

Antimicrobial factors in whole saliva of human infants: a longitudinal study

Tuula Hyyppä, Lic Odont, D Odont Laura Karhuvaara, Lic Odont
Jorma Tenovuo, Lic Odont, D Odont Marianne Lumikari, Lic Odont
Pekka Vilja, PhD

Abstract

Because early childhood is an important period for the colonization of bacteria in the primary dentition, it is possible that antimicrobial factors in saliva may modify these early events. In this study we have followed longitudinally 33 children from predentate to dentate phase and analyzed whole saliva for such salivary factors as lysozyme, lactoferrin, salivary peroxidase, myeloperoxidase, thiocyanate, hypothiocyanite, total IgA, IgG, IgM, and total protein. Children's saliva samples were compared with those from an adult reference group whose samples were collected and analyzed in an identical way. It was observed that salivary thiocyanate and IgG increased and salivary peroxidase decreased significantly from predentate to dentate phase. The other parameters remained unchanged. Children in predentate phase already had reached adult levels of hypothiocyanite and IgM, whereas all the other components were found in significantly lower amounts in children's saliva than in adult saliva. Salivary myeloperoxidase assay is interfered by the thiocyanate ions, and the observed increase in salivary "myeloperoxidase" activity may be due to the simultaneous increase in salivary thiocyanate. Our results indicate that the levels of most antimicrobial substances of children's whole saliva are not significantly affected by tooth emergence (which provides access for serum components into the mouth) and that, with the exception of hypothiocyanite and IgM, the salivary antimicrobial systems are still immature in human infants.

With the exception of salivary immunoglobulins, relatively little is known about the composition of saliva in young children. This is most likely due to difficulties in collecting representative saliva samples, but perhaps also because the functions of saliva are generally thought to be more important at a somewhat older age when oral diseases such as dental caries are more prevalent.

However, early infancy is an important age for

infection by cariogenic bacteria, in particular by *Streptococcus mutans* (Carlsson et al. 1975; Catalanotto et al. 1975; Masuda et al. 1979; Berkowitz et al. 1981; Van Houte et al. 1982; Alaluusua and Renkonen 1983; Köhler et al. 1988). The microorganism is also implicated in subsequent caries development both in primary (Alaluusua and Renkonen 1983; Aaltonen et al. 1987) and permanent (Alaluusua et al. 1987) dentitions. Therefore, it is of interest to study all those factors which may influence the colonization of the primary teeth by cariogenic bacteria.

Human saliva is known to affect bacterial adherence, multiplication, and metabolism (Mandel 1979; Tenovuo et al. 1987a). Therefore, individual variations in salivary antimicrobial factors may affect the nature of indigenous oral microflora. It is not known whether salivary antimicrobial factors are able to completely prevent the colonization of primary teeth by *S. mutans*. But since a considerable proportion of those children whose mothers are caries active (and therefore likely to infect their children by cariogenic bacteria) do not become infected (Köhler and Bratthall 1978), it is possible that protective antibodies or other antimicrobial factors of saliva prevent infection (Aaltonen et al. 1988).

Previous studies on salivary antimicrobial factors in young children have been either cross-sectional (Burgio et al. 1980; Ben-Aryeh et al. 1984; Tenovuo et al. 1986, 1987a), or the saliva samples from adult reference groups have been collected in a different way (Ben-Aryeh et al. 1984; Tenovuo et al. 1986, 1987a). To avoid these possible sources of error, in the present study we have followed longitudinally the development of some selected salivary antimicrobial factors from predentate to dentate phase, and compared these values with those measured from an adult reference group whose saliva samples were collected and analyzed in an identical

way. In later studies, an attempt will be made to analyze the possible association between these saliva factors and the colonization of primary teeth by *S. mutans*.

Materials and Methods

Subjects

A total of 33 children (18 girls, 15 boys) comprised the study group. All children were healthy and were not taking medication. The children were selected randomly from those attending annual check-ups at a health center. At the time of first examination 27 children were still breast fed. At the second examination only 5 were breast fed. Four children did not attend the second examination.

The adult reference group comprised 24 dental students (17 girls, 8 boys) whose mean age was 23.3 years (range 21-31 years). None of the adults took medication, smoked, or had any systemic disease. Their oral health was good at the time of sample collections.

