Antimicrobial Factors in Whole Saliva of Human Infants

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Antimicrobial factors were analyzed in samples of whole saliva from 31 children, aged 0.8 to 3.8 years. When compared with the adult reference group, the children displayed similar levels of lysozyme, salivary peroxidase, and hypothyroxin (OSC)−, whereas the amounts of immunoglobulins (isotypes A, G, and M), lactoferrin, myeloperoxidase, thiocyanate (SCN−), amylase, and protein were significantly lower than the adult values. The child’s behavior during the collection period noticeably influenced the composition of the saliva. Children who were restless and crying during the collection had significantly more immunoglobulins, lysozyme, lactoferrin, salivary peroxidase, myeloperoxidase, and protein in their saliva samples, obviously due to the contamination of saliva mixed with nasal or lacrimal secretions. Therefore, the normal values for saliva could be determined for the noncrying children only. These salivary defense systems did not show any relation to the length of breast-feeding or to the previous history of antibiotic treatment. Thus, with the exception of lactoferrin and myeloperoxidase, the nonimmunoglobulin antimicrobial saliva systems studied here seem to be already at the adult level during early childhood, when the protective antibody systems are still immature.

Human whole saliva, which contains a variety of antimicrobial factors, is constantly bathing the teeth and oral mucosal surfaces. These antimicrobial salivary systems contain both nonimmunoglobulin agents and secretory antibodies (for reviews, see references 5, 18, and 28). Numerous unsuccessful attempts have been made to detect qualitative or quantitative differences in these saliva factors between individuals with differing severities of dental or other oral diseases (14, 17, 26). However, positive findings have also been reported, such as higher amounts of lysozyme (32), agglutinins (23), and anti-Streptococcus mutans antibodies of the immunoglobulin A (IgA) isotype (4, 15) in the saliva of caries-resistant compared with caries-susceptible individuals. The whole idea of studying the possible relationship between one particular saliva factor and the prevalence of oral disease may, however, be questioned, since many of the antimicrobial factors in saliva interact with each other (18) and all of them are dependent on the salivary flow rate, which varies from person to person. Hence, only a very pronounced reduction in the flow rate (such as in xerostomia) will impair the salivary defense capacity to such an extent that various oral diseases, especially dental caries, rapidly develop.

Despite the conflicting results obtained from clinical studies, it is well established that saliva can modify in many ways the formation of pellicle and the subsequent attachment to and colonization by bacteria of tooth surfaces. Many antimicrobial salivary proteins (e.g., lysozyme, lactoferrin, peroxidase, and secretory IgA) adsorb to hydroxyapatite (11, 20, 21) and thereby probably affect the colonization of bacteria. Since these salivary factors may be especially important in regulating the colonization by the early microflora of erupting primary teeth, we decided to study the levels of defense factors in the whole saliva of human infants. In early childhood, the amounts of both salivary and serum antibodies are still far below adult levels (7), and it is therefore of particular interest to know the maturity of the nonimmunoglobulin antimicrobial salivary systems. Apart from the preliminary abstract by I. D. Mandel, H. Turett, and J. Alvarez (J. Dent. Res. 62:217, 1983), the present study is, to our knowledge, the first analysis of the various nonimmunoglobulin factors in the saliva of infants. For comparative purposes we also collected whole saliva samples from healthy adults and analyzed them in an identical way. Although there is some indication that salivary amylase may also have an antibacterial effect (19), this enzyme was included in the analysis as a functional reference protein which is present in high amounts in salivary secretions.

MATERIALS AND METHODS

Subjects. A total of 31 children (16 boys and 15 girls), aged 0.8 to 3.8 years (median age, 1.9 years), comprised the study group. Seventeen of the children were patients visiting Turku University Hospital because of various type of infections, in most cases due to recurrent otitis. Fifteen of the children had received more than three courses of antibiotics before examination and sample collection. The other children were randomly selected from patients at a health center. At the time of saliva collection (and for at least 2 days before) none of the children received any medical treatment. Seventeen children had been breast-fed for more than 6 months, but none of them were breast-fed during the last month preceding the saliva collection. Four children had carious lesions extending into the dentin (one to three cavities per child), and twelve harbored S. mutans in their plaque samples (range, 0.1 to 1,250 CFU).

