peptide antibody titer and periodontal status in samples taken from elderly people. Our results provide information for the development of preventive medicines for periodontal diseases.

Materials and Methods

Human subjects

Eighty seven human subjects (2001 subjects, average age, 73 years old; 60 males, 27 females) from Niigata prefecture in Japan in June 2001 participated in study. Further, 3 years later, sixty nine identical subjects (2004 subjects, average age, 76 years old; 46 males, 23 females) participated in cohort study. All were functionally independent and had full dentition. Prior to the study, informed consent was obtained from all subjects and the study was approved by the Ethics Committee of Niigata University. The study was conducted according to the ethical guideline at our institution according to the Helsinki declaration. Dental examinations were conducted under artificial white light by trained dentists. According to WHO criteria [29], decayed teeth (DT), missing teeth (MT), and filled teeth (FT) (DMFT) scores were recorded along with findings of dental caries. Four calibrated dentists assessed subject periodontal conditions in the subjects based on the results from 6 measurements points (mesio-buccal, buccal, disto-buccal,

mesio-lingual, lingual, disto-lingual) around each tooth. Intra- and inter-examiner reliability was confirmed using a kappa statistic (k=0.56-0.92 for attachment loss). To estimate periodontal status, dental calculus and attachment loss were also measured at the same 6 points of each tooth (14). Thereafter, the following indicators were assessed and used to estimate the periodontal status of subject:

rCA: number of sites with dental calculus/ 6 points x 100

AL: attachment loss

rAL4: number of sites with greater than 4 mm of attachment loss/ 6 points x 100

rAL6: number of sites with greater than 6 mm of attachment loss/ 6 points x 100

PD: pocket depth

rPD4: number of sites with greater than 4mm of pocket depth/ 6 points x 100

rPD6: number of sites with greater than 6mm of pocket depth/ 6 points x 100

BOP: number of sites with presence of bleeding on probing/ 6 points x 100

All dental status in 2001 and 2004 subjects were shown in Table 1.

Synthetic peptide

The sequence of PAc(361-386)(NAKATYEAALKQYEADLAAVKKANAA) was derived from the sequence of the PAc gene from *S. mutans* MT8148 as described

by Okahashi *et al.* (16). The peptide was synthesized using a stepwise solid phase procedure at Asahi Techno Glass Co. Inc. (Tokyo, Japan). Synthesized peptide samples were subsequently purified by reversed-phase high-performance liquid chromatography (HPLC) on a TSK-GEL column (1 x 30 cm)(TOSO, Tokyo, Japan) with a 10% to 45% acetonitrile gradient in 0.1% TFA, and developed over 50 minutes at a flow rate of 5 ml/minute. Purity was determined as greater than 95% in each tube by HPLC analysis. To confirm the amino acid sequences of the synthetic peptides, several samples were randomly selected, and then analyzed using a System 7300 Amino Acid Analyzer (Beckman, NJ) and a Model 477A Protein Sequencer (Applied Biosystems, Foster City, CA). The peptide was used as a coating antigen for ELISA to detect antibody titer in saliva samples.

Human saliva collection

Whole saliva from human subjects was stimulated by chewing paraffin gum and collected into ice-chilled sterile bottles over a period of 5 minutes, and clarified by centrifugation at 10 000 x g for 10 minutes at 4°C. Saliva samples were also collected in plastic tubes and stored at –80°C, then defrosted just prior to measuring the antibody levels.

