A survey on the risk factors for the prevalence of dental caries among preschool children in Japan

Yoshiaki Nomura, Shinpei Tsuge, Masaki Hayashi, Masahiro Sasaki,
Tetsuya Yamauchi, Nobuyoshi Ueda and Nobuhiro Hanada

Reprinted from

PEDIATRIC DENTAL JOURNAL 14(1): 79–85, 2004

PEDIATRIC DENTAL JOURNAL 14(1): 79–85, 2004

A survey on the risk factors for the prevalence of dental caries among preschool children in Japan

Yoshiaki Nomura[1,2], Shinpei Tsuge[3], Masaki Hayashi[3], Masahiro Sasaki[3], Tetsuya Yamauchi[3], Nobuyoshi Ueda[3] and Nobuhiro Hanada[1]

[1] Department of Oral Health, National Institute of Public Health
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, JAPAN
[2] Department of Preventive Dentistry and Public Health, Tsurumi University School of Dental Medicine
2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, JAPAN
[3] Gifu Dental Association
1-8 Kano-Jounan-dori, Gifu-shi, Gifu 500-8486, JAPAN

Abstract The etiology of dental caries has been suggested to be multifactorial. We conducted a cross-sectional study to investigate the contribution of the risk factors for dental caries, surveying a total of 645 preschool children at medical check-ups. Among 10 factors investigated in this study, salivary flow, amount of Lactobacillus, amount of total Streptococci, amount of mutans streptococci, and daily number of times of sweet soft drinks correlate with the prevalence of dental caries. Multivariate Logistic regression analysis using the five factors that correlated produced only two factors, salivary levels of mutans streptococci and Lactobacillus, which correlated with the prevalence of dental caries. Furthermore, clear dose-response relationships were observed in these two factors. We therefore suggest that cariogenic bacteria are the most important risk factor for dental caries among preschool children in Japan.

Key words
Lactobacilli,
Mutans streptococci,
Preschool children,
Risk factors,
Saliva

Introduction

Dental caries has been suggested to be a multifactorial disease. Keyes has proposed three main factors in the etiology of dental caries: host, substrate, and microflora[1]. Furthermore, the mechanism of dental caries was theoretically explained[2]. Some clinical trials have shown that controlling these factors suppresses the incidence of dental caries. For the host factors, sodium fluoride has been suggested as useful for preventing dental caries both clinically and economically[3]. For the substitute factors, diet sugar consumption is correlated with the incidence of new dental caries[4,5], and restricting the intake of diet sugar has been suggested to reduce the incidence of dental caries[6]. Recently, xylitol has been shown to be useful as an alternative sucrose, and its efficiency was confirmed[7]. For microflora factors, plaque control has conventionally been used as the primary preventive method. Many clinicians have used plaque control as a tool for fighting both dental caries and also periodontal disease. Furthermore, application of anti-microbial drugs could reduce the number of mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) in saliva or plaque for a few months[8].

These factors are all correlated with the etiology of dental caries. However, the weight of these factors contributing to the prevalence or incidence of dental caries has not been clarified. These factors may confound each other in attempts to explain the etiology of dental caries, and some studies have shown that controlling only one of these factors could not suppress the incidence of dental caries completely.

In this study, we obtained clinical samples and information about the etiology of dental caries by questionnaires at preschool medical check-ups. The aim of this study was to analyze the contribution