Collection and Treatment of Saliva Samples

Both children's and adults' whole saliva samples (1-3 ml) were collected from the floor of the mouth by aspiration using a soft, disposable plastic pipette (Tenovuo et al. 1986, 1987a). During the collection, the subject was in a sitting position and no artificial stimulation of salivary flow was utilized. No drinking or eating was allowed for 1 hr before the collection. The saliva samples were collected between 8 and 11 a.m. and were treated and stored as described by Tenovuo et al. (1986). Because the child's behavior during the collection period noticeably influences the composition of the saliva (Tenovuo et al. 1986), samples from children who were restless or crying during the collection were excluded.

The first saliva samples from children were collected when they were all still edentulous (age 2-6 months, mean 4.3 months). Identical sample collection was performed from the same children (except 4) almost a year later when their age ranged from 12 to 19 months (mean 12.7 months). At the time of the second saliva collection all of the children's first primary teeth had erupted (mean number 5.9, range 2 to 8 teeth). Three children had received one antibiotic course between the examinations.

Immediately after the collection, a 120 μ l portion of uncentrifuged saliva was taken for hypothiocyanite (OSCN⁻) and lysozyme assays. The activities of salivary peroxidase and myeloperoxidase were determined after centrifugation (18,800 \times g, 15 min 4°C) from fresh samples. Salivary immunoglobulins, lactoferrin, thiocyanate (SCN⁻), and total protein were assayed from centrifuged samples which were stored at -20°C a few weeks before analysis.

Chemical Assays

The lysozyme level was estimated with micrococcus diffusion plates (Lysozyme Kit[®] — Kallestad Laboratories; Chaska, MN) with lyophilized human urine lysozyme used as a reference. The lactoferrin concentration in saliva was analyzed by a noncompetitive avidin-biotin enzyme immunoassay (Vilja et al. 1985). Human colostrum lactoferrin (Sigma Chemical Co; St. Louis, MO), which was further purified by affinity chromatography, was used as a standard. Avidin DH (Vector Laboratories; Burlingame, CA), biotinylated peroxidase (Vector Laboratories; Burlingame, CA), and the biotinyl-N-hydroxysuccinimide ester (Calbiochem-Behring; San Diego, CA) were used in the assay of lactoferrin.

Salivary peroxidase activity was measured at 22°C by following the rate of oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5-dithiobis-(2-nitrobenzoic acid) (Nbs)₂ (Aldrich Chemical Co; Milwaukee, WI) by OSCN⁻ ions generated during the oxidation of SCN⁻ by salivary peroxidase (Wever et al. 1982). Details of our assay system have been described previously (Tenovuo et al. 1986). The replacement of SCN⁻ by Cl⁻ in the assay mixture makes the method suitable for determination of myeloperoxidase activity in human saliva (Mansson-Rahemtulla et al. 1986), since Cl⁻ is oxidized to OCl⁻ by myeloperoxidase, but not by salivary peroxidase (Mansson-Rahemtulla et al. 1986).

Hypothiocyanite (OSCN⁻) ions were assayed by reaction with the colored anionic monomer of (Nbs)₂, as described previously (Tenovuo et al. 1982). The thiocyanate concentrations were determined by the ferric nitrate method (Betts and Dainton 1953), and the total protein concentration according to the method of Lowry et al. (1951).

The total concentrations of salivary IgA, IgG, and IgM were assayed with a "trapping antibody"-type enzyme immunoassay, as described in detail earlier (Lehtonen et al. 1984). Rabbit anti-human IgA, IgG, and IgM as well as horseradish peroxidase-conjugated antihuman immunoglobulins (Dako Immunoglobulins a/s; Copenhagen, Denmark) were used in the analysis. The immunoglobulin standards were purified from human serum (Behringwerke AG; Marburg, Federal Republic of Germany). The absorbances in the immunoglobulin and lactoferrin analyses were read by an automatic photometer (Titertek Multiskan — Eflab Oy; Helsinki, Finland).

Results

Whole saliva concentrations of lysozyme and lactoferrin did not differ between pre-eruptive and eruptive phase of children (Fig 1, next page). When compared with adults, these proteins were present in

