determined to be 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 in enzyme-linked immunosorbent assay (ELISA) examinations to determine the antibody titer in the saliva samples.

Human saliva collection

Whole saliva samples were collected on swabs after stimulating by biting paraffin gum for 5 minutes and placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate buffered saline), to determine the numbers of mS and total streptococci (tS) organisms, which was performed by Bio Medical Laboratory (BML, Tokyo, Japan). Other saliva samples was also collected after stimulation by biting paraffin gum and placed into ice-chilled sterile bottles over a period of 5 minutes, which were then clarified by centrifugation at 10 000 x g for 10 minutes, filter-sterilized, and used immediately for measuring the antibody levels.

ELISA

For enumeration of the IgA specific to *S. mutans,* 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μl of PAc (361-386) and PAc (346-364) peptides (concentration 20 μg/ml) or skim milk (as a control) in coating buffer at pH 9.6, and then subjected to ELISA (25). 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 100 μl aliquot of a 1/4 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 100 μl of alkaline phosphatase-conjugated goat anti-human immunoglobulin A (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 100 μl of para-nitrophenyl phosphate at 3 mg/ml as a substrate and incubation for 90 minutes at 37°C. Absorbance at 405 nm was measured with a microplate reader (Multiskan Bichromatic Laboratory Japan, Tokyo, Japan). The experiments were performed independently 3 times, with similar results obtained in each.

Bacteria counting

Cotton swabs containing saliva samples from the elderly subjects were placed in transport fluid and taken to BML for analysis. Each sample was poured onto Mitis-Salivarius agar (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) or modified Mitis-Salivarius agar containing 0.2 U/ml of bacitracin (MMTSB) (30) using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain), and incubated at 37°C under anaerobic conditions for 48 hours, before counting the total numbers of streptococci (tS) and mutans streptococci (mS) organisms. MMTSB is known to be extremely precise for the counting of MS colonies (30). Following anaerobic inoculation for 48 hours at 37°C, the numbers of colony-forming units (CFU) 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. All bacteria counting was performed by BML.

Various parameters in blood

Ten milliliters of blood was extracted from each of the elderly subjects, placed in sterilized glass tube including heparin, and sent to Niigata Rinsyo Laboratory (Niigata, Japan) to determine the following blood parameters; leukocyte count, erythrocyte count,

hemoglobin concentration, hematocrit count, platelet count, mean cellular volume (MCV), mean cellular haemoglobin (MCH), mean cellular haemoglobin concentration (MCHC), and total serum IgA. A percentage of the different types of leukocytes was generated based on the counts of eosinophils, basophils, lymphocytes, monocytes, and neutrophils.

Comparisons between 2 groups

The subjects were divided into 2 groups according to ELISA antibody titer, those with a PPA level $\leq$ 0.1(PPA not detected group) and those with a PPA level > 0.1 (PPA detected group). This grouping by antibody level has been used in previous epidemiological studies (30). ANOVA was used to compare periodontal status between the 2 groups. Differences at the .05 level were considered to be significant. StatView for Macintosh (Version 10.0) was used for all statistical analyses.

Results

There were no significant differences for the various parameters (DMFT, tooth number, sound tooth number, saliva volume) between the 2 groups (data not shown). Table 1 shows the periodontal status of the 2 groups based on antibody level, as determined by ELISA. The average rAL6 result (8.7±14.1) in the PPA detected group was significantly lower than that (14.7±22.9) in the PPA not detected group. Further, females in the PPA detected group showed significantly lower values for average BOP, AL, rAL6, and rPD (8.9±11.1, 3.4±1.0, 9.0±13.8, and 2.2±0.5, respectively) as compared to those in the non-detected group (16.8±17.2, 4.1±1.7, 22.1±32.6, and 2.5±0.7, respectively)(Table 2). Antibody specificity to the PAc (361-386) peptide was also confirmed by comparisons between PPA and the anti-PAc (346-364) peptide antibody (positive control) (25), and the anti-skim milk antibody (negative control). The PAc (346-364) peptide has an antigenic epitope that recognizes human antibodies (25), however, it did not show a correlation with the numbers of mS, tS, and LB organisms in saliva or the various periodontal status parameters (data not shown). There were no significant differences between males in the 2 groups for any of the periodontal parameters. As for female subjects, to clarify the systemic association of PPA with periodontal status, various blood status parameters, such as leukocytes, erythrocytes,

