Salivary antimicrobial proteins and *mutans* streptococci in tonsillectomized children

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Abstract

Whole saliva from 53 children who had been tonsillectomized when they were younger than 4 years old was analyzed for selected antimicrobial proteins and oral mutans streptococci 3–4 years after the operation. The results were compared with those from age- and gender-matched control children with no history of tonsillectomy. The salivary analyses comprised both immune (total IgA, IgG and IgM) and selected nonimmune (lactoferrin, myeloperoxidase, salivary peroxidase) antimicrobial proteins. Specific IgA and IgG antibodies against viral antigens (adeno-, cytomegalo-, respiratory syncytial- and Epstein-Barr-viruses) and against Streptococcus mutans cells were quantitated in both groups. The tonsillectomized children had statistically significantly higher concentrations of all immunoglobulin isotypes (P 0.001) as well as of lactoferrin ($P < 0.005$), and myeloperoxidase ($P < 0.001$) in saliva. However, no differences were found in the numbers of cariogenic mutans streptococci or in the total oral aerobic flora. In line with the streptococcal counts, no differences existed in anti-S. mutans IgA or IgG titers between the groups. Most antibodies against viruses, especially of IgG isotype, were significantly ($P < 0.001$) higher in saliva of tonsillectomized children than in that of the controls. The results suggest that, within a long run, the humoral immune status of human saliva is not weakened by tonsillectomy. Also, mainly serum-derived antimicrobial proteins (myeloperoxidase, lactoferrin, IgG) exist in high concentrations in whole saliva after tonsillectomy. (Pediatr Dent 14:86–91, 1992)

Introduction

The human mouth is characterized by a dense, indigenous microbial flora which colonizes oral and pharyngeal mucosal surfaces and the dentition. In a clinically healthy situation, the number of bacteria shed into mixed saliva is about 100 billion per day, and in plaque deposits on tooth surfaces the microorganisms may achieve densities of 100 million per milligram wet weight.[1] The human mouth also acts as a port of entry for foreign pathogens into the body, so that mouth-to-mouth transmission of virulent bacteria[2] or viruses[3] may cause oral or systemic infections.

This extensive microbial flora, both indigenous and foreign, normally is controlled by salivary flow (resulting in microbial clearance) and by various oral defense systems which are either innate (nonspecific) or acquired (immunoglobulins).[4,5] Oral antimicrobial agents either are synthesized in the salivary glands or leak into the mouth from blood, usually via inflamed gingival crevices. Examples of purely glandular antimicrobial products are secretory IgA, salivary peroxidase and histidine-rich polypeptides, whereas lysozyme, lactoferrin, and IgM may originate from both saliva and gingival fluid.[5,6] Salivary IgG is almost entirely of crevicular origin.[7] Also oral polymorphonuclear (PMN) leukocytes can release considerable amounts of myeloperoxidase, lysozyme, and lactoferrin[5] contributing to the overall levels of these antimicrobial proteins in mixed saliva. Thus, human saliva forms a first line of defense against exogenous antigens entering the human body via oral contacts. Therefore, proper function of salivary factors is essential, not only for oral health,[6] but also for cytoprotection of the upper digestive tract.[8]

The lymphoid nature of the nasopharyngeal tonsils and their role as part of the secretory immune system are likely to contribute to the protective functions in the oral and nasopharyngeal area.[9] However, very limited information exists about the role of tonsils in the immune and nonimmune protection of the oropharyngeal area. The data of salivary IgA levels before[10,11] and after tonsillectomy[11–14] are controversial. To our knowledge, no previous report exists about the nonimmunoglobulin defense factors as related to tonsillectomy. Therefore, in this study, we report how tonsillectomy, the most frequent operation performed on children, affects both immune (salivary IgA, IgG, and IgM) and some selected nonimmune (lactoferrin, salivary peroxidase, myeloperoxidase) antimicrobial proteins in saliva. Furthermore, the levels of cariogenic *Streptococcus mutans* cells and specific anti-*S. mutans* and several antiviral IgA and IgG antibodies were assayed from both tonsillectomized and non-tonsillectomized children.