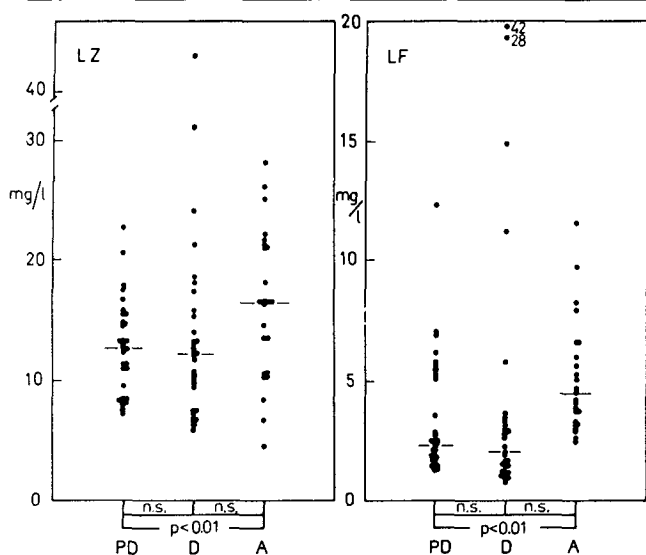


FIG 1. Whole saliva concentrations (mg/l) of lysozyme (LZ) and lactoferrin (LF) in saliva samples from children at predentate (PD) and dentate (D) phase, as well as from an adult (A) reference group. The horizontal lines in all figures indicate the median values.

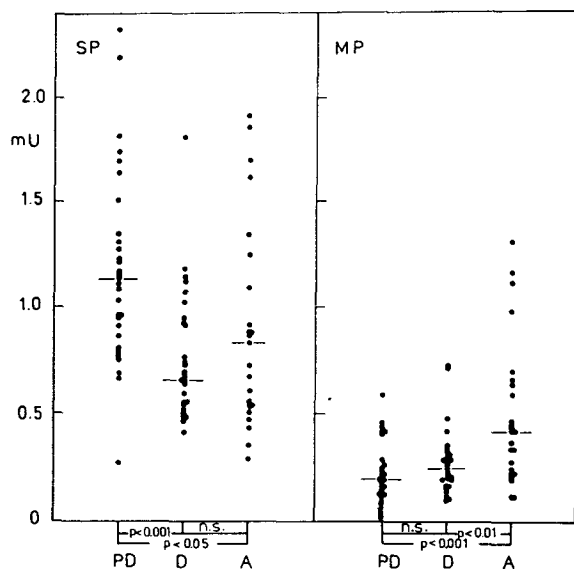


FIG 2. Activities (mU) of salivary peroxidase (SP; Nbs-SCN⁻ assay) and myeloperoxidase (MP; Nbs-Cl⁻ assay) in whole saliva samples from children at predentate (PD) and dentate (D) phase, and from the adult (A) reference group.

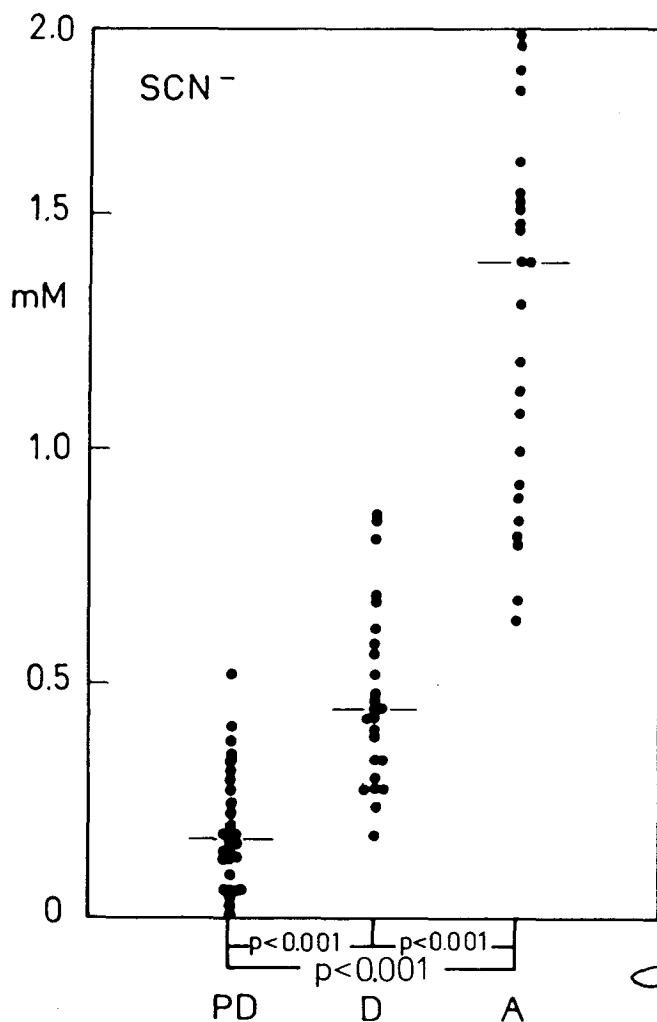


FIG 3. Concentration of thiocyanate (SCN⁻) ions (mM) in whole saliva samples from children at predentate (PD) and dentate (D) phase, as well as from an adult (A) reference group.

