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H. 知的財産件の出願・登録状況
該当なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
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IV. 研究成果の刊行物・別刷

Gerodontology

Original article

The role of anti-PAc (361–386) peptide SIgA antibody in professional oral hygiene of the elderly

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The role of anti-PAc (361–386) peptide SIgA antibody in professional oral hygiene of the elderly

Objective: Measurement of salivary IgA antibody (PAc-peptide antibody, PPA) to amino acid residues 361–386 of *Streptococcus mutans* PAc, which possess a multiple binding motif to various HLA-DR molecules and a B-cell epitope that recognises the inhibiting antibody to *S. mutans*, is an indicator for the population numbers of mutans streptococci (MS) in human saliva. The purpose of this study was to clarify the role of PPA in infection control of MS after professional oral hygiene care.

Materials and methods: Thirty-nine dependently living institutionalised elderly subjects (75.9 ± 7.5 years; 10 males, 29 females) participated in the study. The measurements of PPA, MS, total streptococci (TS) and lactobacilli (LB) were performed by ELISA and culture techniques from saliva, plaque and tongue samples from the elderly.

Results: After treatment using professional oral care, the numbers of MS decreased significantly at 6 months in saliva and tongue samples from the group not having PPA in comparison with the primary data; whereas in the PPA-detected group, a significant decrease in MS number was shown immediately following professional care at 1–12 months in all samples. There was little difference in the numbers of LB at any of the time points. The numbers of TS decreased rapidly in PPA-not detected group in comparison with the PPA-detected group.

Conclusion: PPA may be more effective for controlling MS number in the oral cavity after professional treatment. The measurement of PPA may be used for preventive instruction to dental caries at the chair side in the clinical setting.

Keywords: PAc (361–386) peptide, salivary IgA, oral hygiene, mutans streptococci, elderly.

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Introduction

Dental caries is one of the most common diseases of the oral cavity, and is a significant problem for the elderly where they are associated with tooth loss[1,2]. Although various species of bacteria have been found in dental plaque, mutans streptococci (MS), *Streptococcus mutans* and *Streptococcus sobrinus* are shown to be the principal causative pathogens related to dental caries in humans[3–5]. Reports have shown that institutionalised elderly individuals have poorer oral health than those who live independently at home[6–8]. The oral hygiene of the bedridden elderly individual is often wholly inadequate[7], and this promotes the formation of dental plaque containing opportunistic pathogens[7–9] that have been isolated[10–12]. The bacterial plaque formed on teeth and denture surfaces is thought to play a role in many diseases particularly aspiration pneumonia and septicaemia as well as dental diseases such as caries[12]. Therefore, the oral cavity is thought to be a potential reservoir of dental and systemic disease pathogens in the institutionalised elderly[13,14].

Mutans streptococci are thought to play a central role in the development of the biofilm and dental caries. They produce insoluble glucans incorporating other bacteria to form the mature biofilm[13–15]. Furthermore, MS may be an indicator for pathogenic biofilm formation on tooth surfaces where

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salivary levels of MS are shown to be correlated with coronal and root caries incidence in the institutionalised elderly[16–19].

The function of the cell surface protein antigen of *S. mutans* (known as PAc[20], AgI/II[21], PI[22] and B[23]) is essential for colonisation by the bacterium on tooth surfaces via its interaction with the salivary pellicle that coats the dental enamel[24–26]. The A-region of the PAc peptide (361–386) contains the epitope shown to induce an antibody that inhibits the interaction of *S. mutans* with salivary components on tooth surfaces[27,28], and includes the multiple binding motif (L–V-K–A) that reacts with human leucocyte antigens *DRB1*0802*, **1101*, **1402* and **1405*[29]. The concentration of secretory IgA (SIgA) antibody reacting with the PAc (361–386) peptide may be an indicator of the population and proportion of MS where low and high concentrations of the salivary antibody were found to be correlated positively and negatively, respectively, with the concentrations of MS in the saliva of human subjects[30].

Professional care is an important practice to maintain the oral health of the elderly[8,31]. Little is known about how oral care controls MS colonisation and infection in the oral cavity and the components present in saliva that are antimicrobial agents. To determine the most effective dental caries prevention strategy to maintain oral health of the elderly, the study examined the combined role of the anti-PAc (361–386) peptide SIgA antibody (PPA) during professional oral care and physical effects of professional care, and the effects of antibody function to reduce MS in the oral cavity following short and long duration care. Here we studied two groups of elderly patients either having or not having anti-PAc (361–386) peptide SIgA antibody in their saliva. The data suggest that the presence of SIgA against PAc is an important diagnostic tool for the development of preventive medicine for dental caries.

Materials and methods

Human subjects

Thirty-nine elderly residents from Itabashi-ward, Tokyo nursing home (75.9 ± 7.5 years old; 10 males, 29 females), who were dependently living in long-term nursing care participated in this study. The study was conducted from June 2006 to June 2007. The subjects were randomly selected from the residents and made blind to the investigators. Subjects with more than three teeth were selected. Prior to the survey, the aim and details of the study were explained and consent was obtained from all subjects before registration. The study was approved by the Ethics Committee of the Tokyo Medical and Dental University and performed according to the rules of the Helsinki Declaration. Dental examinations to determine the presence of dental caries, periodontal disease, denture usage, level of oral hygiene, coated tongue and other typical oral conditions were performed under artificial white light by trained dentists before the study and routine professional oral care began. Oral examinations were performed according to WHO oral examination procedures[32]. Coronal caries status indicators were: the number of coronal and root decayed teeth, the number of coronal and root filled teeth (FT), the number of coronal and root surface decayed teeth and the number of coronal and root surface filled teeth (DFT). The general oral status of subjects before investigation is shown in Table 1. The caregivers and other staff in the institution did not change during the study period.