Received on September 22, 2003
Accepted on January 14, 2004

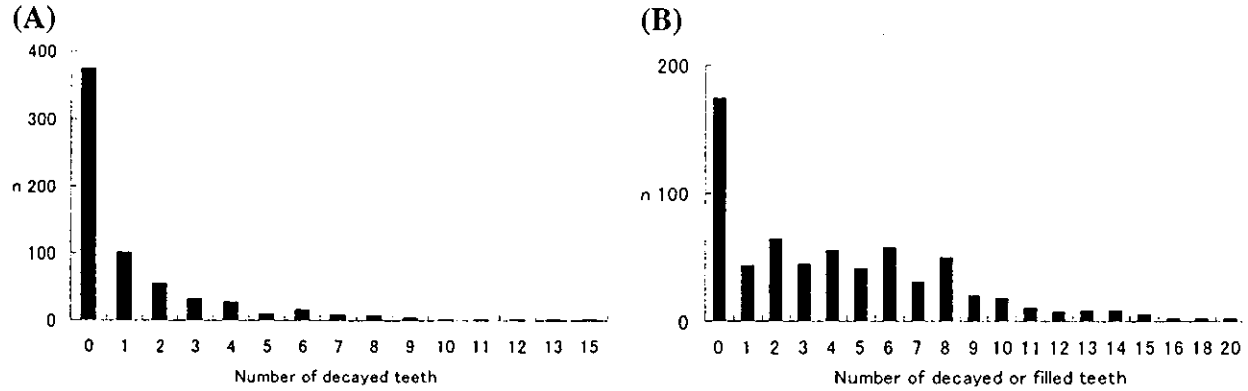

Fig. 1 The distribution of dt and dft

The distribution of the number of decayed deciduous teeth (A) and decayed or filled teeth (B) in this study. The median of the decayed teeth was 0 and decayed or filled teeth was 3. With the erupted permanent teeth, no decayed of filled teeth was observed.

of these factors in children by multiple Logistic regression analysis to clarify their importance.

Materials and methods

Study population

The study population was sampled from pre-elementary school children (five or six years old) residing in the Ena and Nakatsugawa areas of Gifu Prefecture, Japan. The fluoride concentration of drinking water in this area is less than 0.8 ppm. Thirteen of the thirty elementary schools, 9 from Ena area and 4 from Nakatsugawa, were selected to sample the population. Children were informed of the survey by the letter from the municipal office announcing their entry into elementary school and a total of 645 children participated in this study. Twelve children dropped out, primarily because of relocation and missing check ups due to illness. We obtained clinical samples and questionnaires during the pre-school medical check-ups; we obtained informed consent at the collection of the questionnaires.

Clinical examination and clinical samples

Dentists conducted oral examinations under a light and using dental mirrors. Teeth examined for dental caries were scored as sound, decayed or filled. The decayed or filled teeth were identified according to the WHO standard method and criteria[9].

Saliva samples were obtained by having subjects chew a gum base that contains no taste or flavor additives for 3 mins. The 3-min stimulated salivary flow and salival buffering capacity were evaluated by pH testing paper (Toyoroshi, Tokyo, Japan).

Microbial procedures

To quantify the total Streptococci, mutans streptococci, and Lactobacilli in saliva, we performed microbial procedures according to the method described previously[10]. Saliva samples (50μl) were sonicated by ultrasonic dispersion (60 power output) for 10 seconds and poured onto Mitis-Salivarius agar (MS, Gibco, Tokyo, Japan) plates for total Streptococci, improved Mitis-Salivarius agar plates containing 0.02 M bacitracin (Wako, Osaka, Japan) (MSB) and 2 ug/ml of Gramidine[11] for mutans streptococci and Rogosa SL agar plates (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) for Lactobacilli using an EDDY JET spiral system (Gunze Sangyo, Inc., Tokyo, Japan). In the improved MSB plate, the growth of the mutans streptococci are higher than that in the conventional MSB plate[12]. All samples were then incubated for 48 hours anaerobically. After the anaerobic incubation, we counted the colonies on each agar plate and calculated the number of bacteria per ml whole-saliva.