Salivary total IgA levels were significantly higher in adults than in children (Fig 5), whereas no significant differences between the groups were observed in salivary IgM values (Fig 6). Although IgM levels tended to be higher during dentate than predentate phase, the difference was not statistically significant. On the other hand, salivary IgG levels were significantly elevated among dentate children when compared to predentate phase, and tended to be even higher among the adult study group (Fig 6, page 34).

Salivary total protein content was also significantly higher among adults than with children whose values did not change from predentate to dentate phase (Fig 7, page 34).

Discussion

In general, the present results are in agreement with the previous studies, usually cross-sectional by nature.

slightly but statistically significant lower amounts in saliva of children (Fig 1). Salivary peroxidase activities were highest among predentate children, even higher than among adults (Fig 2). Myeloperoxidase activities increased in line with age (Fig 2), which was also true for salivary SCN⁻ ions (Fig 3). Hypothiocyanite (OSCN⁻) levels in saliva were almost identical in all study groups (Fig 4).

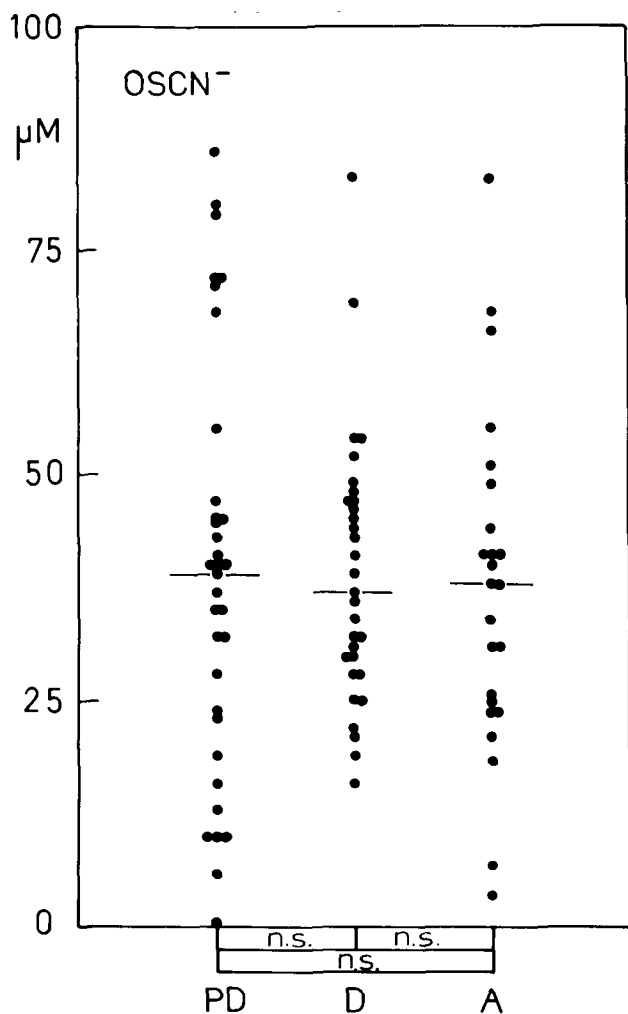


FIG 4. Hypothiocyanite (OSCN⁻) concentrations (µM) in whole saliva samples of children at pre-eruptive (PD) and eruptive (D) phase, and of an adult (A) reference group.

Also in this longitudinal study, comparison of saliva samples from pre-eruptive to eruptive phase should be done with caution, since any observed differences also may be due to general ontogeny and growth — not just because of possible leakage of serum-derived components into whole saliva via newly formed gingival crevices.

Whole saliva levels of salivary IgG and SCN⁻ increased from pre-eruptive to eruptive phase. At the same time salivary peroxidase activities decreased, whereas all the other parameters remained unchanged. In the present study we did not find the previously reported increase in total protein content of saliva during the first years of life (Tenovuo et al. 1987a); instead, our observations agree with those of Ben-Aryeh et al. (1984).