Oral care

During the initial examination, all patients had a routine dental and medical check. Oral care techniques by dental hygienists were standardised before beginning oral care. For daily oral care, subjects who were able to use the sink facilities in their room performed daily care two to three times a day after each meal. In addition, dental hygienists provided professional care for 20 min once per week, i.e. dental brushing of tooth surfaces, mucosal cleaning with a sponge brush (Toothette; Inoue Attachment, Tokyo, Japan), denture cleaning and oral washing with tap water in addition to the daily oral care. The routine oral care including professional care was performed for 12 months. Subjects received no standard or professional care from entering the institution to the first sampling. No antibiotic therapy was administered during the 2 weeks before this study and during the 12 month study period; and none of the subjects suffered from severe infections. There was no significant difference in systemic disease incidence between the two groups before beginning the study (Table 1). However, there was no information concerning antibiotic therapy of the subjects before entering the institution. One subject at 6 months and two subjects at 12 months, after the beginning of the study, moved to other institutions in PPA-not detected group; no other subjects dropped out.

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Table 1 Oral state before investigation.

	Total	*PPA-not-detected*	*PPA-detected*
Tooth brush/day	2.50 ± 1.05	2.70 ± 1.22	2.32 ± 0.820
Existing tooth	19.0 ± 7.43	19.9 ± 7.20	18.0 ± 7.73
Decayed tooth	0.79 ± 2.17	0.90 ± 2.67	0.68 ± 1.53
Missing tooth	8.97 ± 7.48	8.10 ± 7.20	9.89 ± 7.84
Filled tooth	9.27 ± 5.45	10.6 ± .45	7.93 ± 5.28
Partial denture usage			
Do	18	7	11
Do not	21	13	8
Status of oral hygiene			
Good	10	4	6
Moderate	26	15	11
Bad	3	1	2
Inflammation of gingivae			
No	0	0	0
Mild	18	9	9
Moderate	18	10	8
Heavy	3	1	2
Coating of tongue			
–1/3	11	7	4
1/3–2/3	16	10	6
2/3–	12	3	9
Oral dryness			
Normal	25	16	9
Mild	1	1	0
Moderate	6	1	5
Severe	7	2	

Values indicate mean ± SD of numbers for tooth brush/day, existing tooth, and decayed, missing and filled tooth. Other values indicate number of persons. PPA, PAc-peptide antibody.

Sampling of the saliva, plaque and tongue

Subjects were asked not to brush their teeth 2 h before sampling. All sampling was performed before professional oral care at 0, 1, 2, 3, 6 and 12 months after beginning the study. Whole saliva was stimulated by chewing paraffin gum. For bacterial counts, a sterile cotton swab (Seedswab No. 1; Eiken Chemical Co., Ltd, Tokyo, Japan) was immersed in the saliva for 10 s and placed in a transport fluid (0.4% agar, 0.15% thioglycolate/phosphate). Supragingival plaque samples were collected from the postero-anterior buccal surface of the upper right second premolar and first molar five times with a cotton swab. Subjects having partial dentures (n = 29) and not having the above teeth were sampled from the opposite side or other remaining teeth. Tongue samples were collected from the centre of the tongue by swabbing five times. After placing in a transport fluid, all samples were immediately taken to the Biomedical Laboratory (BML, Tokyo, Japan) for analysis to detect MS, total streptococci (TS) and lactobacilli (LB).

Synthetic peptide

The sequence of PAc (361–386) (NAKATYEAALKQYEADLAAVKKANAA) was derived from the sequence of the PAc gene of *S. mutans* MT8148 as described by Okahashi *et al.*[33]. The peptide was synthesised using a step solid phase procedure at Asahi Techno Glass Co., Inc. (Tokyo, Japan). The synthesised peptide was subsequently purified with reversed-phase high-performance liquid chromatography (HPLC), a TSK-GEL column (1 × 30 cm; TOSO, Tokyo, Japan) with a 10–45% acetonitrile gradient in 0.1% trifluoroacetic acid for 50 min at a flow rate of 5 ml/min. Purity was determined to be >95% using HPLC analysis. To confirm the amino acid sequence of the synthetic peptides, several samples were randomly selected and sequenced using a System 7300 Amino Acid Analyzer (Beckman, Somerset, NJ, USA) and a Model 477A Protein Sequencer (Applied Biosystems, Foster City, CA, USA). The peptide was used as the coating antigen for the ELISA to detect antibody titre in the saliva samples.

