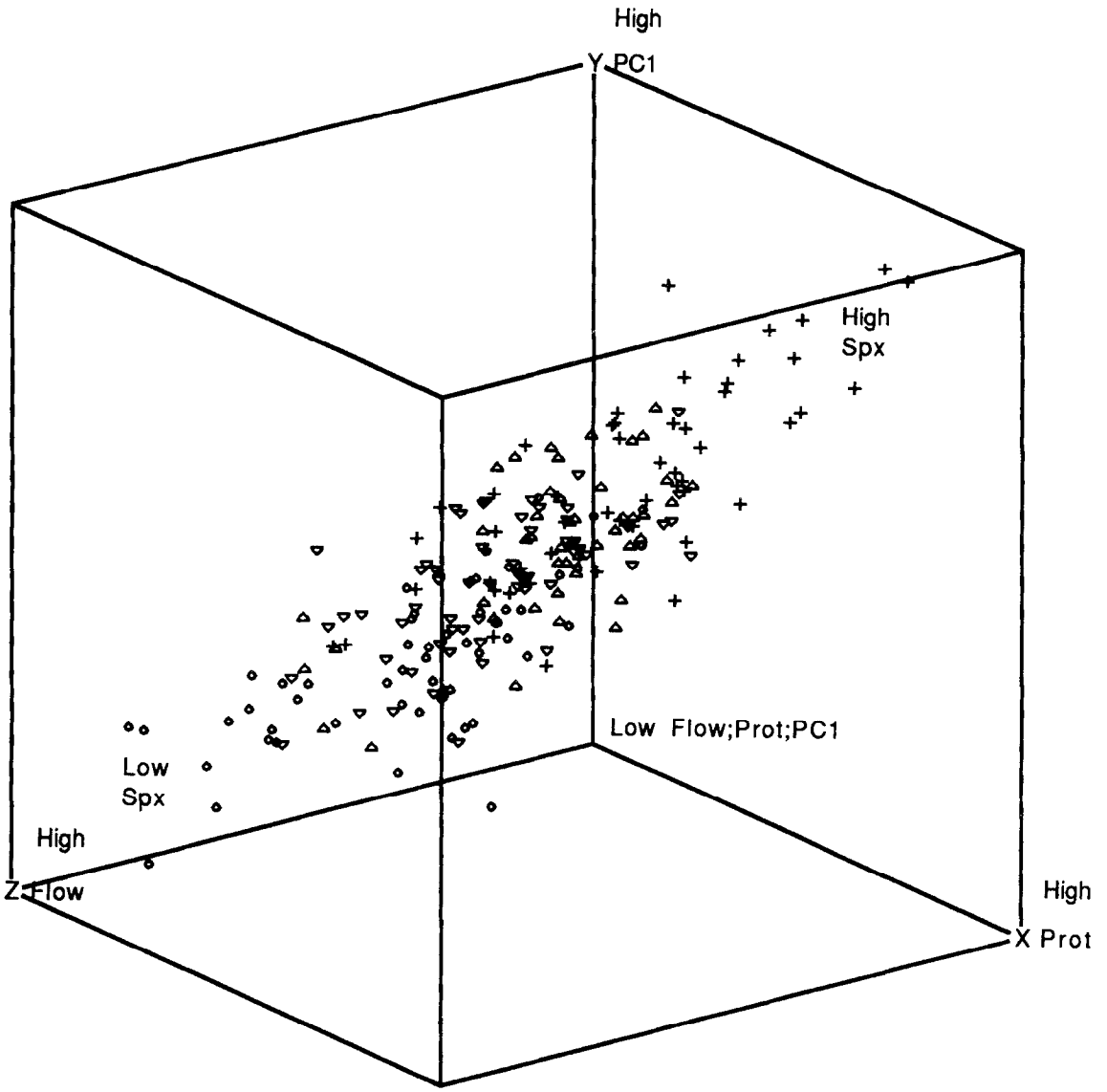

Fig. 1. Multidimensionsal plot illustrating relationships among saliva variables. *X* axis is total protein (Prot); *Y* axis is principal-component scores (PC1); *Z* axis is flow rate (Flow). Origin of axes is indicated by (Low), denoting lowest values; locations of highest values are indicated by (High). Total peroxidase (Spx) is included as a fourth variable denoted by symbols; circles indicate the first quartile; inverted triangles the second quartile, triangles the third quartile, and crosses the fourth quartile. The plot shows the inverse relationship between flow rate and total protein, peroxidase, and principal-component scores; also the positive relationship among total protein, peroxidase, and principal-component scores.