More than 80% of salivary IgG is derived from gingival fluid (Challacombe et al. 1978; Grönblad and Lindholm 1987) where IgG concentrations are similar to

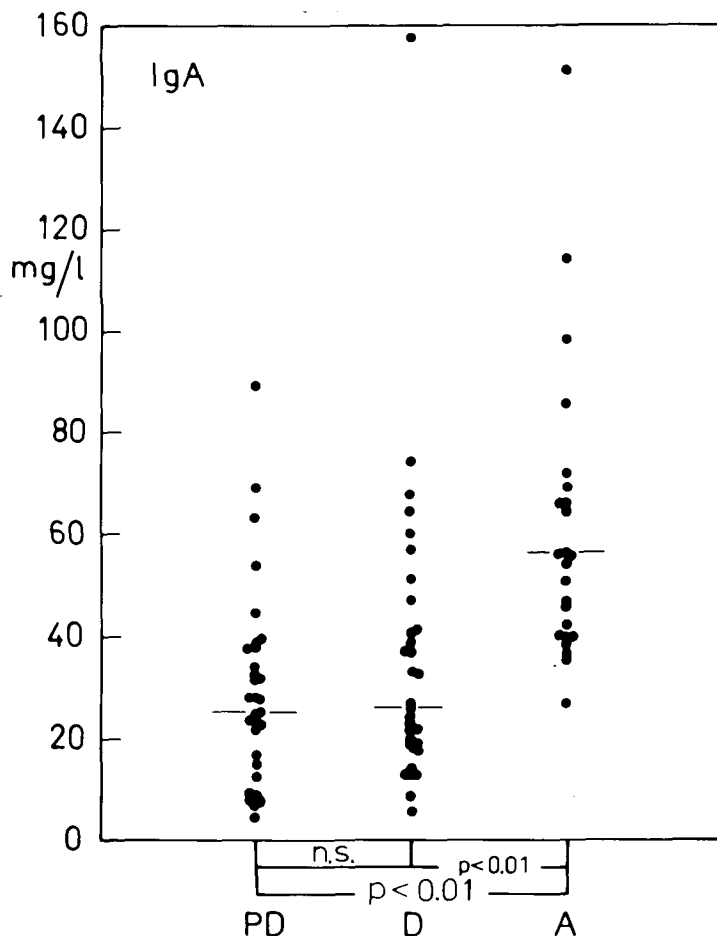


FIG 5. Whole saliva concentrations (mg/l) of total IgA among children at pre-eruptive (PD) and eruptive (D) phase, as well as among adult (A) reference group.

those in serum (Holmberg and Killander 1971), i.e., about 1000 times that of saliva (Tenovuo et al. 1987a). Thus, it is very likely that the observed significant increase in salivary total IgG during tooth emergence is due to the leakage from serum. Because IgA and IgM are present in very low concentrations in infants' sera (Lehtonen et al. 1987), they do not seem to elevate salivary levels in a similar way.

The access of serum-derived IgG antibodies into the mouth seems to be very important for prevention of early colonization of teeth by *S. mutans* (Tenovuo et al. 1987b) as well as for later caries development (Aaltonen et al. 1987), provided that specific antibodies against *S. mutans* are present in children's sera at the time of tooth emergence (Aaltonen et al. 1987; Tenovuo et al. 1987b). If present in high amounts, these specific IgG antibodies against *S. mutans* seem to protect the primary dentition from dental caries (Aaltonen et al. 1987; Tenovuo et al. 1987b). Because secretory IgA system is still quite immature at this early age (Hanson et al. 1980), specific antibodies of IgA isotype against *S. mutans* are rare in

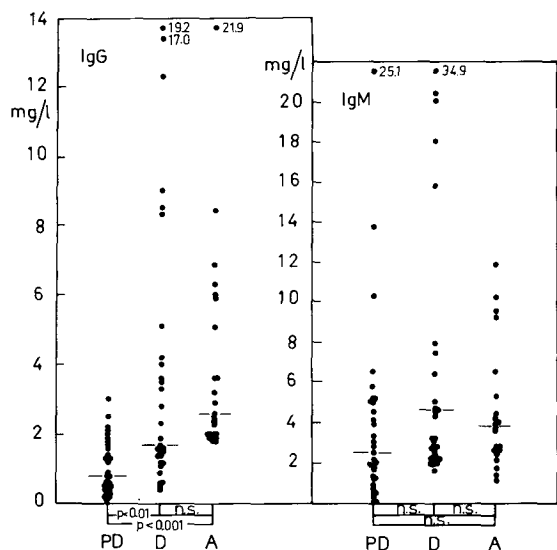


FIG 6. Whole saliva concentrations (mg/l) of total IgG and IgM in samples from children at pre-tentate (PD) and dentate (D) phase, as well as from an adult (A) reference group.