ELISA

For an enzyme-linked immunosorbent assay (ELISA), 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100μl of PAc (361-386) peptide (concentration 20μg/ml) or skim milk (as a control) in coating buffer at pH 9.6 for enumeration of the IgA specific to *S. mutans* (22). The plates were washed with PBS containing 0.1% (vol/vol) Tween 20 (PBST) and blocked with 1% (wt/vol) skim milk in PBST for 1 hour at 37°C. Excess skim milk was removed by washing 3 times with PBST, and then a 100μl aliquot of a twofold serial dilution of saliva was added to the wells and the mixtures were incubated for 1 hour at 37°C. The wells were then washed 5 times with PBST and further incubated for 1 hour at 37°C with 100μl of alkaline phosphatase-conjugated goat anti-human immunoglobulin A or G (both heavy and light chains) antibodies (Zymed Laboratories, South San Francisco, CA). After 5 washes with PBST, bound antibodies were detected after the addition of 100μl of 3 mg/ml para-nitrophenyl phosphate as a substrate and incubation for 90 minutes at 37°C. Absorbance at 405 nm (A_{405}) was measured with a microplate reader (Multiskan Bichromatic; Laboratory Japan, Tokyo, Japan). The ELISA antibody titer was expressed as the reciprocal (Log_2) of the highest dilution giving an A_{405} of 0.1 above that of the control (skim milk) after 1 hour of incubation with the substrate. The experiments were

performed independently 3 times, with similar results obtained in each.

Bacteria counting

All bacteria counting was performed by the Laboratory of Bacteriology (BML). Saliva samples from 2004 subjects were gently shaken and inoculated onto Mitis-Salivarius agar (MTS, Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) using an EDDY JET spiral plating system (IUL, S.A., Torrent, Spain), to count total streptococci (tS). Modified MTSB (MMTSB) was prepared by a classic modification of MTS agar plates containing 0.02 M bacitracin (MTSB, Sigma Chemical Co., St. Louis, MO), and used for detection and counting of mutans streptococci (mS) organisms (28). Following anaerobic inoculation for 48 hours at 37°C, the colony-forming units (CFU) of every group were counted. Colonies of mS were identified by their characteristic appearance and the mS ratio was calculated as colony numbers of mS/colony numbers of tS x 100.

Comparison between 2 groups

The subjects were divided into 2 groups according to ELISA antibody titer, the antibody not detected group [anti-PAc (361-386) peptide antibody titer $\leq 2^2$] and the antibody detected group [anti-PAc (361-386) peptide antibody titer $>2^2$]. This grouping by antibody titer has been used in previous epidemiological studies (28). Since, the numbers of MS and MS/total streptococci ratios in saliva samples were lower in the antibody detected group ($2.1 \pm 5.5 \times 10^4$ and 0.5 ± 1.0, respectively) than in the antibody not detected group ($2.7 \pm 5.2 \times 10^4$ and 0.8 ± 1.5, respectively), a cutoff point of 2^2 was used for the grouping in the present study. Antibody specificity to PAc(361-386) peptide was also confirmed by comparing between anti-PAc(361-386) peptide antibody and anti-PAc(346-364) peptide antibody (positive control)(24) or anti-skimmilk antibody (negative control). A Mann-Whitney U-test was used to compare periodontal status between the 2 groups. Differences at the .05 level were considered to be significant. SPSS for Windows (Version 10.0) was used for all statistical analyses.

Results

There was no significant difference in various parameters between 2001 and 2004 subjects (Table 1). Table 2 shows the periodontal status of 2001 subjects determined by ELISA antibody titer. The average rCA (1.3 ± 2.0) result in the antibody detected group was 4 times lower than that (5.4 ± 6.9) in the antibody not detected group in 2001 subjects. The 4-times lower difference was kept until 3 years later but not significant in the identical 2004 subjects (Table 3). The antibody detected group showed significantly lower values (20.4 ± 19.7 and 2.4 ± 4.6) for average rAL4 and rAL6 than the antibody not detected group (38.2 ± 27.7 and 16.6 ± 16.6) in 2001 subjects. In the 2004 subjects, the antibody detected group showed lower value in average rAL4 but not that in average rAL6 than the antibody not detected group. In addition to rAL4, the maximum AL and average AL were also lower in antibody detected group than antibody not detected group in the 2004 subjects. The different levels of maximum AL, average AL and average rAL in 2001 subjects expanded in 2004 subjects.. Thus, it was indicated that anti- PAc(361-386) IgA antibody in saliva responded to expansion of attachment loss in the cohort study.