Antimicrobial proteins in relation to health status and plaque

Principal-components analysis was used to generate scores that incorporated each subject's scores for lysozyme, lactoferrin, peroxidase, sIgA, total protein and flow rate into a single number (Norusis, 1985). Those scores (and original variables) were used in comparisons of subjects grouped by health status. They also were used in correlation analyses of associations between saliva and plaque variables. Results were matched against cluster comparisons. Principal-components scores and cluster assignments were based on pooled data, but separate analyses were done for each year. Persons who reported non-compliance with oral hygiene instructions or had used antibiotics were excluded from analyses of plaque variables. No significant differences were found between healthy subjects and those with reported symptoms or clinical signs of respiratory or gastrointestinal infection. Few subjects reported chronic disease, tobacco use, or use of drugs that might affect the composition or flow of saliva. The distribution of such subjects appeared random with respect to saliva variables, but numbers were too small for statistical analysis. Neutrophil myeloperoxidase was used as an indicator of oral inflammation, which might contribute to elevated values for lysozyme, lactoferrin and total peroxidase. Estimated myeloperoxidase activities were generally low (Table 1). There were pronounced yearly differ-

Table 5. Relationships between saliva variables and proportions of *Strep. sanguis* in year 1 ($n = 87$) and year 2 ($n = 85$)[a]

Variable	*Summary statistics for Strep. sanguis proportions* Mean ± SD	Skewness	Range	Transform
% of total flora[b]				
Year 1	7.24 ± 8.40	1.54	0–41.18	Square root
Year 2	9.84 ± 14.95	1.01	0–92.98	Square root
% of total streptococci[b]				
Year 1	28.18 ± 29.50	2.91	0–100.0	Square root
Year 2	25.31 ± 26.89	0.90	0–93.59	Square root

Correlations of transformed proportions with saliva variables (Pearson's r)

Variable	% of total flora Year 1	% of total flora Year 2	% of total streptococci Year 1	% of total streptococci Year 2
sIgA	−0.10	−0.10	−0.02	−0.13
Lysozyme	−0.08	−0.23[c]	0.07	−0.26[cd]
Lactoferrin	0.18	−0.31[cd]	−0.02	−0.34[cd]
Salivary peroxidase	0.02	−0.23[c]	−0.03	−0.30[c]
$OSCN^-$	0.04	0.15	−0.07	0.07
Flow rate	0.22[c]	0.24[c]	0.12	0.24[c]
Total protein	−0.11	−0.34	−0.07	−0.37[cd]
Principal component	−0.04	−0.29[c]	−0.01	−0.33[cd]

[a] Excludes subjects reporting non-compliance with oral hygiene instructions or antibiotic use.

[b] Transormed proportions of *Strep. sanguis* showed no significant differences between years ($\alpha = 0.05$) by Student's t-test (Sokal and Rohlf, 1981).

[c] Correlations statistically significant at $\alpha = 0.05$.

[d] Year 2 coefficients significantly different from year 1 coefficients ($\alpha = 0.05$) by a test of the hypothesis that both coefficients could occur by chance in samples drawn from the same population (Sokal and Rohlf, 1981).

ences for correlations between myeloperoxidase activity and saliva variables. In year 1, myeloperoxidase was significantly correlated with all saliva variables, most strongly with total peroxidase ($r = 0.50$; $p < 0.001$). There were no significant correlations between myeloperoxidase and any saliva variable including total peroxidase ($r = 0.18$) in year 2. Clinical signs of oral inflammation were rare in either year, and they did not appear to be associated with elevations in myeloperoxidase. The discrepancy between years thus suggested that myeloperoxidase activity could not be distinguished from total peroxidase activity with the spectrophotometer used in year 1.

PHP-M plaque index scores showed no significant associations with saliva variables. Likewise, no associations with saliva variables were found for total blood counts, total streptococci, *Strep. salivarius* and *Strep. mutans* or *Strep. sanguis* expressed as counts. However, pronounced yearly differences were found when *Strep. sanguis* was considered as a proportion of total streptococci (Table 5). No significant correlations with saliva variables were found in year 1. In year 2, the proportions of *Strep. sanguis* were significantly and negatively correlated with peroxidase, lysozyme, lactoferrin, total protein and principal-components scores. Flow rate was significantly and positively correlated with year 2 of proportions *Strep. sanguis*: there were no significant correlations with sIgA or $OSCN^-$. Differences between year 1 and year 2 correlations were large enough to be statistically significant. Year 2 samples also showed significant differences in proportions of *Strep. sanguis* between high flow-rate/low-protein and low flow-rate/high-protein clusters. Interviews with each year's enumerator suggested that the yearly differences might be related to the introduction of the API Rapid Strep system, which led to identification of a wider range of colonies as *Strep. sanguis* in year 2.