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ELISA

Whole saliva from the subjects was stimulated by chewing paraffin gum and collected into ice-chilled sterile bottles over a period of 5 min. They were then clarified by centrifugation at 10 000 *g* for 10 min at 4°C. Saliva samples were also collected in plastic tubes and clarified by centrifugation at 10 000 *g* for 20 min at 4°C and stored at −80°C before being thawed just prior to measuring the antibody levels. To determine whether antibody was present in the saliva sample, 96-well microtitre H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μl of albumin-conjugated PAc (361–386) peptides 100 μg/ml in 50 mM carbonate buffer pH 9.6. The plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and blocked with 1% skimmed milk in PBST for 1 h at 37°C. Excess skimmed milk was removed by washing three times with PBST, a 100 μl aliquot of a 1:2, 1:4, 1:8 and 1:16 dilution of whole saliva added and the mixtures were then incubated for 1 h at 4°C. The plates were then washed three times with PBST, and further incubated for 1 h at 37°C with 100 μl alkaline phosphatase-conjugated goat anti-human IgA (heavy and light chains) (Zymed Laboratories, South San Francisco, CA, USA). After three washes with PBST, the bound antibodies were detected using the addition of 100 μl of para-nitrophenyl phosphate at 3 mg/ml as a substrate and incubation for 60 min at 37°C. Absorbance at 405 nm was measured using a microplate reader (Multiskan Bichromatic Laboratory Japan, Tokyo, Japan). The experiments were performed independently on two occasions and similar results were obtained. After the subjects were screened, they were selected and divided into two groups using the ELISA antibody titre: those with an anti-PAc (361–386) peptide SIgA antibody (PPA) level ≤0.11 OD (PPA-not detected group, $n = 20$) and those with a PPA level >0.11 OD (PPA-detected group, $n = 19$) as in a previous epidemiological study[34].

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) to count the TS, modified Mitis-Salivarius agar containing 0.2 U/ml bacitracin to count MS, and Rogosa selective lactobacillus agar to count LB using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain). These were incubated at 37°C under anaerobic conditions for 48 h[30] and 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 × 100.

Statistical procedures

All data were analysed using the Statistical Package for SPSS for Windows (version 100; Chicago, IL, USA). The repeated data of bacterial number (log 10) and ratio (MS/TS CFU) were compared using one-way ANOVA. *t*-Test with Bonferroni method was used to compare two groups. The data of other parameters from two groups in Table 1 were compared using a chi-squared test for equal and unequal variations. *p*-values less than 0.05 were considered to be significant.

Results

For systemic diseases such as sinus problem, pneumonia, cardiac disorder, diabetes mellitus, etc., there were no significant differences between the PPA-detected and PPA-non-detected groups (data not shown). The results for dental status of the PPA-detected and PPA non-detected groups are shown in Table 1. There were no significant differences between the two groups in all parameters. The anti-PAc (361–386) peptide SIgA antibody value (mean range of absorbance value at 405 nm, 0.095–0.130 or 0.159–0.260) using the ELISA did not change significantly for all sampling times (0, 1, 2, 3, 6 and 12 months) after treatment with professional oral care in PPA-not detected or PPA-detected groups (data not shown).

The roles of anti-PAc (361–386) SIgA antibody against the oral pathogenic bacteria, MS, MS ratio, LB and TS were studied at various time points after professional oral care from the saliva, plaque and tongue samples of both groups. Significant decreases for the repeated data of MS number were indicated by one-way ANOVA in all samples from both groups (Table 2). However, the variance level of repeated data to decreasing numbers of MS was higher in all samples from the detected PPA elderly patients than in those from the non-detected PPA elderly patients. Furthermore, in the two data comparison using *t*-test with the Bonferroni method, the numbers of MS decreased significantly at only 6 months after treatment in the saliva and tongue samples from the non-detected PPA elderly

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Table 2 Number of MS in 'PPA-not-detected' and 'PPA-detected' elderly subjects.

Subject group	*After start of professional oral care (month)*						*One-way*
Samples	*0*	*1*	*2*	*3*	*6*	*12*	ANOVA *(p)*
PPA-not-detected							
Saliva	5.1 ± 2.0	4.8 ± 1.4	4.7 ± 1.8	4.7 ± 1.5	4.1 ± 1.5*	4.3 ± 1.7	0.013*
Plaque	5.3 ± 2.0	5.0 ± 1.5	5.0 ± 1.9	4.8 ± 1.9	4.1 ± 2.0	4.4 ± 2.0	0.011*
Tongue	5.1 ± 2.0	4.7 ± 1.3	4.5 ± 1.6	4.4 ± 1.5	4.0 ± 1.3*	3.8 ± 1.8	0.003**
PPA-detected							
Saliva	5.1 ± 1.9	3.7 ± 1.8*	4.2 ± 1.6	3.8 ± 1.8*	3.3 ± 1.5**	3.2 ± 2.1**	0.000**
Plaque	5.5 ± 1.7	4.5 ± 1.9*	4.6 ± 2.2	4.2 ± 2.3*	3.7 ± 2.2**	3.9 ± 2.2**	0.000**
Tongue	5.3 ± 1.9	3.8 ± 1.7*	3.9 ± 1.6*	3.7 ± 1.8**	3.1 ± 1.8**	3.0 ± 2.0**	0.000**

Results are expressed as mean ± standard deviations for number (log 10/ml) of mutans streptococci. Asterisks indicate significant differences between 0 month and other months in *t*-test with Bonferroni method and among 0–12 months in one-way ANOVA (*$p < 0.05$, **$p < 0.01$). MS, mutans streptococci; PPA, PAc-peptide antibody.

patients in comparison with the primary data (at 0 month); in the PPA-detected group, a significant decrease in MS number was also observed throughout the study from 1 to 12 months in all samples. Significant differences for the repeated data of MS ratio were not indicated in all samples from both groups (Table 3). However, the MS ratio increased at only 2 and 3 months in saliva sample, and 1, 2, 3 and 6 months in plaque samples from the non-detected PPA elderly, in comparison with the primary data (Table 3). The MS ratio increased at 2 and 3 months in plaque samples, from the PPA-detected elderly, in comparison with the primary data. Significant decreases for the repeated data of LB were indicated in the plaque and tongue samples from PPA-not detected group (Table 4). Furthermore, there were significant differences in LB numbers at 6 h in the plaque and at 3 h in the tongue sample. By contrast, a significant increase for the repeated data of LB was not indicated in all samples from the PPA-detected group. The LB numbers increased significantly at only 3 months in the saliva sample from the PPA-detected group. Significant decreases for the repeated data of TS numbers were indicated in all samples from both groups (Table 5). The variance level of repeated data was similar in all samples from the detected PPA elderly patients and those from the non-detected PPA elderly patients. The numbers of TS decreased significantly at 3, 6 and 12 months in the saliva; at 2, 3, 6 and 12 months in plaque; and at 6 months on the tongue from the non-detected PPA group (Table 5); whereas in the detected PPA group, the TS numbers decreased significantly only at 6 months in all samples, by comparison with time zero.