Subjects and Methods

Study Population

The study group comprised 53 children (27 boys and 26 girls), with a mean age of 7 years 9 months (range 5 years 2 months — 8 years 9 months), who had been tonsillectomized at the Department of Otolaryngology, University Central Hospital, Turku, Finland, during the years 1984–85 when they were younger than 4 years old. Detailed descriptions of the children and their diseases, medications, social background and day care are presented in a separate report.[15] Clinical data on caries and/or gingival health could not be obtained for the tonsillectomized children who resided in a large area of southwestern Finland. The control group (N = 69, 35 boys and 34 girls) was collected from two schools in Turku, and none of them had undergone any tonsillar operations. Their mean age was 8 years 4 months with a range from 7 years 10 months to 8 years 10 months. The study was approved by the Ethical Committee of the Turku University Hospital and the Health Authorities of the City of Turku. Informed consent was obtained from the parents. Analyses of blood assays are presented in another report.[15]

Collection and Treatment of Saliva Samples

Whole saliva samples (1–2 ml each) were collected from the floor of the mouth using a graduated tube (Mucus Extractor, Uno Plast A/S, Hundested, Denmark) connected to a suction device. The children were not allowed to eat or drink for 1 hr before collection. Immediately after collection, 100 µl of uncentrifuged saliva was transferred into a plastic tube containing 1 ml of tryptic soy broth (Oxoid, Basingstoke, United Kingdom) supplemented with 20% glycerol (used for microbiologic analysis). The samples were stored frozen (–20°C) prior to bacterial cultivations. The concentrations of salivary peroxidase, myeloperoxidase, lactoferrin, and total and specific IgA, IgG, and IgM antibodies were determined after centrifugation (18,800 g, 20 min, 4°C) of the saliva samples that had been stored for 1–2 weeks at –20°C before analysis.

Chemical Assays

The total concentrations of salivary IgA, IgG, and IgM were assayed with the "trapping antibody"-type enzyme immunoassay.[16] In this assay, immobilized isotype-specific antihuman immunoglobulins catch sample antibodies, which are detected by enzyme-conjugated antibodies. Antibodies (IgA and IgG) reactive with formalin-treated cells of *S. mutans* NCTC 10449 (serotype c, ATCC 25175) were estimated with a modified enzyme-linked immunosorbent assay.[16] Rabbit anti-IgA, IgG, and IgM and the corresponding reagents conjugated with horseradish peroxidase were from Dako-Immunoglobulins a/s, Copenhagen, Denmark. The rabbit anti-IgA, IgG, and IgM antibodies were all heavy chain specific. Human control serum for NOR-Partigen from Behringwerke AG, Marburg, Germany, was used as a standard for the total IgA, IgG, and IgM immunoassays. The substrate for enzyme immunoassay, 1, 2-phenylendiamine, was purchased from Sigma, St. Louis, MO.

IgG- and IgA-class antibodies against adenoviruses (Adeno), respiratory syncytial virus (RSV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) were measured by enzyme immunoassay. Polystyrene microtiter strips (Microstrip , Eflab, Helsinki, Finland) were coated with viral antigens[17–19] except for EBV where commercial plates (Virotech , System-Diagnostica GmbH, Rüsselsheim, Germany) were used. Saliva specimens were diluted 1:2 in phosphate-buffered saline (PBS) containing 5% normal pig serum and 0.05% Tween-20 (PBS-PT), and 100 µl in duplicate were added to wells of antigen-coated microtiter strips. After 2 hr incubation at 37°C, the wells were washed three times with PBS containing 0.05% Tween-20 (PBS-T). Next, 100 µl of horseradish peroxidase-labelled antibodies against human IgG (Medac GmbH, Hamburg, Germany) or IgA (Cappel Inc. West Chester, PA), diluted 1:20,000 or 1:4,000, respectively, in PBS-Tween were added to each well. The wells were incubated at 37°C for 1 hr and washed three times with PBS-T, then 100 µl of fresh substrate solution (0.3% 1,2-phenylene-diamine and 0.02% hydrogen peroxide in citrate-phosphate buffer, pH 5.5) were added. After 30 min incubation, the reaction was stopped by adding 150 µl 1M hydrochloric acid to each well. The absorbances were measured at 492 nm using an automatic eight-channel spectrophotometer (Titertek Multiscan, Labsystems, Helsinki, Finland) The saliva samples were considered positive when the A_{492} 0, 15 for IgG antibodies, A 492 0, 3 for Adeno-, CMV-, and RSV-, IgA antibodies, and 0, 60 for EBV IgA antibodies.