The adult group comprised 30 dentate subjects, 19 males and 11 females. The mean age was 28.2 years (range, 17 to 39). None of the adults had received any medication or had systemic disease. Ten of them were regular smokers. Their oral health was generally good; the DMFS (decayed, missing, filled surfaces)- and DS-indices were 39.8 ± 18.4 and 3.5 ± 3.4, respectively (mean ± standard deviation). All of these subjects harbored oral S. mutans with a median value of 64 × 103 CFU/ml of saliva (range, 5 × 103 to 990 × 103 CFU/ml). The amount of S. mutans in plaque and saliva samples was analyzed as described earlier (1).

Collection and treatment of saliva samples. With children,

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whole saliva samples (1 to 2 ml) were collected from the floor of the mouth by the aspiration technique using a plastic pipette. During the collection the child was in the sitting position. No stimulation was used but the children constantly moved their tongues and lips, as well as bit the pipette, which caused some mechanical stimulation of saliva flow. Before the collection the subjects did not eat or drink anything for 1 h. All samples (from both children and adults) were collected between 8 and 11 a.m. The adult subjects provided a 5-ml sample of paraffin-stimulated whole saliva for analysis. Because it is impractical to determine reliably the flow rate of the saliva in human infants, comparisons between adults and children should be done cautiously. The salivary levels of the antimicrobial factors studied here are influenced somewhat by the flow rate (9, 14, 29).

Immediately after the collection, a 120-μl portion of uncentrifuged saliva was taken for hypothyocyanite (OSC(N−)) and lysozyme assays. The activity of salivary peroxidase was determined after centrifugation (18,000 × g, 15 min, 4°C) of fresh samples (ABTS method) and samples which had been stored frozen (−20°C) for about 3 months (Nbs-SCN− method). Salivary immunoglobulins, lactoferrin, thiocyanate, and total protein were assayed from uncentrifuged samples which had been stored frozen (−20°C) for 5 to 6 weeks before analysis. All assay mixtures used for the colorimetric examination were clarified by centrifugation before the spectrophotometric measurements. The spectrophotometric measurements were made with a Bausch & Lomb spectrophotometer.

Chemical assays. The lysozyme level was estimated with Micrococcus diffusion plates (Lysozyme Kit; Kallestad Laboratories, Inc., Chaska, Minn.) using lyophilized human urine lysozyme as a reference. The lactoferrin level was determined using a noncompetitive avidin-biotin enzyme immunoassay (34). Human colostral lactoferrin (Sigma Chemical Co., St. Louis, Mo.), which was further purified by affinity chromatography, was used as a standard. The amylase content was determined by the Phadebas method (Pharmacia, Uppsala, Sweden).

Salivary peroxidase activity was measured using two different methods. The first assay was done using ABTS [2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid)] as a donor (25). This method has been widely used in recent studies on salivary peroxidases (14, 27, 29), but it is interfered with to a significant extent by salivary SCN− ions leading to an underestimation of the true peroxidase activity (B. Mansson-Rahemtulla, K. M. Pruitt, and F. Rahemtulla, J. Dent. Res. 64:378, 1985). The second assay, which is not interfered with by SCN− ions, is based on the oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5-dithiobis-(2-nitrobenzoic acid) ([Nbs]2) by OSC(N−) ions generated during the oxidation of SCN− by salivary peroxidase (36). The replacement of SCN− by Cl− in the assay mixture makes the method suitable for determination of specific myeloperoxidase activity in human saliva since Cl− is oxidized to OCl− by myeloperoxidase, but not by salivary peroxidase (Mansson-Rahemtulla et al., 1985).