Table 3 MS ratio in 'PPA-not-detected' and 'PPA-detected' elderly subjects.

Subject group	*After start of professional oral care (month)*						*One-way*
Samples	*0*	*1*	*2*	*3*	*6*	*12*	ANOVA *(p)*
PPA-not-detected							
Saliva	1.2 ± 1.4	1.6 ± 1.4	2.6 ± 2.6*	3.1 ± 5.0*	3.2 ± 3.9*	1.8 ± 2.3	0.054
Plaque	1.5 ± 1.6	3.4 ± 3.2**	4.9 ± 4.1**	4.2 ± 4.2*	9.4 ± 16.1*	3.2 ± 3.5	0.110
Tongue	1.4 ± 1.5	1.1 ± 1.1	1.7 ± 1.9	1.0 ± 1.3	1.4 ± 2.0	1.0 ± 2.4	0.273
PPA-detected							
Saliva	0.6 ± 1.0	0.5 ± 0.6	0.9 ± 1.4	1.0 ± 1.9	1.2 ± 2.7	1.0 ± 2.1	0.579
Plaque	1.3 ± 1.5	1.9 ± 2.1	3.0 ± 2.9*	3.3 ± 3.8*	3.6 ± 5.6	2.8 ± 4.1	0.091
Tongue	0.4 ± 0.8	0.5 ± 0.7	0.5 ± 0.8	0.5 ± 1.0	0.8 ± 1.5	0.3 ± 0.6	0.372

Results are expressed as mean ± standard deviations for number (%) of MS ratio. Asterisks indicate significant differences between 0 month and other months in *t*-test with Bonferroni method and among 0–12 months in one-way ANOVA (*$p < 0.05$, **$p < 0.01$). MS, mutans streptococci; PPA, PAc-peptide antibody.

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Table 4 Number of LB in 'PPA-not-detected' and 'PPA-detected' elderly subjects.

Subject group	*After start of professional oral care (month)*						*One-way*
Samples	*0*	*1*	*2*	*3*	*6*	*12*	ANOVA *(p)*
PPA-not-detected							
Saliva	3.7 ± 1.9	3.8 ± 1.5	3.6 ± 1.9	3.5 ± 2.0	3.2 ± 1.5	3.5 ± 1.7	0.155
Plaque	3.7 ± 1.5	3.4 ± 1.8	3.5 ± 1.9	3.2 ± 1.9	2.8 ± 1.7*	2.9 ± 2.1	0.003**
Tongue	3.8 ± 1.2	3.8 ± 1.3	3.3 ± 1.7	3.2 ± 1.9*	3.3 ± 1.7	3.0 ± 1.7	0.013*
PPA-detected							
Saliva	2.6 ± 1.9	3.0 ± 1.7	3.1 ± 1.7	3.3 ± 1.5*	2.8 ± 1.6	3.0 ± 1.8	0.430
Plaque	2.5 ± 2.0	2.8 ± 1.8	2.8 ± 2.0	3.1 ± 2.0	2.8 ± 1.7	2.9 ± 1.7	0.603
Tongue	3.0 ± 1.8	3.1 ± 1.9	3.3 ± 1.4	3.3 ± 1.5	2.7 ± 1.8	2.9 ± 1.6	0.643

Results are expressed as mean ± standard deviations for number (log 10/ml) of lactobacilli. Asterisks indicate significant differences between 0 month and other months in *t*-test with Bonferroni method and among 0–12 months in one-way ANOVA (*$p < 0.05$, **$p < 0.01$). LB, lactobacilli; PPA, PAc-peptide antibody.

Table 5 Number of TS in 'PPA-not-detected' and 'PPA-detected' elderly subjects.

Subject group	*After start of professional oral care (month)*						*One-way*
Samples	*0*	*1*	*2*	*3*	*6*	*12*	ANOVA *(p)*
PPA-not-detected							
Saliva	7.1 ± 0.2	7.2 ± 0.2	7.0 ± 0.3	6.9 ± 0.3**	6.2 ± 0.5**	6.9 ± 0.4*	0.000**
Plaque	7.2 ± 0.2	7.2 ± 0.2	7.1 ± 0.4*	7.0 ± 0.4*	6.1 ± 0.8**	6.8 ± 0.5**	0.000**
Tongue	7.1 ± 0.3	7.2 ± 0.2	7.1 ± 0.2	7.1 ± 0.2	6.4 ± 0.3**	7.0 ± 0.4	0.000**
PPA-detected							
Saliva	7.0 ± 0.3	7.1 ± 0.2	7.2 ± 0.2	7.0 ± 1.5	6.4 ± 0.4**	6.8 ± 0.6	0.000**
Plaque	7.1 ± 0.3	7.2 ± 0.2	7.2 ± 0.2	7.1 ± 0.3	6.4 ± 0.8**	6.8 ± 0.6*	0.000**
Tongue	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.1 ± 0.2	6.6 ± 0.5**	7.1 ± 0.3	0.000**

Results are expressed as mean ± standard deviations for number (log 10/ml) of total streptococci. Asterisks indicate significant differences between 0 month and other months in *t*-test with Bonferroni method and among 0–12 months in one-way ANOVA (*$p < 0.05$, **$p < 0.01$). TS, total streptococci; PPA, PAc-peptide antibody.