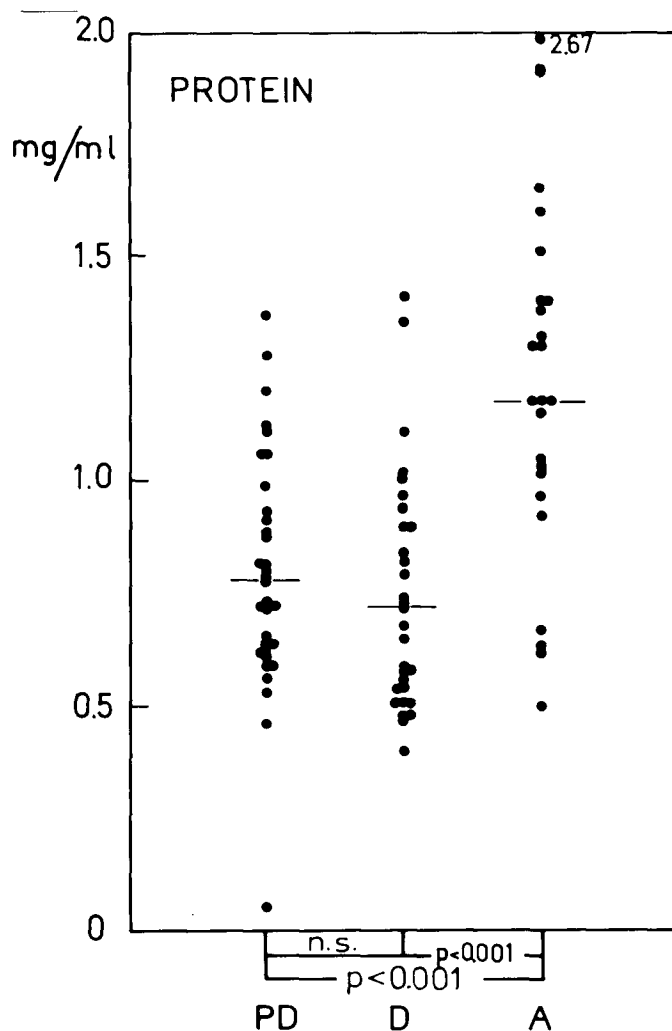


FIG 7. Total protein content (mg/ml) in whole saliva samples of children at pre-tentate (PD) and dentate (D) phase, as well as of adults (A).

early infancy (Gahnberg et al. 1985; Smith et al. 1987).

The reason for the observed increase in salivary SCN^- concentrations by age is not known. However, because the main sources of SCN^- ions in serum, as well as in various secretions, are diet and tobacco (Wood 1975), it is likely that the increase in SCN^- is related to the increasing intake of vegetables which are particularly rich in SCN^- ions (Wood 1975). Interestingly, even the low amount of SCN^- in pre-tentate children is high enough to generate adult levels of hypothiocyanite (OSCN^-) — the peroxidase-catalyzed oxidation product of SCN^- . Hypothiocyanite ions are the actual antimicrobial factors in salivary peroxidase systems (Tenovuo et al. 1987a); thus, it seems that this system is fully mature already in early infancy when the antibody systems are still immature.

In accordance with the previous studies (Gothefors and Marklund 1975; Tenovuo et al. 1986, 1987a), salivary peroxidase activities were higher in pre-tentate babies than at the older age. This is most likely due to the presence of peroxidase activity in human milk (Gothefors and Marklund 1975); this milk-derived and very surface-active enzyme may contribute to the activities found in saliva of breast-fed pre-tentate infants.

It is tempting to speculate that the observed increase in myeloperoxidase activity is due to the enhanced influx of polymorphonuclear leukocytes into the mouth by age. PMN cells are practically the only source for myeloperoxidase, and therefore this enzyme is very unusual in pure salivary secretions (Mandel 1979; Tenovuo et al. 1987a). Myeloperoxidase is present in

gingival crevicular fluid, in particular in connection with PMN cells and gingival inflammation (Kowolik and Grant 1983). The available assay for salivary myeloperoxidase, the Nbs-Cl⁻ assay, is, however, influenced by the simultaneous presence of SCN^- ions so that the more SCN^- present, the higher the "myeloperoxidase activity" in the saliva sample (Mansson-Rahemtulla et al. 1986). Our observation of the increased myeloperoxidase activity by age may thus be significantly affected by the simultaneous increase in salivary SCN^- content. The development and actual presence of myeloperoxidase in human saliva should be analyzed in more detail in future studies.

This study was supported by grants from the Turku University Society and the Finnish Dental Society.