In our assay system, the reaction was carried out in a 0.1 M phosphate buffer, pH 6.0, which was 4.2 mM in SCN− (salivary peroxidase assay) or 150 mM in Cl− (myeloperoxidase assay) (calculated final concentrations). Myeloperoxidase may contribute somewhat to the Nbs-SCN− assay because SCN− is also oxidized by myeloperoxidase (36). The Nbs was prepared as described previously (29), and its concentration was estimated using an extinction coefficient of 12.266 M−1 cm−1 at 412 nm and at pH 5.6 (22). The reaction was started by adding H2O2 (final concentration, 100 μM), and the enzyme activity calculations were based on the first 15 s of the reaction, during which the A412 versus time curves were linear. If necessary, the rates were corrected for nonenzymatic oxidation of SCN− or Cl− by H2O2. One enzyme unit corresponds to the amount of enzyme catalyzing the oxidation of 1 mol of substrate per minute under the assay conditions.

Hypothyocyanite (OSC(N−)) ions were assayed by reaction with the colored anionic monomer of (Nbs)2 as originally described by Thomas et al. (31). A slight modification of this method (29) was used in this study. The thiocyanate (SCN−) concentrations were determined using the ferric nitrate method (3). The total protein content was measured according to the method of Lowry et al. (16).

The total amounts of salivary IgA, IgG, and IgM antibodies were assayed by using a “trapping antibody”-type enzyme immunoassay as described in detail earlier (15).

Reagents. ABTS and catalase (from bovine liver) were obtained from the Sigma Chemical Co. Hydrogen peroxide was purchased as a 30% solution (Perhydrol; E. Merck AG, Darmstadt, Federal Republic of Germany) and stored at 4°C. The reduction of the (Nbs)2 (Aldrich Chemical Co., Milwaukee, Wis.) to Nbs with 2-mercaptopethanol was done as described previously (29). Avidin DH and biotinylated peroxidase, which were used in the lactoferrin assays, were obtained from Vector Laboratories (Burlingame, Calif.), and the biotinyl-N-hydroxysuccinimide ester was obtained from Calbiochem-Behring (San Diego, Calif.). Rabbit anti-human IgA, IgG, and IgM and horseradish peroxidase-conjugated anti-human immunoglobulins were purchased from Dako-Immunoglobulins, Copenhagen, Denmark. The immunoglobulin standards were purified from human serum (Behringwerke AG, Marburg, Federal Republic of Germany). The absorbances in lactoferrin and the immunoglobulin analyses were read by an automatic photometer (Titertek Multiskan; Eflab Oy, Helsinki, Finland).

RESULTS

As expected, the behavior of the children varied during the saliva collection. About half of the children were restless and crying, and their saliva samples were clearly more turbid than those from children behaving normally. Chemical analysis confirmed that the child’s behavior noticeably affected the composition of the saliva: lysozyme, lactoferrin, salivary peroxidase, myeloperoxidase, and protein levels were significantly higher in crying children, whereas the SCN−, OSC(N−), and amylase levels were unaffected (Table 1). A similar difference was found for salivary immunoglobulins (Fig. 1). Hence, to analyze the actual differences between infants and adults in the amounts of salivary defense factors, only values from noncrying children were included in the comparison (Table 2, Fig. 2).

No significant differences in salivary lysozyme, peroxidase, and OSC(N−) levels were observed between children and adults; however, the lactoferrin and SCN− concentrations in children’s saliva were only about one-third of those measured in adults’ saliva (Table 2). Also, the activities of myeloperoxidase and amylase, as well as the immunoglobulin and protein content of whole saliva, were lower in children than in adults (Table 2, Fig. 2). No differences between the sexes could be observed in any of the parameters studied, and none of the factors showed any clear age-dependency within the study groups. The length of breast-feeding (<6 versus ≥6 months) or a history of fre-
TABLE 1. Whole saliva levels of lysozyme, lactoferrin, peroxidase system components, amylase, and total protein in human infants as affected by behavior during collection of the saliva samples