Discussion

The human saliva defence factors that control colonisation and infections by oral micro-organisms include histatins, lactoferrin, lactoperoxidase, lysozyme, defensin, statherin, SIgA and amylase[35–41]. Reports show that SIgA is an important factor for host defence against infection[40,41]. This has focused interest on the development of mucosal vaccines[42,43] as well as their possible therapeutic use in the treatment of infection[28,41,44]. Additionally, saliva SIgA concentrations are known to be associated with caries protection[45,46] and SIgA antibodies are reported to play an important role against *S. mutans* to prevent dental caries through bacteriostasis[45,47].

In this study we show that decreasing MS numbers using professional oral care occurred rapidly in elderly patients with the salivary anti-PAc (361–386) peptide SIgA antibody in comparison with the elderly patients having no antibody. The application of mouse monoclonal antibody to PAc (361–386) peptide inhibits the interaction of *S. mutans* to salivary components *in vitro*[48,49] and the recolonisation of *S. mutans* on the tooth surface in rats[49]. Therefore, it could be suggested that the antibody reduces MS infection and attachment in the oral cavity that is further reduced in the biofilm following professional oral care. Possibly the biofilm bacteria induce a low phagocyte response that provides a barrier to the antibody, complement and the cells of the immune system[50]; and the oral biofilm blocks the salivary antibody allowing bacterial infection. Oral hygiene procedures are used to remove the biofilm from teeth and mucosal surfaces to create a healthy environment in the oral cavity. Salivary PAc (361–386) peptide SIgA antibody may be more effective in controlling oral

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micro-organisms and supports the effect of professional care in the oral cavity by a hygienist rather than using conventional care that does not completely remove the biofilm.

In unpublished data, subjects with anti-PAc (361–386) peptide SIgA had a significantly lower incidence of new caries than those that were SIgA negative. The antibody is thought to be responsible for the inhibition of the caries. Tsuha *et al.*[30] reported that the SIgA antibody titre against the PAc (361–386) peptide of *S. mutans* showed a significant negative correlation with the level of *S. mutans*. This suggests a negative correlation between SIgA antibody titre against the PAc (361–386) peptide of *S. mutans* and the incidence of caries. However, the numbers of MS were not significantly lower than in the PPA-detected group compared with the PPA non-detected group in our primary data before beginning of professional oral care. A previous report shows similar results in the elderly without such care[34]. This may be caused by the combination of a positive response to MS infection with a low antibody titre and the negative effect by direct reaction of SIgA antibody to the MS infection with a high antibody titre in the PPA-detected elderly group who had >0.11 OD in ELISA. Our data suggest that the measurement of salivary anti-PAc (361–386) peptide SIgA antibody is a useful tool to predict positive outcomes to prevent infection and attachment of MS in patients as well as predicting a lower dental caries incidence in future. Previous reports described the immune response against cariogenic bacteria in the oral cavity[41,51]. However, this is the first report to show that the natural immune system can support the prevention of cariogenic bacteria with the added use of professional oral care in humans.

Elderly people with no SIgA antibody to the PAc (361–386) peptide showed a higher incidence of periodontal disease indicators such as attachment loss[34]. Therefore, elderly subjects without the antibody may show increased periodontal disease risk as well as caries risk. Patients exhibiting increasing MS with a negative SIgA antibody should alert the clinician to suspect that there is an increased risk of tooth loss.

Therefore, the risk of carries incidence can be assessed by measuring the concentration of inhibiting SIgA against *S. mutans* in saliva. This antibody may suppress the re-attachment of MS by specific binding to the tooth surface. Measurement of MS-specific SIgA titre in saliva may predict the change in MS levels in the oral cavity. This antibody level did not correlate significantly with increasing LB, but not having PPA was implicated in the increasing ratio of MS/TS in elderly people. The decreasing TS numbers that occurred rapidly in elderly patients not having PPA in comparison with the elderly patients having PPA affected the increasing ratio. The antibody titre may not affect decreasing LB numbers or the microbial ratio by MS and TS during treatment.

Recently, a rapid diagnostic system has been, the Saliva check SIgA system, using the PAc (361–386) peptide to measure the salivary antibody. Subjects with a negative Saliva check SIgA showed a significantly higher incidence of DFT and FT during the following year when compared with those with a Saliva check-positive SIgA. Therefore, the measurement of anti-PAc (361–386) peptide IgA antibody may be used for preventive instruction at the chair side in the dental surgery as it provides an immediate evaluation of the potential caries risk.