Discussion

The antibody detected group showed significantly lower rate of sites with dental calculus deposition than the antibody not detected group in fragmental study of 2001 subjects. Moorer reported that the mutans group of streptococci may be involved in dental calculus formation (11), which was confirmed by our results. Further, it has been speculated that the inhibition of adhesion of *S. mutans* to tooth surfaces by the anti-PAc (361-386) peptide antibody leads to suppression of dental calculus formation. Further, the antibody detected group showed significantly lower rate of attachment loss than antibody not detected group. In the antibody detected group, the low frequency of dental calculus deposition may result in a slow progression of attachment loss. There have been several reports on the relationship between dental calculus and attachment loss (1, 3, 13, 27), which are supported by our results. However, in the cohort study, the significant inhibition by the antibody expanded in the attachment loss, but did not reveal in the dental calculus deposition. The influence by antibody may be not consistent to the dental calculus deposition.

Marsh found that the optimal growth condition for *S. mutans* was different from other periodontal pathogens (10), while other studies have shown that the growth of periodontal pathogens is not correlated with or inhibited by *S. mutans*, and suggested

that number of *S. mutans* was not involved in growth condition of periodontal pathogens (5, 7). The anti-PAc (361-386) peptide antibody titer has been speculated to be a negative contributor in proportion to the numbers of *S. mutans* organisms in the oral cavity (28). However, in the present study, there were no significant differences between antibody detected group and antibody not detected group for rCA and rAL but not rPD. Other epidemiological surveys showed that the value of rAL4 was higher in MS carrier than non-carrier in 75 years old elderly people, who grew 2 years old in identical subjects to the present study (unpublished data). However, there was not a significant difference between MS carrier and non-carrier. Together, these results suggest that the present antibody might be related to physical condition rather than substantial condition for growing pathogens in development of periodontal diseases in elderly. Further, the anti-PAc (361-386) peptide antibody may be related to pre- and post-conditioning for the development of periodontal disease, and measurement of production of the antibody may be useful as an indicator for predicting periodontal disease occurrence and progression in elderly patients.

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Table 1 Dental status in 2001 and 2004 subjects

Dental status	Subjects[a]	
	2001	2004
Total number	87	69
Female	27	23
Male	60	46
Age	73	76
Tooth number	18.2 ± 8.1	17.3 ± 9.0
Sound tooth number	5.1 ± 5.4	4.8 ± 4.8
DT	0.3 ± 0.6	0.4 ± 0.7
MT	12.5 ± 8.3	12.8 ± 10.4
FT	10.1 ± 7.0	11.2 ± 7.2
Average rCA	2.4 ± 4.6	2.4 ± 4.6
Maximum AL	3.5 ± 1.2	3.6 ± 1.2
Average AL	7.0 ± 2.2	7.1 ± 2.1
Average rAL4	44.4 ± 28.5	45.4 ± 29.8
Average rAL6	10.5 ± 17.1	10.9 ± 18.8
Maximum PD	5.1 ± 1.4	5.4 ± 1.7
Average PD	2.2 ± 0.5	2.2 ± 0.5
Average rPD4	10.7 ± 11.1	10.9 ± 11.3
Average rPD6	1.0 ± 1.8	1.6 ± 4.3
Average BOP	8.0 ± 10.1	11.0 ± 10.7

a: The subjects were measured for various parameters in 2001 or 2004.

Table 2. Periodontal status of 2001 subjects in low and high group

	Antibody not detected group (n=77)	Antibody detected group (n=10)	p-value*
Average rCA	5.4 ± 6.9	1.3 ± 2.0	0.05
Maximum AL	7.2 ± 3.1	6.2 ± 1.7	0.40
Average AL	3.3 ± 1.0	2.7 ± 0.9	0.17
Average rAL4	38.2 ± 27.7	20.4 ± 19.7	0.04
Average rAL6	11.6 ± 16.6	2.4 ± 4.6	0.02
Average rPD4	11.7 ± 13,3	7.6 ± 1.3	0.22
Average rPD6	2.0 ± 6.2	1.2 ± 2.2	0.60