<table>
<thead>
<tr>
<th></th>
<th>Normal values (mean ± SD; n = 16)</th>
<th>Crying children (mean ± SD; n = 15)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (mg/liter)</td>
<td>10.4 ± 4.1</td>
<td>21.8 ± 8.2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Lactoferrin (mg/liter)</td>
<td>3.0 ± 1.6</td>
<td>11.2 ± 10.4</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Salivary peroxidase (mU)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABTS method</td>
<td>474 ± 181</td>
<td>709 ± 263</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Nbs-SCN− method</td>
<td>0.68 ± 0.28</td>
<td>1.14 ± 0.40</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Myeloperoxidase (mU)</td>
<td>0.19 ± 0.08</td>
<td>0.32 ± 0.12</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Thiocyanate (mM)</td>
<td>0.38 ± 0.18</td>
<td>0.48 ± 0.27</td>
<td>NS</td>
</tr>
<tr>
<td>Hypothiocyanite (μM)</td>
<td>36 ± 12</td>
<td>39 ± 14</td>
<td>NS</td>
</tr>
<tr>
<td>Amylase (U × 10^3)</td>
<td>168 ± 124</td>
<td>262 ± 138</td>
<td>NS</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>1.13 ± 0.28</td>
<td>1.89 ± 0.48</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

* Student’s t test. NS, Not significant.

quent antibiotic treatment showed no relationship to the levels of salivary defense factors (data not shown).

DISCUSSION

The colonization of tooth surfaces by pathogenic bacteria, especially S. mutans, at an early age (<4 years) noticeably promotes caries development in primary dentition (2, 13). Many factors, such as diet, oral hygiene, and the familial occurrence of S. mutans, may affect the colonization of the dentition by cariogenic bacteria, but, because of the low level of secretory antibodies at this age (7), the protective effect of saliva against oral pathogens may also be lowered. However, since acquired antibodies are only a part of the entire repertoire of salivary antimicrobial factors, we also studied the levels of innate, nonimmunoglobulin defense factors in saliva during the early period of tooth emergence.

The fact that restless and crying children displayed significantly higher levels of immunoglobulins, lysozyme, lactoferrin, peroxidases, and protein in the saliva most likely reflects the contamination of saliva samples by nasal or lacrimal secretions. These secretions are very rich in the above components (6, 12, 30), and therefore even a small amount of such secretions may noticeably increase their levels in whole saliva. The large deviations for the saliva values of crying children (Table 1, Fig. 1) suggest that the proportion of nasal and lacrimal fluid varied to a great extent from child to child.

The reason for the large variation in salivary antimicrobial factors has not been mentioned in previous reports on infants’ salivary antimicrobial proteins. Therefore, direct comparison with previous studies may not be reasonable, especially since many of the assay methods used also differ. Mandel et al. (J. Dent. Res. 62:217, 1983), in their study with 1- to 4-month-old infants, observed two- to three-times higher levels of lysozyme and peroxidase in infants’ saliva compared with adults. Also, the lactoferrin levels were already similar to the adult values at this early age. This difference from our observations may, at least in part, result from the contamination of saliva by nasal or lacrimal fluids in previous studies.

In contrast to the other salivary components studied, the amounts of SCN−, OSCN−, and amylase were not affected by the child’s behavior. This indicates that the concentrations of these compounds are low or nil in nasal and lacrimal secretions. No data on nasal secretion is available, but human tears contain only about 0.02 mM SCN− and are completely devoid of OSCN− (30). Although amylase is present in human tear fluid, it is not one of the major proteins in that secretion (33). The finding that the SCN− and OSCN− levels were unaltered in whole saliva despite the increased peroxidase activity in these children agrees with the observation of the dynamic equilibrium state of SCN− peroxidation to OSCN− in vivo (K. M. Pruitt, J. Tenovuo, B. Mansson-Rahemtulla, and P. Harrington, J. Dent. Res. 64:378, 1985). Gothefors and Marklund (10), using pyrogallol as an oxidizable substrate, showed that salivary peroxidase

FIG. 1. Whole saliva levels of immunoglobulins (isotypes A, G, and M) in human infants as affected by their behavior during the collection of the saliva samples (see the text). N, normal values (n = 16); C, crying and restless children (n = 15). The horizontal lines indicate the mean values. The differences between the groups were analyzed by Student’s t test.
TABLE 2. Comparison of the amounts of lysozyme, lactoferrin, peroxidase system components, amylase, and total protein in whole saliva samples of human infants and adults