The lactoferrin levels were determined by a noncompetitive avidin-biotin enzyme immunoassay.[20] Human colostral lactoferrin (Sigma Chemical Co., St. Louis, MO), further purified by affinity chromatography, was used as a standard. Salivary peroxidase and myeloperoxidase concentrations were quantitated with nonisotopic immunometric assays using biotin-labelled antibodies and avidin-alkaline phosphatase label.[21] Antibodies against peroxidases were raised in California rabbits using purified human leukocyte myeloperoxidase[22] and bovine milk lactoperoxidase as antigens. Bovine milk lactoperoxidase and human salivary peroxidase are immunologically cross-reactive.[23] The avidin-alkaline phosphatase conjugate was from Cappel, Organon Teknika Corp., West Chester, PA.

The absorbances in lactoferrin, immunoglobulin, and peroxidase assays were read with an automatic spectrophotometer (Titertek Multiscan, Eflab Oy, Helsinki, Finland).

Quantitation of *Mutans* Streptococci and Total Aerobic Flora

The saliva samples in tryptic soy broth tubes were thawed and mixed vigorously for 20 sec with sterile glass beads. For quantitation of *mutans* streptococci, the suspension was diluted 1:10 and 1:100, and 10 µl was plated in duplicate on mitis-salivarius agar supplemented with 15% sucrose and 0.2 U bacitracin/ml. After three days incubation in candle jars at 37°C, the colonies were counted. The total aerobic flora of mixed saliva was studied by plating duplicate samples on blood agar plates (Oxoid, 5% bovine blood), and incubating for two days at 37°C.

Results

The total concentrations of all studied immunoglobulin isotypes, A, G, and M, were found in significantly higher concentrations in whole saliva of tonsillectomized children than in the controls (Table 1). However, no such difference existed in specific anti-*S. mutans* IgA or IgG antibodies. Lactoferrin and myeloperoxidase concentrations were significantly higher in tonsillectomized children than in the controls, but no difference was found in salivary peroxidase levels (Table 2).

The total count of salivary aerobic bacteria did not differ between the tonsillectomized and the control children (Table 3, next page). The mean number of *mutans* streptococci was approximately the same in both groups, although the number of individuals with detectable levels of *mutans* streptococci in saliva was slightly lower among the tonsillectomized children (Table 3).

IgG antibodies against the viruses were low, and in most cases, below the measuring limit (Table 4, next page). Conversely, most patients had detectable salivary IgA antibodies against the viruses tested. IgG antibodies against adenoviruses were detected significantly more often in tonsillectomized than in control patients. Both EBV IgG and IgA antibody levels of the antibody-positive individuals were significantly lower in tonsillectomized children compared with controls, whereas no significant differences were found in the mean antibody levels against the other viruses (Table 4).

Discussion

Myeloperoxidase in human whole saliva is derived from PMN-leukocytes[24] entering the oral cavity mainly via gingival crevices.[25] Because no myeloperoxidase is synthesized in the salivary glands,[5] myeloperoxidase activity in whole saliva indicates the influx of PMN-cells or serum proteins into the mouth.[26] In our study, myeloperoxidase levels were elevated remarkably among tonsillectomized children, suggesting an enhanced flow of serum material into the oral cavity after tonsillectomy. This is further supported by the high levels of lactoferrin and total IgG in whole saliva of these children, since these proteins are mainly derived from serum, and not from the salivary glands. Also, IgG-type antibodies against viral antigens were more common in whole saliva of tonsillectomized children, although the serum levels were not statistically different.[15]

Table 1. Total and specific antibodies in whole saliva of tonsillectomized and control children