*: Significant difference demonstrated by Mann-Whitney U test

Table 3. Periodontal status of 2004 subjects in low and high group

	Antibody not detected group (n=61)	Antibody detected group (n=8)	p-value*
Average rCA	2.6 ± 4.8	0.6 ± 0.9	0.55
Maximum AL	7.3 ± 2.2	5.6 ± 1.2	0.03
Average AL	3.7 ± 1.0	2.9 ± 0.5	0.03
Average rAL4	48.4 ± 30.0	22.5 ± 15.9	0.02
Average rAL6	12.0 ± 19.7	2.6 ± 3.4	0.12
Average rPD4	11.2 ± 11.6	8.3 ± 8.9	0.51
Average rPD6	1.7 ± 4.5	0.8 ± 1.5	0.42

*: Significant difference demonstrated by Mann-Whitney U test

Role of cariogenic bacteria in periodontal and root status

Hidenobu Senpuku[1], Akio Tada[2], Yasuhiko Saotome[1,3], Nobuhiro Hanada[4], Akihiro Yoshihara[5] and Hideo Miyazaki[5]

[1]*Department of Bacteriology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan.* [2]*Chiba City Health* Center, *Chiba 261-8755, Japan.* [3]*Department of Gerodontology, Tokyo Medical and Dental University. Tokyo 113-8549, Japan.* [4]*Department of Oral Health, National Institute of Public Health, Tokyo 162-8640, Japan.* [5] *Department of Oral Health Science, Graduate School of Medical and Dental Science, Niigata University, Niigata, 951-8514, Japan*

Running title: Relationship between root caries and oral bacteria

Corresponding author: Hidenobu Senpuku, Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640 Japan

TEL 03-5285-1111, FAX 03-5285-1172, E-mail: hsenpuku@nih.go.jp

Abstract

The relationship between periodontal status and prevalence of caries on exposed root surface and oral bacteria in 75-year-old subjects was studied. Three hundred sixty-eight elderly people were examined on pocket depth, greater than 4 mm of attachment loss (rAL4), average of attachment loss of sites measured (aAL), root surface caries, salivary counts of mutans streptococci (MS) and of lactobacilli, saliva flow, and life style concerning dental diseases. The value of aAL, of rAL4 and the rate of decayed root surface was significantly higher in lactobacilli carrier than lactobacilli non-carrier (aAL p=0.018 ; rAL4 p=0.015; decayed root surface p=0.006). The number of decayed root surface was correlated to MS (p=0.006) and lactobacilli level (p<0.001). A stepwise multiple correlations showed that lactobacilli level was selected as the interacting factor for aAL and rAL4. MS and lactobacilli level were also selected as the interacting factor for the number of decayed root surface.

Key words: Elderly, Periodontal status, Attachment loss, Root caries, Mutans streptococci, Lactobacilli

Introduction

Dental caries is a considerable health problem of elderly individuals because dental caries have been shown to be significantly associated with tooth loss (Hand *et al.*, 1991; Locker *et al.*,1996). Epidemiological surveys have confirmed that greater numbers of *Streptococcus mutans* in children are associated with a higher incidence of decayed, missing, and filled teeth (DMFT), i.e. fragment caries experiences (Granath *et al.*, 1993; Kristoffersson *et al.*, 1986; Thibodeau and O'Sullivan 1999; Loesche *et al.*,1986; Zickert *et al.*,1982). In the elderly individuals, several bacteria species, *S. mutans*, *Streptococcus sobrinus*, Lactobacilli, and yeast have been thought to be pathogen of dental caries, but definitive findings has not yet been obtained (Hunt *et al.*,1992; Klock *et al.*,1990; Kohler and Persson 1991; Loesche *et al.*, 1999; Loesche *et al.*,1995).

Periodontitis is another major factor for tooth loss (Ong 1998; Olver and Brown 2000). In Japan, most of elderly persons suffer from periodontitis (*Dental Health division of Health Policy Bureau Ministry of Health and Welfare Japan*). Periodontitis often induces gingival recession, resulting increase of susceptibility to root surface caries by causing exposure of root surface. Moreover, gingival recession change the oral condition which may cause ecological change, resulting microbiological changes,