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ORAL MICROBIOLOGY
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Inhibiting effects of *Streptococcus salivarius* on competence-stimulating peptide-dependent biofilm formation by *Streptococcus mutans*

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Introduction: The effects of *Streptococcus salivarius* on the competence-stimulating peptide (CSP)-dependent biofilm formation by *Streptococcus mutans* were investigated.
Methods: Biofilms were grown on 96-well microtiter plates coated with salivary components in tryptic soy broth without dextrose supplemented with 0.25% sucrose. Biofilm formations were stained using safranin and quantification of stained biofilms was performed by measuring absorbance at 492 nm.
Results: *S. mutans* formed substantial biofilms, whereas biofilms of *S. salivarius* were formed poorly in the medium conditions used. Furthermore, in combination cultures, *S. salivarius* strongly inhibited biofilm formation when cultured with *S. mutans*. This inhibition occurred in the early phase of biofilm formation and was dependent on inactivation of the CSP of *S. mutans*, which is associated with competence, biofilm formation, and antimicrobial activity of the bacterium, and is induced by expression of the *comC* gene. Comparisons between the *S. mutans* clinical strains FSC-3 and FSC-3Δ*glrA* in separate dual-species cultures with *S. salivarius* indicated that the presence of the bacitracin transport ATP-binding protein gene *glrA* caused susceptibility to inhibition of *S. mutans* biofilm formation by *S. salivarius*, and was also associated with the regulation of CSP production by *com* gene-dependent quorum sensing systems.
Conclusion: It is considered that regulation of CSP by *glrA* in *S. mutans* and CSP inactivation by *S. salivarius* are important functions for cell-to-cell communication between biofilm bacteria and oral streptococci such as *S. salivarius*. Our results provide useful information for understanding the ecosystem of oral streptococcal biofilms, as well as the competition between and coexistence of multiple species in the oral cavity.

Key words: cell-to-cell communication; competence-stimulating peptide; GlrA; oral biofilm; *Streptococcus mutans*

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Mutans streptococci, including *Streptococcus mutans* and *Streptococcus sobrinus*, are biofilm-forming bacteria considered to be the primary etiological agents of human dental caries (13, 23). They possess a variety of abilities to colonize tooth surfaces and, under certain conditions, are numerically significant in cariogenic biofilms and also form biofilms with other organisms, including other streptococci and bacteria, such as *Actinomyces*, *Neisseria*, and *Veillonella*, in the oral cavity (5, 23, 30). Dental biofilms are produced by bacterial communities, and have been reported to have a vast biodiversity (>700 bacterial species) (2, 20, 33) and high density [$\sim 10^{11}$ cells/g (wet weight)] (14). Oral streptococci have been shown to

compose from 60% to 90% of supragingival plaque biomass structures within the first 24 h of colonization (26, 30). Although numerous studies have elucidated the mechanisms of initial streptococcal adhesion (9, 17, 31, 40) and coaggregation (12, 18) among oral bacteria, the subsequent process of bacterial interaction, accumulation, and proliferation leading to a functional heterogeneous organized sessile community such as dental biofilm has not been well elucidated. It is likely that cooperative interactions between mutans streptococci and other oral streptococci play important roles in the development of dental biofilm and caries in the oral cavity (19).

Oral streptococci are normal inhabitants of the human oral cavity and play a role in resistance to colonization by invading pathogens (43, 45). However, in spite of increased research into the functions of cariogenic bacteria with those of other oral streptococci, little information is available on the competition between and coexistence of surface-associated communities. *Streptococcus salivarius* is one of several oral commensal bacteria and a major constituent of biofilms that colonize the buccal epithelium, tongue, and dorsal epithelium (4). This organism can comprise the majority of the total cultivable flora on soft tissues of the mouth, and possesses a number of important biological activities for lactose uptake and urease enzymes that are thought to contribute to the stability of oral communities (7, 42). Furthermore, products from *S. salivarius* may control oral biofilm formation over a considerable distance to integrate the whole oral cavity into a single, interacting ecosystem (10, 42). In the present study, we studied cell-to-cell communication of *S. mutans* and *S. salivarius*, and attempted to quantify the effects of *S. salivarius* on the biofilm formation by *S. mutans*, by investigating interspecies interactions in 96-well microtiter plates coated with salivary components.

S. mutans is known to be resistant to bacitracin and a number of mechanisms of that resistance have been reported for a variety of bacteria (6, 32, 35). In the bacitracin-producing organism *Bacillus licheniformis*, resistance is attributed to the *bcrABC* genes, which encode a putative heterodimeric ATP-binding cassette (ABC) transporter that has been proposed to mediate the active efflux of bacitracin and bacitracin resistance (29, 35). Furthermore, homologs of that transporter have been identified in *S. mutans* (8, 44) and the homolog encoding a protein with 60% identity to the BcrA protein was designated GlrA. In our previous study, the *S. mutans glrA*-deficient mutant FSC-3 (FSC-3Δ*glrA*) showed morphological changes in a flow cell system, including a lower level of biofilm formation in the bottom area and a higher level in the top area when attached to a glass surface, when compared with FSC-3 (28). Therefore, *glrA* is a relating gene for biofilm morphology of *S. mutans*. A recent report has proposed competence-stimulating peptide (CSP), which induces competence and antimicrobial activity and is encoded by *comC*, as one of the key factors for streptococcal biofilm formation (34). Our study showed that expression of *glrA* was regulated by CSP in *S. mutans*.

CSP is recognized by the sensor kinase receptor ComD which is autophosphorylated and transfers a phosphoryl group to the ComE response regulator (16). ComE activates a number of early competence genes. ComX, which acts as an alternative sigma factor, activates late competence genes in *S. mutans* and *Streptococcus pneumoniae* (3, 21). Inactivation of the regulators involved in competence, including *comDE* or *comX*, is likely to interfere with the expression of genes which are regulated by CSP.