haemoglobin, hematocrit, and others, were tested and compared between the 2 groups. The PPA detected group showed significant lower values for eosinophils (2.5±1.8%) and basophils (0.5±1.3%) than the PPA not detected group (4.0±2.0% and 0.8±0.3%, respectively) (Table 3). However, there were no significant differences between the 2 groups when only males were analyzed. As for females, PPA showed a positive correlation with LB and tS counts, as well as proportion of basophils (p = 0.0015, 0.0433, and 0.0340, respectively) (Table 4). The number of Ts organisms also showed a positive correlation with LB and MS numbers (p = 0.0004 and 0.0170, respectively), while the number of LB organisms showed the largest correlation with number of MS in all comparisons ($p < 0.0001$). The percentage of eosinophils showed a positive correlation with that of basophils, as well as AL, BOP, and rPL6 (p = 0.029, 0.0439, 0.0475, and 0.0168, respectively). However, there were no significant differences between PPA level and total serum IgA level (data not shown). Thus, it was indicated that the PPA level in saliva responded to the progression of periodontal disease, as well as to the proportions of eosinophils and basophils in the female subjects.

Discussion

The present results suggest that the presence of PPA indicates immunological activities that induce the production of human IgA antibodies to tS, but not those to mS, in elderly subjects. A positive correlation between antibody levels in saliva and past experiences of infection and colonization with tS containing *S. mutans* and *S. sobrinus* in the oral cavity has been speculated. Further, a high concentration of PPA has been speculated to play a role as a negative contributor in proportion to the numbers of mS organisms in the oral cavity (30). However, in the present study the mS ratio was higher in the PPA detected group than the PPA not detected group for all subjects, as well as female and male subjects (1.5±3.7 vs 1.0±1.8, 1.7±3.9 vs 1.3±1.9, and, 1.4±3.4 vs 0.8±1.8, respectively), though the differences between the 2 groups were not significant.

The amino acid residue 365 to 377 [PAc (365-377) peptide] in the A-region is an antigenic epitope for the induction of antibodies that inhibits the interactions of *S. mutans* PAc with human salivary components (26, 27). Further, the common epitope (YEA-L-QY) between the surface protein antigen (PAg) of *Streptococcus sobrinus* (15) and its PAc, as well as its core B-cell epitope (-Y---L--Y----) are essential sequences in the antigenic epitopes of the surface proteins of oral streptococci that are specifically recognized by the antibody (35). The antibody to the core epitope in PPA may serve as

an indicator of infection by tS as well as mS colonization. Since PPA plays roles as a positive indicator and negative effector of MS infection, the non-correlation between PPA level and MS count in saliva seen in our study might have been caused by the mixture of positive and negative effects. Further, the correlations between numbers of LB organisms and PPA, tS, and MS indicate that LB may be incorporated with tS biofilm formation.

Periodontitis often induces bone absorption and gingival recession through gingival inflammation. In addition, gingival recession changes the oral condition, which may cause ecological changes, resulting in microbiological changes such as the development of supragingival plaque containing streptococcal bacteria (36, 37). Marsh found that the optimal growth condition for streptococci was different from other periodontal pathogens (38), while other studies have shown that the growth of periodontal pathogens is not correlated with or inhibited by *S. mutans*, and also suggested that number of streptococci was important for growth conditions for periodontal pathogens (39, 40). Therefore, an increase in number of tS may produce PPA and inhibit the growth of microorganisms that are associated with the progression of periodontal diseases.