	Tonsillectomy•	(N)	*Controls*•	(N)	*Significance*†
Total IgA (µg/ml)	69.1 ± 39.3	(51)	45.6 ± 26.2	(67)	$P < 0.001$
Total IgG (µg/ml)	13.0 17.1	(52)	4.54 7.72	(67)	$P = 0.001$
Total IgM (µg/ml)	9.74 10.4	(52)	4.49 2.98	(67)	$P < 0.001$
Anti-*S. mutans* IgA (arbitrary units)	27.7 24.3	(52)	24.0 23.0	(67)	NS
Anti-*S. mutans* IgG (arbitrary units)	4.09 ± 4.40	(51)	3.39 ± 4.10	(67)	NS

•Mean ± SD. †Student's *t*-test; NS = not significant.

Table 2. Some nonimmunological antimicrobial proteins in whole saliva of tonsillectomized and control children

	Tonsillectomy•	(N)	*Controls*•	(N)	*Significance*†
Lactoferrin (mg/l)	9.22 ± 7.77	(53)	6.07 ± 3.75	(68)	$P < 0.005$
Salivary peroxidase (µg/l)	400 108	(51)	362 107	(64)	NS
Myeloperoxidase (µg/l)	231 ± 201	(51)	91 ± 94	(64)	$P < 0.001$

•Mean ± SD. †Student's *t*-test; NS = not significant.

Table 3. The number of *mutans* streptococci (MS) and total aerobic flora in whole saliva samples of tonsillectomized children and their controls

	Tonsillectomy (N = 53)	*Controls** (N = 69)
MS-negative samples (N)	18 (34%)	16 (23%)
MS-positive samples (N)	35 (66%)	53 (77%)
MS (log CFU/ml)†	2.60 ± 0.28	2.94 ± 0.21
Total aerobes (log CFU/ml)†	7.05 ± 0.15	6.86 ± 0.07

* No significant differences were found between the groups.

† Mean ± SD.

The concentration of salivary peroxidase, which is a product of the major salivary glands and does not exist in serum,[5] was not altered after tonsillectomy. Because we could not get clinical data on possible oral inflammations of the tonsillectomized children (such as gingivitis), it is impossible to assess the route of enhanced serum influx. However, all children in both groups attended public dental health care yearly, and it is unlikely that any major differences existed in the degree of gingival inflammation. Also, none of the children had any systemic disease which could have influenced the salivary analyses.

Similar oral status also is indicated by salivary counts of *mutans* streptococci whose levels did not differ between the groups. Salivary *mutans* streptococci are significantly elevated in caries-active young children compared to those with no, or only low, caries experience.[27, 28] *Mutans* streptococci reside almost exclusively on tooth surfaces and their numbers in saliva reflect the number of colonized sites in the dentition.[29] The analyses of these bacteria and specific anti-*S. mutans* antibody levels were considered interesting since tonsils form part of the oral lymphoid tissues modifying the immunoglobulin production in the oropharyngeal area.[9] The numbers of oral *mutans* streptococci are regulated by both locally derived specific secretory IgA antibodies[30] and by serum-derived specific IgG antibodies.[28] In accordance with the actual bacterial counts, the titers of these specific bacterial antibodies in saliva did not differ between the study groups. Viral antibodies, both of IgA and IgG type, existed in notably high concentrations in whole saliva of tonsillectomized children. All these observations suggest that tonsillectomy as an operation does not weaken the humoral immunity against oral bacteria or viruses, nor do the numbers of microorganisms increase after the operation.

Cantani et al.[11] observed significantly decreased secretory IgA levels 1–4 months after tonsillectomy, whereas after a few years, the IgA values in saliva returned to levels comparable to those in nontonsillectomized controls.[13, 14, 31] We observed increased levels of salivary IgA after tonsillectomy, which is in contrast to serum IgA levels, which are lower after tonsillectomy.[11, 14, 15] Because serum and secretory IgA systems operate under different immune mechanisms,[4,32] it is not surprising that secretory immunity is normal, or even elevated, if antibody levels in serum are low.[33] IgA deficiency, assessed from serum, also leads to a compensatory elevation of nonimmunoglobulin antimicrobial proteins in saliva.[33]

Some bacterial species, such as *Haemophilus influenzae, S. sanguis, Capnocytophaga ochracea, S. pneumoniae, and Bacteroides melaninogenicus* have been isolated from the tonsils[34] and all these microorganisms are able to cleave IgA_1, the most common subtype of IgA in human saliva.[35] Removal or reduction in numbers of these pathogens along with the operation also may be involved in the presence of higher whole saliva immunoglobulin concentrations after tonsillectomy.