In the present study, to investigate the influences of *S. salivarius* to CSP-dependent biofilm formation and *glrA*-dependent morphological changes of biofilm by *S. mutans*, FSC-3Δ*glrA*, and *comC*, *comD*, *comE*, and *comX* mutants, and wild-type strains were used in the present experiments. Our results provide useful information for understanding the ecosystem of oral streptococcal biofilm, as well as competition between and coexistence of multiple species in the oral cavity.

Materials and methods

Bacterial strains and culture conditions

S. mutans GS5 and UA159, and *S. salivarius* HT9R were used in this study. The *S. mutans* clinical strain FSC-3 and its isogenic mutant FSC-3Δ*glrA* (28), as well as UA159 *com* mutants, were also used to compare dual-species biofilm formation in combination cultures with *S. salivarius*. GS5Δ*comC* was also used for comparison of bacteriocin production in combination culture with FSC-3 and FSC-3Δ*glrA*. The group C streptococcal strain RP66 was used in some assays as an indicator of the bacteriocin activity (47). The tested mutants and wild-type strains are listed in Table 1. All bacteria were grown in brain–heart infusion broth (BHI, Difco Laboratories, Detroit, MI) in an aerobic atmosphere of 5% CO_2, 75% N_2, and 20% O_2 (GasPack CO_2, Becton/Deckinson, Sparks, MD) at 37°C before incubation in 96-well microtiter plates.

Human saliva collection

Whole saliva samples were collected from five healthy human subjects (28–30 years old) after stimulation by chewing paraffin gum and were pooled into ice-chilled sterile bottles over a period of 5 min. The samples were clarified by centrifugation at 10,000 *g* for 20 min at 4°C, filter sterilized, and used immediately for biofilm assays in 96-well microtiter plates.

Biofilm formation assays

Biofilm formation by each strain was assayed using a method described previously (28). To evaluate biofilm formation by mixed cultures of *S. mutans* GS5, FSC-3, FSC-3Δ*glrA*, UA159, and UA159 *com* mutants with *S. salivarius*, 20 μl of each cell suspension [4.0×10^4 colony-forming units (CFU)] was mixed with 160 μl tryptic soy broth without dextrose (TSB, Difco Laboratories) supplemented with 0.25% sucrose (TSBS) in the wells of a 96-well (flat bottom) microtiter plate

Table 1. Bacterial strains

Strain	Genotype or phenotype	Source or reference
Streptococcus mutans		
GS5	Erms, serotype c human isolate	SUNYaB[a]
GS5 *comC* mutant	Ermr, GS5 derived, comC deficient	Ref. 46
UA159	Erms, serotype c human isolate	ATCC[b]
UA159 *comC* mutant	Ermr, UA159 derived, comC deficient	This study
UA159 *comD* mutant	Ermr, UA159 derived, comD deficient	This study
UA159 *comE* mutant	Ermr, UA159 derived, comE deficient	This study
UA159 *comX* mutant	Ermr, UA159 derived, comX deficient	This study
FSC-3	Erms, human isolate	Ref. 28
FSC-3 *glrA* mutant	Ermr, FSC-3 derived, *glrA* deficient	Ref. 28

[a]SUNYaB: the culture collection in Department of Oral Biology, State of New York, Buffalo, NY.
[b]ATCC: American Type Culture Collection, Manassas, VA.

(Sumitomo Bakelite, Tokyo, Japan). Single culture assays of the tested strains were also performed as controls by mixing 20 μl of each cell suspension (4.0×10^4 CFU) with 180 μl TSBS. To more closely approximate human oral conditions, salivary components, including antimicrobial agents and receptors of streptococcal adhesions (9), were used to coat the polystyrene surfaces before the addition of the bacterial cell suspension into the wells. The coating of whole saliva was performed for 60 min at 4°C. In some of the experiments with single and dual-species cultures, after the plates were incubated at 37°C for 4, 8, 12, 16, and 20 h under 5% CO_2 aerobic conditions, liquid medium was removed and the wells were rinsed a second time with sterile distilled water (dH_2O). The plates were air-dried and stained with 0.25% safranin and 0.5% ethanol in H_2O for 15 min, then rinsed with dH_2O to remove excess dye and air-dried. The biofilm mass was measured using a microplate reader (Thermo Bioanalysis Japan, Tokyo, Japan) without dissolving with solvent because the biofilm was formed uniformly on the bottoms of the wells in the 96-well plates (28). Quantification of stained biofilm on the bottom was performed by measuring absorbance at 492 nm.

CSP (amino acid sequence, SGSLSTFFRLFNRSFTQALGK) (22) was synthesized by Asahi Techno Glass (Tokyo, Japan). To analyse the effects of CSP on the cell growth of *S. mutans* UA159 and UA159Δ*comC*, 10 μl of each cell suspension (2.0×10^4 CFU) was mixed with 90 μl TSBS involving 0, 0.2, 0.5, 1.0, 2.0, 5.0, 7.0, or 10.0 μM CSP in the 96-well microtiter plate and incubated for 0, 2, 5, 8, 10, 12, 14, and 16 h at 37°C under 5% CO_2 aerobic conditions. The cell growth level at each culture time was determined by measuring absorbance at 540 nm. Furthermore, to clarify the effects of CSP on biofilm produced by *S. salivarius* HT9R, 20 μl of culture supernatant (0, 6.25, 12.5, 25.0, 50.0, and 100 μg/ml) from HT9R filtered through 0.22-μm pore-size filters was mixed and incubated with 20 μl exogenous CSP (70 μM) at 37°C for 1 h. Then, 40 μl CSP solution treated in this way was added to *S. mutans* UA159Δ*comC* (4.0×10^4 CFU) in 160 μl fresh TSBS to a final concentration of 7 μM and incubated at 37°C for 16 h. In other experiments performed for clarification of the inhibition effects of CSP on *S. salivarius*, exogenous CSP was added to experiments of mixed cultures of UA159 or UA159Δ*comC* and *S. salivarius* HT9R, and of FSC-3 or FSC-3 *glrA* and *S. salivarius* HT9R, with a final concentration of CSP at 1 or 10 μM.