Questionnaires

Questionnaires were distributed by mail with the announcement of school participation and collected at preschool medical check-ups. The questionnaires consisted of five items concerning fluoride usage and diet. Fluoride usage was evaluated by daily usage of fluoride containing dentifrices (yes or no), the experience of fluoride varnish at private dental office or usual health care check-ups (yes, experienced or never) and daily use of mouthwash with fluoride (yes, experienced or never). The questionnaire on

Table 1 Descriptive analysis of each factors

	This study		National average in Japan
	Mean	SD	
Number of decayed teeth	1.25	2.19	2.02
Number of filled teeth	2.90	3.26	2.37
Number of df teeth	4.15	4.01	4.38
Salivary flow (ml/3 mins)	2.96	1.74	—
Salivary pH	7.276	0.198	—
Total Streptococci (CFU/ml, $\log_{10}$ count)	6.887	0.261	—
Lactobacillus (CFU/ml, $\log_{10}$ count)	2.561	2.35	—
mutans streptococci (CFU/ml, $\log_{10}$ count)	4.208	2.305	—

Mean and standard deviation of the data from the oral examination and bacterial cultures obtained in this study. The mean number of decayed or filled teeth was below the average of a national survey in Japan. The data of the national average of Japan were obtained from The Survey of Dental Diseases by Health Policy Bureau Ministry of Health and Welfare Japan (1999).

diet sugar intake consisted of two items, the number of daily intakes of sweet juice and the daily intake of sweet snacks (once, twice, three times or more than four times).

Statistical analysis

Before the analysis, patients were divided into two groups: subjects free from dental caries and subjects with at least one decayed or filled tooth (df teeth). For the high risk children, subjects were divided into two groups by the 75th percentile of the distribution by of the df teeth. As with microbiological factors, the bacteriological counts were $\log_{10}$-transformed prior to statistical analysis to normalize the variances. After evaluation of the distribution, amount of the mutans streptococci and Lactobacillus were categorized into four groups by the 25th, 50th and 75th percentile of the distribution.

Logistic regression analysis was used to evaluate the crude or adjusted odds ratios and their associated 95 percent confidence intervals. To eliminate the confounding factors, multiple Logistic regression analysis was used for factors correlated to the prevalence of dental caries. To confirm the dose-response relationships, the final factors correlated with the prevalence of the dental caries were classified according to the distribution.

Then two-way ANOVA was used to investigate the co-effect for the mutans streptococci and Lactobacillus for the df teeth.

Results

Forty-two percent of the children participating in this study had decayed teeth, and 73.1% had df teeth. The distribution of results is shown in Fig. 1. Table 1 shows the mean and SD of the number of the decayed, filled, and df (decayed or filled) teeth, and the salivary flow, salivary pH and salivary levels of the bacteria investigated in this study. Table 2 shows the categorized results from questionnaires. Salivary levels of mutans streptococci were not detected

Table 2 Results of the data obtained from questionnaires in this study

	n	%
Usage of fluoride containing dentifrice		
Yes	404	61.1%
No	245	37.1%
Experience of fluoride varnish		
Regularly	382	57.8%
Experienced	235	35.6%
Never	33	5.0%
Experience of fluoride mouth rinse		
Regularly	38	5.7%
Experienced	108	16.3%
Never	495	74.9%
Sweet soft drink intake (daily)		
Once	325	49.2%
Twice	228	34.5%
Three times	79	12.0%
Four or more times	17	2.6%
Sweet snack intakes (daily)		
Once	34	5.1%
Twice	393	59.5%
Three times	198	30.3%
Four or more times	24	3.7%