In the present study, the PPA detected group showed a significantly lower

number of sites with rAL6 than the PPA not detected group, while the PPA detected group also showed a significantly lower rate of AL in female subjects. Several studies have reported the relationship between CA and AL (24, 41-43), however, the PPA not detected group showed a significant progression of AL, but did not of CA deposition, in the present subjects. Therefore, there may be contrasting variables involved in the relationships between the antibody and AL increases and reduction with aging. We performed a comparison between PPA and periodontal status in a small number of elderly subjects over a 4-year period (initial study in 2001; average age, 73 years old; 60 males, 27 females). Two years after obtaining baseline data, 62 of the subjects from the original cohort participated in follow-up examinations (2003 study; average age, 75 years old; 45 males, 17 females) and 1 year later 69 subjects from the original cohort (2004 study; average age 76 years old; 46 males, 23 females) also participated, with periodontal status continuously observed in PPA detected and PPA not detected groups. We found that AL was significantly lower in the PPA detected group in all subjects at every examination (data not shown), indicating a consistent association of AL with the induction of PPA.

In the present study, eosinophils were correlated with periodontal status during the progression of periodontitis, while basophils were also correlated with eosinophil

and PPA levels. Eosinophils and basophils are known to be associated with most inflammatory and infectious disorders associated with allergic manifestations. The eosinophil fraction in gingival crevicular fluid (GCF) from periodontitis patients has been reported to range from 6% to 10%, which is much higher than that in circulating blood (44), while GCF specifically yielded a higher rate of activated eosinophils in another study (45). A striking common feature of many autoimmune and inflammatory diseases in humans is that females are more susceptible to specific immunological disorders than males (46). Our results support those data, and also suggest that eosinophils and basophils play important roles in host immune and defensive systems in response to periodontitis in elderly females.

The present findings suggest that the anti-PAc (361-386) peptide antibody is responsible for physical status in periodontal tissues, as well as systemic condition in regards to eosinophil and basophil proportions among leukocytes during the development of periodontal disease. They also imply that the induction of PPA may be indirectly correlated with that of some factors that inhibit periodontal pathogens. We concluded that this antibody is useful as a predictor of periodontal diseases in elderly patients.

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Table 1. Periodontal status of subjects in antibody detected and non-dtected group

Periodontal status	Antibody non-detected group (n=40)	Antibody detected group (n=241)	*p*-value*
rCA	1.6 ± 4.6	1.1 ± 3.3	0.435
BOP	11.4 ± 13.1	8.9 ± 11.5	0.215
AL	3.7 ± 1.3	3.4 ± 1.0	0.145
rAL4	44.1 ± 32.6	37.1 ± 28.1	0.160
rAL6	14.7 ± 22.9	8.7 ± 14.1	0.029*
rPD	2.2 ± 0.1	2.2 ± 0.5	0.733
rPD4	11.6 ± 13.9	10.4 ± 11.9	0.579
rPD6	2.4 ± 4.4	2.0 ± 4.5	0.303

*: Significant difference demonstrated by Mann-Whitney U test

Table 2. Periodontal status of female subjects in antibody detected and non-dtected group

Periodontal status	Antibody non-detected group (n=13)	Antibody detected group (n=105)	*p*-value*
rCA	2.9 ± 5.5	1.3 ± 4.2	0.210
BOP	16.8 ± 17.2	8.9 ± 11.1	0.027*
AL	4.1 ± 1.7	3.4 ± 1.0	0.022*
rAL4	51.5 ± 35.0	38.1 ± 27.0	0.117
rAL6	22.1 ± 32.6	9.0 ± 13.8	0.010*
rPD	2.5 ± 0.7	2.2 ± 0.5	0.038*
rPD4	15.9 ± 16.6	10.4 ± 10.9	0.113
rPD6	3.2 ± 5.6	1.8 ± 3.3	0.203

*: Significant difference demonstrated by Mann-Whitney U test