Dr. Hyypä is acting associate professor, periodontology, Drs. Karhuvaara and Lumikari are instructors, and Dr. Tenovuo is a

- professor, cariology, University of Turku. Dr. Vilja is laboratory supervisor, biomedical sciences, University of Tampere, Finland. Reprint requests should be sent to: Dr. J. Tenovuo, Dept. of Cariology, Institute of Dentistry, University of Turku, Lemminkäisenkatu 2, SF-20520 Turku, Finland.
- Aaltonen AS, Tenovuo J, Lehtonen O-P: Increased dental caries activity in preschool children with low baseline levels of serum IgG antibodies against the bacterial species *Streptococcus mutans*. *Arch Oral Biol* 32:55-60, 1987.
- Aaltonen AS, Tenovuo J, Lehtonen O-P: Antibodies to the oral bacterium *Streptococcus mutans* and the development of caries in children in relation to maternal dental treatment during pregnancy. *Arch Oral Biol* 33:33-39, 1988.
- Alaluusua S, Renkonen O-V: *Streptococcus mutans* establishment and dental caries experience in children from 2 to 4 years old. *Scand J Dent Res* 91:453-57, 1983.
- Alaluusua S, Kleemola-Kujala E, Nyström M, Evälahti M, Grönroos L: Caries in primary teeth and salivary *Streptococcus mutans* and *Lactobacillus* levels as indicators of caries in permanent teeth. *Pediatr Dent* 9:126-30, 1987.
- Ben-Aryeh H, Lapid S, Szargel R, Benderly A, Gutman D: Composition of whole unstimulated saliva of human infants. *Arch Oral Biol* 29:357-62, 1984.
- Berkowitz RJ, Turner J, Green P: Maternal salivary levels of *Streptococcus mutans* and primary oral infection of infants. *Arch Oral Biol* 26:147-49, 1981.
- Betts RH, Dainton FS: Electron transfer and other processes involved in the spontaneous bleaching of acidified solutions of ferric thiocyanate. *J Am Chem Soc* 75:5721-27, 1953.
- Burgio GR, Lanzavecchia A, Plebani A, Jayakar S, Ugazio AG: Ontogeny of secretory immunity: levels of secretory IgA and natural antibodies in saliva. *Pediatr Res* 14:1111-14, 1980.
- Carlsson J, Gråhnén H, Jonsson G: Lactobacilli and streptococci in the mouth of children. *Caries Res* 9:333-39, 1975.
- Catalanotto FA, Shklair IL, Keene HJ: Prevalence and location of *Streptococcus mutans* in infants and children. *J Am Dent Assoc* 91:606-9, 1975.
- Challacombe SJ, Russell MW, Hawkes JE, Bergmeier LA, Lehner T: Passage of immunoglobulins from plasma to the oral cavity in rhesus monkeys. *Immunology* 35:923-31, 1978.
- Gahnberg L, Smith DJ, Taubman MA, Ebersole JL: Salivary IgA antibody to glucosyltransferase of oral microbial origin in children. *Arch Oral Biol* 30:551-56, 1985.
- Gothefors L, Marklund S: Lactoperoxidase activity in human milk and in saliva of newborn infants. *Infect Immun* 11:1210-15, 1975.
- Grönblad E, Lindholm K: Salivary immunoglobulin concentrations in predecidate and edentulous mouths. *Scand J Dent Res* 95:27-31, 1987.
- Hanson LÅ, Carlsson B, Dahlgren U, Mellander L, Svanborg Eden C: The secretory IgA system in the neonatal period, in *Perinatal Infections*, Ciba Foundation Symposium 77. Amsterdam, Excerpta Medica, 1980 pp 187-204.
- Holmberg K, Killander J: Quantitative determination of immunoglobulins (IgG, IgA, and IgM) and identification of IgA-type in the gingival fluid. *J Perio Res* 6:1-8, 1971.
- Köhler B, Bratthall D: Intrafamilial levels of *Streptococcus mutans* and some aspects of the bacterial transmission. *Scand J Dent Res* 86:35-42, 1978.
- Köhler B, Andreen I, Jonsson B: The earlier the colonization by *Streptococcus mutans*, the higher the caries prevalence at 4 years of age. *Oral Microbiol Immunol* 3:14-17, 1988.
- Kowolik MJ, Grant M: Myeloperoxidase activity in human gingival crevicular neutrophils. *Arch Oral Biol* 28:293-95, 1983.
- Lehtonen O-P, Gråhn E, Ståhlberg TH, Laitinen LA: Amount and avidity of salivary and serum antibodies against *Streptococcus mutans* in two groups of human subjects with different dental caries susceptibility. *Infect Immun* 43:308-13, 1984.
- Lehtonen O-P, Tenovuo J, Aaltonen AS, Vilja P: Immunoglobulins and innate factors of immunity in saliva of children prone for respiratory infections. *Acta Pathol Microbiol Immunol Scand, Sect C* 95:35-40, 1987.
- Lowry OH, Rosebrough AL, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-75, 1951.
- Mandel ID: In defense of the oral cavity, in *Proceedings, Saliva and Dental Caries*, Kleinberg I et al., eds (spec suppl Microbiology Abstracts). New York; Information Retrieval Inc, 1979 pp 473-91.
- Mansson-Rahemtulla B, Baldone DC, Pruitt KM, Rahemtulla F: Specific assays for peroxidases in human saliva. *Arch Oral Biol* 31:661-68, 1986.
- Masuda N, Tsutsumi N, Sobue S, Hamada S: Longitudinal survey of the distribution of various serotypes of *Streptococcus mutans* in infants. *J Clin Microbiol* 10:497-502, 1979.
- Smith DJ, Taubman MA, Ebersole JL: Ontogeny and senescence of salivary immunity. *J Dent Res* 66:451-56, 1987.
- Tenovuo J, Pruitt KM, Thomas EL: Peroxidase antimicrobial system of human saliva: hypothyocyanite levels in resting and stimulated saliva. *J Dent Res* 61:982-95, 1982.
- Tenovuo J, Lehtonen O-P, Aaltonen AS, Vilja P, Tuohimaa P: Antimicrobial factors in whole saliva of human infants. *Infect Immun* 51:49-53, 1986.
- Tenovuo J, Gråhn E, Lehtonen O-P, Hyyppä T, Karhuvaara L, Vilja P: Antimicrobial factors in saliva: ontogeny and relation to oral health. *J Dent Res* 66:475-79, 1987a.
- Tenovuo J, Lehtonen O-P, Aaltonen AS: Serum and salivary antibodies against *Streptococcus mutans* in young children with and without detectable oral *S. mutans*. *Caries Res* 21:289-96, 1987b.
- Van Houte J, Gibbs G, Butera C: Oral flora of children with "nursing bottle caries." *J Dent Res* 61:382-85, 1982.
- Vilja P, Krohn K, Tuohimaa P: A rapid and sensitive non-competitive avidin-biotin assay for lactoferrin. *J Immunol Methods* 76:73-83, 1985.