Antiviral IgG class antibodies in whole saliva originate from gingival crevicular fluid, and most likely leak from serum, transmucosally or via the salivary glands.[36] Although the IgG viral antibodies in saliva are low

Table 4. Salivary IgG and IgA antibodies against viruses in tonsillectomized children and their controls

	Tonsillectomy		*Controls*		
	Positive/ tested	*Relative concentration**	*Positive/ tested*	*Relative concentration**	*Significance†*
Adeno IgG	22/52	301 ± 255	6/67	366 ± 294	$P < 0.001$‡
Adeno IgA	42/52	111 90	42/67	127 ± 66	NS
CMV IgG	7/52	130 56	1/67	74	NS
CMV IgA	32/52	99 58	50/67	153 ± 105	NS
EBV IgG	17/51	105 55	6/65	554 ± 456	$P < 0.001$‡
EBV IgA	39/51	166 72	48/65	262 ± 192	$P < 0.005$§
RSV IgG	6/51	143 71	0/65	—	NS
RSV IgA	25/51	97 ± 25	25/65	135 ± 87	NS

* Mean absorbance of specific antibodies/total Ig-class concentration x 10.000 ± SD.

† Student's *t*-test; NS = not significant.

‡ The proportion of antibody positive individuals (Z-test).

§ The relative concentrations.

compared to serum, they have been reported to reflect the titers present in serum regardless of the dental status of the patient.[36] The IgA antibodies against viruses are likely to emerge by the common mucosal immune system.[32]

EBV principally infects B-lymphocytes, and it is thus possible that removing lymphoid tissue might play a role in the observed lower antibody level in tonsillectomized patients compared with controls. However, because of the higher total immunoglobulin levels in the tonsillectomized children, the calculation of relative antibody concentrations exaggerates the differences in these antibody levels. A somewhat surprising finding was the more common appearance of Adeno IgG antibodies in the tonsillectomized children compared with that in controls. We have no explanation for this finding, but it is unlikely that the exposure to adenoviruses would have differed markedly between the groups. IgG antibodies to all viruses tested were more common in the tonsillectomized children; thus, this finding may be due partially to the influx of serum antibodies into saliva.

Although it seems that from the immunological point of view tonsillectomy does not impair the antimicrobial capacity of human saliva, much more research has to be conducted to reveal the interaction between tonsillectomy and the humoral immune status.

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Bicycle helmets can save lives

Perhaps one life a day could be saved if all bicyclists in the United States wore helmets, according to a study published in the *Journal of the American Medical Association.*

The study examined the number of United States residents who reportedly died as a result of bicycle-related head injuries from 1984 to 1988. Universal use of helmets by all bicyclists could have prevented as many as 2500 deaths and 757,000 head injuries (i.e., one death each day and one head injury every 4 min), according to the authors, Jeffrey J. Sacks, MD, MPH, of the Centers for Disease Control, Atlanta, and colleagues.

The authors collected data from the National Center for Health Statistics and the National Electronic Injury Surveillance System.

From 1984 to 1988, bicycling accounted for 2985 head injury deaths (62% of all bicycling-related deaths) and 905,752 head injuries (32% of bicyclists treated at emergency departments). Forty-one per cent of head injury deaths and 76% of head injuries occurred among children younger than 15 years old.

The authors noted that helmet use in the younger-than-15 age group is low. They suggested that targeting this group for helmet campaigns could significantly increase its use of helmets.

In an accompanying editorial, Barry D. Weiss, MD, of the University of Arizona Health Sciences Center, Tucson, noted that fewer than 10% of bicyclists wear helmets, and children are especially less likely to wear them. He recommended that all health care providers should strongly advise their patients who bicycle to wear helmets.