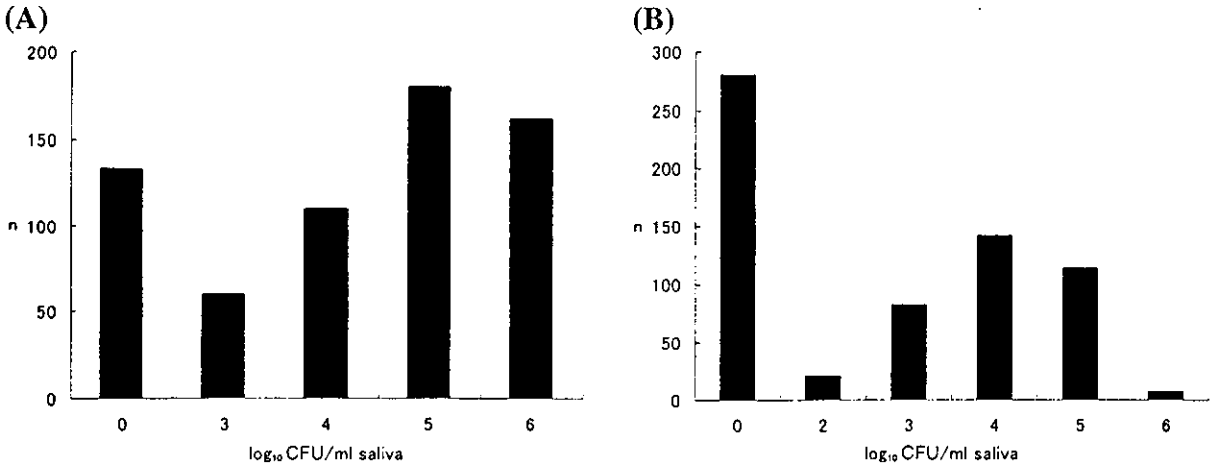

Fig. 2 The distribution of the microflora

The distribution of the mutans streptococci (A) and Lactobacillus (B) in this study. 20.6% of children could not detected mutans streptococci from their saliva, however, from 25.1% of children, mutans streptococci could be detected more than 10^6 CFU/m*l* saliva.

Table 3 The odds ratios for the dental caries

(A)

	Crude odds ratio	95% CI	*P*-value	Adjusted odds ratio	95% CI	*P*-value
Salivary flow rate	0.753	0.605–0.936	0.011	0.866	0.677–1.107	0.249
Salivary pH	0.607	0.250–1.477	0.272			
Lactobacillus (log_{10} count)	1.643	1.449–1.864	<0.001	1.405	1.222–1.617	<0.001
Total Streptococci (log_{10} count)	1.835	1.334–2.523	<0.001	1.156	0.806–1.658	0.431
mutans streptococci (log_{10} count)	1.406	1.297–1.525	<0.001	1.246	1.135–1.368	<0.001
Dentifrice containing fluoride	0.837	0.585–1.196	0.328			
Fluoride varnish	1.343	0.570–3.164	0.500			
Fluoride mouth rinse	0.500	0.205–1.219	0.127			
Juice intake	1.409	1.107–1.794	0.005	1.236	0.950–1.607	0.114
Sweet snack intake	1.106	0.839–1.459	0.475			

(B)

	Crude odds ratio	95% CI	*P*-value	Adjusted odds ratio	95% CI	*P*-value
Salivary flow rate	0.640	0.410–0.999	0.049	0.651	0.402–1.054	0.081
Salivary pH	0.323	0.086–1.206	0.093			
Lactobacillus (log_{10} count)	1.566	1.290–1.900	<0.001	1.342	1.082–1.663	0.007
Total Streptococci (log_{10} count)	1.821	1.158–2.864	0.009	1.197	0.723–1.981	0.485
mutans streptococci (log_{10} count)	1.771	1.354–2.315	<0.001	1.378	1.029–1.874	0.032
Dentifrice containing fluoride	0.879	0.506–1.527	0.647			
Fluoride varnish	1.031	0.666–1.595	0.892			
Fluoride mouth rinse	0.521	0.215–1.256	0.150			
Juice intake	1.247	0.915–1.700	0.163			
Sweet snack intake	1.251	0.830–1.884	0.284			

Crude and multivariate adjusted odds ratios for subjects with df teeth or not (A) and for the high risks (B). Odds ratios were calculated by Logistic regression analysis. *P*-values were calculated by the Wald test. Among the 10 factors investigated in this study, only two factors such as salivary levels of mutans streptococci and Lactobacillus had statistically significant correlation with the dental caries conditions.