Wever R, Kast WM, Kasinoedin JH, Boelens R.: The peroxidation of thiocyanate catalysed by myeloperoxidase and lactoperoxidase. *Biochim Biophys Acta* 709:212-19, 1982.

Wood JL: Biochemistry, in *Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives*, Newman AA ed. New York; Academic Press, 1975 pp 156-221.

1990 dental health goals

The Centers for Disease Control (CDC) recently issued a report on the year 1990 dental health goals set for the nation a decade ago. The 12 goals were formulated from a 1979 report from the U.S. Surgeon General. CDC said two objectives were achieved, four are probably attainable, three probably won't be met, and data are insufficient for three.

A review of all 1990 health goals is nearing completion and the U.S. Public Health Service hopes to issue new goals for the year 2000 soon.

Goals achieved

- By 1990 at least 40% of 9-year-old children should be caries free in their permanent teeth
- By 1990 at least 7% of adults should be aware of the necessity for both thorough personal oral hygiene and regular professional care in the prevention and control of periodontal disease.

Goals probably attainable

- Reducing gingivitis and destructive periodontal disease in adults to 20 and 21% respectively
- Making 95% of school children and parents aware of dental disease risk factors and the importance of fluoride and other preventive
- Creating a system to monitor the nation's dental health
- Improving personal oral hygiene of children.

Goals which probably won't be met

- Too little information is available to measure progress on reducing gingivitis in children aged 6-17, removing sugared snacks from schools and providing optimally fluoridated drinking water for children in fluoride-deficient areas, CDC said.
- Routine use of mouthguards by student athletes, nationwide water fluoridation and reduced consumption of highly cariogenic foods are goals that probably won't be reached.
- Only three sports require use of mouthguards — football, ice hockey and men's lacrosse. The nation is far from a 1990 goal of having 95% of community water supplies fluoridated. In 1985, said CDC, an estimated 61.9% of the population using public water systems had access to drinking water with fluoride levels capable of preventing dental caries. This represented 54.5% of the total population.
- Opposition to fluoridation remains strong and is concentrated on local and state governments. The fluoridation of school water systems also has been stymied by regionalization of public water supplies, (regionalization includes schools formerly having independent water supplies).
- Reduced consumption of sugary foods has been deemphasized because of difficulty in determining and stating the caries potential of foods. It's not likely to be included in new goals for the year 2000.