DISCUSSION

Our findings indicate that relationships among antimicrobial proteins differ in expectorated whole and stimulated parotid saliva. Rudney and Smith (1985) and Rudney (1989) found two axes of common variation among stimulated parotid saliva proteins. The first axis was positively correlated with total protein, and defined primarily by salivary peroxidase. The second axis was negatively correlated with flow rate, and defined primarily by sIgA. Lysozyme and lactoferrin were more strongly associated with the sIgA axis when a weak stimulus was used (Rudney and Smith, 1985), and more strongly associated with the peroxidase axis when a strong stimulus was used (Rudney, 1989). In expectorated whole saliva, lysozyme, lactoferrin, peroxidase, and sIgA all were associated within a single axis that was positively correlated with total protein and negatively correlated with flow rate.

It is striking that total protein and peroxidase both showed significant negative correlations with flow rate in expectorated whole saliva. We did not find that pattern in either previous study of stimulated parotid saliva (Rudney and Smith, 1985; Rudney, 1989). Total protein appears to be dominated by acinar cell products in stimulated parotid saliva (Oberg, Izutsu and Truelove, 1982), and histochemical studies suggest that salivary peroxidase is exclusively an acinar cell product (Riva *et al.*, 1978). The negative correlation between total protein and flow rate thus

might reflect a greater contribution of non-acinar proteins to expectorated whole saliva. However, negative correlations with flow rate generally have not been reported for peroxidase (Azen, 1978; Lamberts *et al.*, 1984). That correlation probably cannot be explained by any contribution of neutrophil peroxidase to total peroxidase activity. Valid measurement of myeloperoxidase appears to have been achieved in year 2. Those results suggested that myeloperoxidase activity was minimal in this population of healthy young adults. Moreover, we found that year 2 peroxidase still showed a negative correlation with flow rate when estimated myeloperoxidase activity was subtracted out ($r = -0.40$; $p < 0.001$).

Rudney (1990) has recently reported that parotid saliva proteins which were negatively correlated with flow rate showed corresponding positive correlations with saliva collection time. We here observed the same pattern for peroxidase, total protein, and all other proteins of expectorated whole saliva (data not shown). Strong positive correlations with collection time may indicate passive release of proteins from secretory cells (Rudney, 1990). *In vitro* studies have demonstrated stimulus-independent constitutive secretory pathways in acinar cells. Those pathways promote release of the contents of secretory granules, even in the presence of adrenergic blockade (Castle, 1990). The negative correlation between peroxidase and flow rate seen here may thus denote constitutive secretion from acinar cells. Lysozyme and lactoferrin are mainly products of intercalated duct cells, while sIgA is translocated mostly through duct cells (Korsrud and Brandtzaeg, 1982). The four proteins we studied were nevertheless correlated, and those correlations appeared to be a function of common correlation with total protein and flow rate. That might indicate common regulation of constitutive secretion in a variety of salivary gland cells. Our studies of parotid saliva suggest that common regulation does not persist once stimulation begins.

Common regulation suggests a limited range of interaction among antimicrobial proteins in resting whole saliva. That has implications for clinical studies of antimicrobial protein action, as unstimulated secretion predominates at most times (Dawes, 1987). It may be most useful to compare subjects at opposite extremes of the flow rate/concentration continuum. Subjects closer to population means are not likely to differ if subjects with extreme values for all proteins do not. Cluster and principal-components analyses facilitate identification of extreme subjects. However, that in itself is a first step. It also is necessary to identify variables that measure the action of antimicrobial proteins. The outcome variables we used were presence/absence of signs and symptoms of respiratory or gastrointestinal infection, one-day plaque accumulation, and counts/proportions of common oral streptococci.