Table 4 Dose-response relationships of the odds ratios

(A)

		with or without dental caries			high risks		
	n	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
0	280	reference group			reference group		
$0<\log_{10}$ CFU/m*l*$<10^4$	103	3.112	1.809–5.355	<0.001	1.938	1.003–3.746	0.049
$10^4<\log_{10}$ CFU/m*l*$<10^5$	141	4.057	2.429–6.774	<0.001	4.331	2.515–7.457	<0.001
$10^5<\log_{10}$ CFU/m*l*	121	6.812	3.587–12.938	<0.001	6.327	3.665–10.992	<0.001

(B)

		with or without dental caries			high risks		
	n	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
0	133	reference group			reference group		
$0<\log_{10}$ CFU/m*l*$<10^5$	170	2.824	1.753–4.549	<0.001	1.139	0.552–2.315	0.725
$10^5<\log_{10}$ CFU/m*l*$<10^6$	180	3.651	2.248–5.929	<0.001	2.063	1.060–4.015	0.033
$10^6<\log_{10}$ CFU/m*l*	162	11.746	6.162–22.390	<0.001	5.649	2.981–10.706	<0.001

Dose-response relationships of the salivary levels of Lactobacillus (A) and mutans streptococci (B) for dental caries. Clear dose-response relationships were observed in this study.

Table 5 Cross table of the number of df teeth by the salivary levels of mutans streptococci and Lactobacillus

Lactobacillus			mutans streptococci				
			0	$0<\log_{10}$CFU/m*l*$<10^5$	$10^5<\log_{10}$CFU/m*l*$<10^6$	$10^6<\log_{10}$CFU/m*l*	Total
0		mean ± SD	1.943 ± 2.917	2.471 ± 2.917	3.303 ± 3.371	4.048 ± 3.498	2.586 ± 3.148
		n	107	85	67	21	280
$0<\log_{10}$CFU/m*l*$<10^4$		mean ± SD	3.250 ± 2.990	3.447 ± 3.244	4.108 ± 3.478	7.077 ± 5.327	4.147 ± 3.820
		n	8	45	37	13	103
$10^4<\log_{10}$CFU/m*l*$<10^5$		mean ± SD	3.636 ± 4.501	4.516 ± 4.007	4.578 ± 3.621	7.250 ± 3.832	5.434 ± 4.058
		n	11	32	47	51	141
$10^5<\log_{10}$CFU/m*l*		mean ± SD	2.857 ± 2.642	6.000 ± 3.640	5.103 ± 3.880	7.080 ± 4.341	6.277 ± 4.276
		n	7	8	29	75	121
Total		mean ± SD	2.212 ± 3.070	3.280 ± 3.410	4.096 ± 3.610	6.726 ± 4.312	4.139 ± 3.989
		n	133	170	180	162	645

Dose-response relationship was observed by the mutans streptococci and Lactobacillus for df teeth. By two-way ANOVA analysis, *P*-values for the mutans streptococci was <0.001 and Lactobacillus <0.001. However, *P*-values for the mutans streptococci* Lactobacillus was 0.769, so interaction of the mutans streptococci and Lactobacillus for the number of df teeth was not observed in this study.

from 20.6% of the children, and salivary levels of Lactobacillus were not detected from 43.4% of the children (Fig. 2). However, more than 10^6CFU/m*l* salivary mutans streptococci were detected from 25.1% of children.

To correlate the prevalence of the number of df teeth and risk factors investigated in this study, we performed Logistic regression analysis to calculate the odds ratio for df teeth. Table 3 shows the result of the crude odds ratios. Among 10 factors investigated in this study, salivary flow, amount of Lactobacillus, amount of total Streptococci, amount of mutans streptococci and daily number of sweet soft drinks taken were correlated with the prevalence