<table>
<thead>
<tr>
<th>Whole saliva levels of:</th>
<th>Children (mean ± SD; n = 16)</th>
<th>Adults (mean ± SD; n = 30)</th>
<th>Significancea</th>
</tr>
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<tr>
<td>Lysozyme (mg/liter)</td>
<td>10.4 ± 4.1</td>
<td>10.8 ± 3.3</td>
<td>NS</td>
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<td>Lactoferrin (mg/liter)</td>
<td>3.0 ± 1.6</td>
<td>8.5 ± 4.0</td>
<td>P &lt; 0.001</td>
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<td>ABTS method</td>
<td>474 ± 181</td>
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<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Nbs-SCN− method</td>
<td>0.68 ± 0.28</td>
<td>0.80 ± 0.23</td>
<td>NS</td>
</tr>
<tr>
<td>Myeloperoxidase (mU)</td>
<td>0.19 ± 0.08</td>
<td>0.39 ± 0.13</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Thiocyanate (mM)</td>
<td>0.38 ± 0.18</td>
<td>1.22 ± 0.47</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Hypothiocyanite (µM)</td>
<td>36 ± 12</td>
<td>27 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Amylase (U x 10^3)</td>
<td>168 ± 124</td>
<td>276 ± 144</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>1.13 ± 0.28</td>
<td>1.58 ± 0.40</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

* Student’s t test. NS, Not significant.
+ n = 20.
\( n = 20 \) (smokers excluded).

Values were highest during the first weeks after delivery and decreased to adult levels within 9 weeks.

However, it should be stressed that all of the peroxidase assays using organic donors or halides as substrates are influenced by salivary SCN− ions (Mansson-Rahemtulla et al., 1985). Thus, the higher amount of SCN− in the adults’ saliva compared with that of children results in peroxidase levels for the adults that are too low. This may be the reason why in previous studies (10; Mandel et al., 1983) the salivary peroxidase activity has been reported to be higher among infants than among adults. We also observed the same phenomenon when ABTS was used as an organic donor (Table 2). However, with the Nbs-SCN− assay, which is not interfered with by SCN− ions, the values were not significantly different for infants and adults.

Myeloperoxidase activity in human whole saliva is derived from leukocytes entering the oral cavity mainly via gingival crevices (28). As expected, salivary myeloperoxidase activity was significantly lower among infants than among adults, most likely due to the presence of fewer teeth and better gingival health. Although myeloperoxidase activity in saliva reflects the number of inflammatory cells (28), myeloperoxidase is also able to catalyze the oxidation of SCN− to OSCI− (36), thereby contributing to the antimicrobial capacity of human saliva.

The reported values for human salivary lysozyme are extremely variable (8, 20, 26, 32, 35), probably due to the differences in the assay methods and in the pretreatment of the samples (35). Both long-term storage and immediate centrifugation result in a considerable reduction of the enzyme levels (35); we therefore performed our lysozyme assays using fresh, uncentrifuged samples. However, our results may still have been influenced by the presence of mucins, which may form complexes with lysozyme (20). The dissociation of these complexes by acidification (35) or treatment with NaCl (J. W. Jenzano, S. Hogan, and R. L. Lundblad, J. Dent. Res. 64:328, 1985) significantly increases the measurable values of salivary lysozyme. Identical treatment of the saliva samples from children and adults, however, makes it possible to compare the free-lysozyme levels in our two study groups.

The salivary protein levels we measured are in line with the previous reports in which the Lowry method was used. However, within the first month after birth the protein levels are higher (2.4 to 2.9 mg/liter) and then decrease to values comparable with our measurements (24). Cole et al. (8) reported significantly higher protein values in their study of 7- to 12-year-olds, but the assay method was different. The lactoferrin levels among 7- to 12-year-old children are already similar to those of adults, i.e., about 10 µg/ml (8, 9).

Our results show that, with the exception of lactoferrin and myeloperoxidase, the innate defense systems present in whole saliva seem to be at the adult level when the primary
teeth erupt. Because these systems possess many antimicrobial properties, such as bacteriostatic and bactericidal effects, the inhibition of bacterial acid production, and the capacity to agglutinate bacteria and viruses, it seems likely that they provide protection against pathogenic microorganisms at the critical time when the antibody systems are still immature.

ACKNOWLEDGMENT
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LITERATURE CITED