We found no indication that persons with high levels of antimicrobial proteins were more likely to have experienced recent or concurrent infection. That contradicts our previous findings for stimulated parotid saliva (Rudney, 1989). Our symptom questionnaire was revised for this study, and that might have affected subject responses. Subjects knew that they would be examined for signs of infection, and that also might have affected questionnaire responses. Even if reports of symptoms were consistent, infectious agents with different effects on salivary gland function might have been present at the times that the parotid and whole samples of saliva were taken. Another explanation might be that infection has different effects on resting and stimulated rates of antimicrobial protein synthesis and secretion. Similarly, the parotid gland might respond differently to infection than other glands. Finally, the association with symptoms seen in stimulated parotid saliva was statistically significant, but weak. Weak associations are vulnerable to sampling error, and that might have prevented detection of a genuine relationship in whole saliva. These issues are complex, and our data do not support a clear conclusion. However, other researchers have reported associations between antimicrobial proteins and infection, and the question may warrant further study (Cockle and Harkness, 1978; Tabak *et al.*, 1978; Skurk, Krebs and Rehberg, 1979).

Measures of dental plaque are more directly relevant to questions of antimicrobial protein action in the mouth. We found no association between concentrations of antimicrobial proteins and plaque accumulation. That agrees with the findings of Simonsson *et al.* (1987), who compared levels of lysozyme, lactoferrin, peroxidase and sIgA in whole saliva of heavy and light plaque-formers. However, our findings for the year 2 proportions of *Strep. sanguis* may indicate an effect of antimicrobial proteins on plaque composition. Those results seem related to the introduction of the API Rapid-Strep system in year 2. That system has been applied successfully in taxonomic studies of oral streptococci (Coykendall, 1989), and the year 2 proportions of *Strep. sanguis* may have greater validity than those based solely on colony morphology. However, taxonomic considerations also limit conclusions that can be drawn from year 2 data. Strains traditionally placed in *Strep. sanguis* have recently been reclassified into four distinct species (Kilian, Mikkelsen and Henrichsen, 1989). Each species contains multiple biotypes, and additional species may yet be identified (Coykendall, 1989). Sanguis-group species appear to differ in expression of traits such as amylase binding and IgA-protease production (Kilian *et al.*, 1989; Scannapieco *et al.*, 1989) and interstrain variability in susceptibility to antimicrobial proteins also occurs *in vitro* (Arnold, Brewer and Gauthier, 1980; Iacono *et al.*, 1980; Laible and Germaine, 1982; Iacono *et al.*, 1985; Pruitt and Reiter, 1985; Twetman, Lindqvist and Sund, 1986). The effects of such variation are difficult to discern in these correlations of concentrations with crude proportions. As we used the API system as an adjunct to observation of colony morphology, full classification of the strains counted will require further work with stored isolates obtained from culture plates. Those studies should improve our understanding of the effects of antimicrobial proteins on different strains and species, and may allow more complete interpretation of year 2 findings.

Interpretation of those findings also will require improved understanding of other issues. For example, peroxidase was correlated with the proportions of *Strep. sanguis*, but hypothiocyanite ion, the bacteriostatic product of peroxidase, was not. Similarly, peroxidase and $OSCN^-$ were not correlated with each

other, and that is consistent with suggestions that H_2O_2 of bacterial origin is the rate-limiting factor in the production of $OSCN^-$ (Pruitt *et al.*, 1982). These observations make it difficult to understand the association between peroxidase and the proportions of *Strep. sanguis*. One answer may lie in the common correlation of all antimicrobial proteins with total protein. Associations of various proteins with general measures of antimicrobial action may be likely when all proteins tend to vary together. Such circumstances will require development of methods for identifying important proteins in a context of common variation where interaction also may determine importance.

That task is further complicated by the common influences of flow rate on the concentrations of antimicrobial proteins. Low flow rates were associated with low proportions of *Strep. sanguis* in year 2, and it is difficult to isolate passive effects of low saliva flow from active effects of correspondingly high levels of antimicrobial proteins. The general effects of saliva flow are not likely to be simple. Clearance of saliva from the mouth may be an important determinant of the time available for interaction between molecules of antimicrobial protein and plaque bacteria (Rudney, 1990). Current protocols for saliva collection do not provide for measurement or control of that variable, and it is possible that clearance times may differ among persons with similar concentrations of antimicrobial proteins (Rudney, 1990). Similarly, rates of saliva clearance appear to vary between oral sites (Dawes *et al.*, 1989). This suggests that the effects of saliva flow on antimicrobial proteins might not be the same in all parts of the mouth.

Variation among oral sites or bacterial strains may be important sources of inconsistency in the results of previous clinical studies. Study designs that take those factors into account could greatly aid the progress of research in this area. The statistical approach we have developed does allow a large population to be prescreened for subjects at the extremes of antimicrobial protein concentration. Site- and strain-specific comparisons could then be used to obtain a clearer picture of antimicrobial protein action *in vivo*.

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