of dental caries. To eliminate the confounding factors, we performed multivariate Logistic regression analysis using the six factors that correlated with the dental caries. Table 3 shows the results of multivariate Logistic regression analysis. Only two of the five factors, amounts of mutans streptococci and Lactobacillus, were correlated with the prevalence of dental caries. For the medians of the decayed teeth, multivariate adjusted odds ratio were 1.259 by Lactobacillus and 1.105 by mutans streptococci; for the medians of the decayed or filled teeth, were 1.236 by Lactobacillus and 1.160 by mutans streptococci. The same tendencies were observed in the crude odds ratio if the children were divided into groups with or without decayed teeth or divided into two groups by the 75th percentile of the decayed or filled teeth (Table 3-B). To confirm the dose-response relationships, we categorized these factors by the distribution and then performed Logistic analysis again. Table 4 shows the dose-response relationships for these factors. Clear dose-response relationships were observed for the salivary levels of mutans streptococci and Lactobacillus. Then to check the co-effect of the mutans streptococci and Lactobacillus for dental caries, two-way ANOVA analysis was carried out. As shown in Table 5, no co-effects were found in mutans streptococci and Lactobacillus for dental caries.

Discussion

The etiology of dental caries has been suggested to be classified into three main categories, and this has been confirmed by laboratory investigation and clinical studies. In particular, mutans streptococci and Lactobacillus have been intensively studied as microflora factors. The nature of the acid production and biofilm formation by cariogenic microorganism has been studied[13,14]. However, clinical studies have shown that some subjects have decayed teeth even though these bacteria were below detection levels in the oral cavity[15,16]. These results have shown that the etiology of the dental caries cannot be explained easily. In contrast, some studies have shown that, as a substrate factor, dietary intervention to restrict the sucrose consumption has reduced the prevalence or incidence of the dental caries[6]. However, in this study, no correlation was found in daily sucrose consumption and the prevalence of the decayed teeth, filled teeth or df teeth. This may be because the method of surveying sucrose consumption by questionnaire does not reflect the actual conditions for the substitute factors. However, in present, there is no other method to survey for these factors.

The difference in the results may thus be due to the method of surveillance.

This study found no correlation in fluoride usage by dentifrice and mouth rinse and varnish. Clearly, fluoride has been used to reduce the prevalence of dental caries. In this study, the institutions that applied the fluoride were varied. This may also be due to the methods of the investigation. The method of the fluoride application and intervals of application may be reflected in the result.

Some studies have shown that salivary flow rate and salivary buffering capacity or salivary pH contribute to the incidence of dental caries[17,18], while others found no correlation between these factors and dental caries[16,19]. In this study, we could not find any statistically significant correlation between dental caries prevalence and salivary factors. It is generally considered that salivary flow rate was affected by the side effects of medication or systemic diseases. In general, the salivary flow rate of children is high, and few children take drugs affecting the sympathetic nerve system and reducing the salivary flow rate. Sgan-Cohen *et al.* found a significant correlation of the salivary flow and dental caries, however the correlation was weaker for other factors such as microflora[21]. This may be the main reason for the contradictory result.

In conclusion, of the three main factors suggested by Keyes, the microflora factors are strongly correlated with the prevalence of dental caries for the preschool children in Japan. In the future, controlling this factor for the cohort may lead to strong strategies for preventing dental caries through community-based prevention programs.

References

1) Keyes, P.H.: Present and future measures for dental caries control. *J Am Dent Assoc* **79**: 1395–1404, 1969.
2) Seow, W.K.: Biological mechanisms of early childhood caries. *Community Dent Oral Epidemiol* **26**: 8–27, 1998.
3) Clarkson, J.J. and McLoughlin, J.: Role of fluoride in oral health promotion. *Int Dent J* **50**: 119–128, 2000.
4) Sundin, B. and Granath, L.: Sweets and other sugary products tend to be the primary etiologic factors in dental caries. *Scand J Dent Res* **100**: 137–139, 1992.
5) Newbrun, E.: Sugar and dental caries. *Clin Prev*