To confirm which species formed biofilms in dual-species cultures with *S. mutans* GS5 and *S. salivarius* HT9R, 200 μl of each cell suspension (4.0×10^5 CFU) and 1600 μl TSBS were mixed in the wells of a 24-well (flat bottom) microtiter plate (Corning Incorporated, Corning, NY) with a coating of saliva. After culturing for 4, 8, 12, 16, and 20 h, pH in the supernatant was determined. After washing three times with sterilized phosphate-buffered saline (PBS), the biofilm cells were scraped with a sterilized scraper into 1 ml sterilized PBS. The scraped biofilm was collected and that remaining was collected using a pipette with sterilized PBS, after which 0.25% safranin was added to the well after scraping. No or only slightly stained biofilm remaining was used to confirm the efficacy of the scraping. The biofilm suspension was sonicated using ultrasonic dispersion (60 W power output) for 10 s, then shaken, diluted 1/1000 in PBS, and poured into a Mitis-Salivarius agar plate (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) using an EDDY JET spiral system (Gunze Sangyo, Inc., Tokyo, Japan) (25). Following aerobic incubation with 5% CO_2 for 48 h, *S. mutans* and *S. salivarius* colonies were identified by their characteristic appearance.

Preparation of inhibiting substance

S. salivarius HT9R was incubated aerobically in extra BHI medium after dialysis on a cellulose membrane (passage molecular weight, 14,000, Viskase Companies, Ind., Darien, IL) at 37°C overnight, and then supernatant samples were precipitated by salting out utilizing ammonium sulfate. Next, the precipitates were collected by centrifugation at 10,000 *g*, and suspended and dialysed in 20 mM Tris–HCl buffer (pH 7.4) at 4°C. Samples greater than 10 kDa were condensed by ultrafiltration using a centrifugal filter device in the sample solution (Amicon Ultra, Millipore, Billerica, MA). Each supernatant sample was then applied to a Sepharose 4B column (26 × 100 cm, Amersham Pharmacia Biotech, Buckinghamshire, UK) pre-equilibrated with the same buffer. After washing the column extensively, 7-ml fractions were collected, then monitored for ultraviolet absorbance and suppression effects toward the formation of biofilm by *S. mutans* GS5. Active fractions were pooled and the proteins were again precipitated by salting out using ammonium sulfate. Then the precipitates were collected by centrifugation at 10,000 *g*, and suspended and dialysed in PBS (pH 7.4). Protein concentrations in each sample solution were determined using a protein assay kit (BioRad, Richmond, CA) and the samples were also used as supernatant samples in biofilm experiments.

Construction of *com* mutants

The *comCDEX* genes were identified in the *S. mutans* UA159 database (http://www.genome.ou.edu/smutans.html) and mutants were constructed by double-crossover homologous recombination via insertion of an erythromycin resistance determinant into each gene. The plasmids used for disruption of the *comCDEX* genes were prepared as follows. The polymerase chain reaction (PCR) fragments of the upstream

Table 2. Primer list

Primer	Nucleotides sequence	Amplicon
ComCAF (Kpn)	5′-CGGGTACCAAATCTGAACAAGCAGGGG-3′	comCA
ComCAR (Bam)	5′-CGGGATCCGATAGTGTTTTTTTCATTTTATATCTCC-3′	
ComCBF (Bam)	5′-CGGGATCCTGGGAAAAT-3′	comCB
ComCBR (Xba)	5′-CGTCTAGACAGGACATCAATTGCAGGA-3′	
ComXAF (Kpn)	5′-CGGGTACCGGGCTAATGGTTTCTCCTT-3′	comXA
ComXAR (Bam)	5′-CCGGATCCAATCTTCACGAGTCCACA-3′	
ComXBF (Bam)	5′-CCGGATCCGGGAACATCCAGAATTAGAA-3′	comXB
ComXBR (Xba)	5′-CGTCTAGACATTTAAATCAACACTGGCC-3′	
ComDAF (Kpn)	5′-CGGGTACCCCATTCATCTGAAACTCAGT-3′	comDA
ComDAR (Bam)	5′-CCGGATCCAACAGGCAGCAGACCATAA-3′	
ComDBF (Bam)	5′-CCGGATCCGGCGGGCAATCATATTCTT-3′	comDB
ComDBR (Xba)	5′-CGTCTAGATCCTGCAATTGATGTCCTG-3′	
ComEAF (Kpn)	5′-CGGGTACCGCTGCTTTATCTTGGACAG-3′	comEA
ComEAR (Bam)	5′-CCGGATCCGGTTTCAAGACGTCCTTGT-3′	
ComEBF (Bam)	5′-CCGGATCCTTATTGACGCTATCCCTG-3′	comEB
ComEBR (Xba)	5′-CGTCTAGAGCTCTCCTTTGATCAGCAA-3′	

Endonuclease recognition sequences are underlined.
A, upstream; B, downstream; F, forward; R, reverse.