Dent **4**: 11–14, 1982.
6) Karjalainen, S., Sewon, L., Soderling, E., Lapinleimu, H., Seppanen, R. and Simell, O.: Oral health of 3-year-old children and their parents after 29 months of child-focused antiatherosclerotic dietary intervention in a prospective randomized trial. *Caries Res* **31**: 180–185, 1997.
7) Makinen, K.K., Bennett, C.A., Hujoel, P.P., Isokangas, P.J., Isotupa, K.P. and Pape, H.R.: Xylitol chewing gums and caries rates: a 40-month cohort study. *J Dent Res* **74**: 1904–1913, 1995.
8) Takeuchi, H., Senpuku, H., Matin, K., Kaneko, N., Yusa, N., Yoshikawa, E., Ida, H., Imai, S., Nishizawa, T., Abei, Y., Kono, Y., Ikemi, T., Toyoshima, Y., Fukushima, K. and Hanada, N.: New dental drug delivery system for removing mutans streptococci from the oral cavity: effect on oral microbial flora. *Jpn J Infect Dis* **53**: 211–212, 2000.
9) World Health Organization: Oral Health Survey: Basic Methods. 4th ed. World Health Organization, Geneva, 1997.
10) Hanada, N., Nomura, Y., Takeuchi, H., Senpuku, H., Ida, H., Yoshikawa, E. and Kumagai, T.: New dental drug delivery system for removing mutans streptococci. *J Dent Res* **80**: 567, 2001.
11) Suzuki, T., Tagami, J. and Hanada, N.: Role of F1F0-ATPase in the growth of *Streptococcus mutans* GS5. *J Appl. Micro Boil* **8**: 555–562, 2000.
12) Hanada, N., Imai, S., Nishizawa, T. and Mukasa, H.: Clinical Biology of the mutans streptococci. Quintessence Co., Tokyo, 2003, pp. 90–97. (in Japanese)
13) de Soet, J.J., Nyvad, B. and Kilian, M.: Strain-related acid production by oral streptococci. *Caries Res* **34**: 486–490, 2000.
14) Rozen, R., Bachrach, G., Bronshteyn, M., Gedalia, I. and Steinberg, D.: The role of fructans on dental biofilm formation by *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus gordonii* and *Actinomyces viscosus*. *FEMS Microbiol Lett* **195**: 205–210, 2001.
15) Alaluusua, S., Kleemola-Kujala, E., Gronroos, L. and Evalahti, M.: Salivary caries related test as predictors of future caries increment in teenagers. *Oral microbiology and immunology* **5**: 77–81, 1990.
16) Gabris, K., Nagy, G., Madlena, M., Denes, Z., Marton, S., Keszthelyi, G. and Banoczy, J.: Associations between microbiological and salivary caries activity tests and caries experience in Hungarian adolescents. *Caries Res* **33**: 191–195, 1999.
17) Holbrook, W.P.: Dental caries and cariogenic factors in pre-school urban Icelandic children. *Caries Res* **27**: 431–437, 1993.
18) Sundin, B., Granath, L. and Birkhed, D.: Variation of posterior approximal caries incidence with consumption of sweets with regard to other caries-related factors in 15–18-year-olds. *Community Dent Oral Epidemiol* **20**: 76–80, 1992.
19) Dodds, M.W., Johnson, D.A., Mobley, C.C. and Hattaway, K.M.: Parotid saliva protein profiles in caries-free and caries-active adults. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **83**: 244–251, 1997.
20) Pattanaporn, K. and Navia, J.M.: The relationship of dental calculus to caries, gingivitis, and selected salivary factors in 11- to 13-year-old children in Chiang Mai, Thailand. *J Periodontol* **69**: 955–961, 1998.
21) Sgan-Cohen, H.D., Steinberg, D., Zusman, S.P. and Sela, M.N.: Dental caries and its determinants among recent immigrants from rural Ethiopia. *Community Dent Oral Epidemiol* **20**: 338–342, 1992.

Controlling cariogenic bacteria by the regular check-up system

Yoshiaki Nomura, Akihisa Tsurumoto, Fusao Nishikawara, Mizuho Motegi, Nobuhiro Hanada and Takashi Kumagai

Reprinted from

PEDIATRIC DENTAL JOURNAL 14(1): 95–101, 2004

PEDIATRIC DENTAL JOURNAL 14(1): 95–101, 2004

Controlling cariogenic bacteria by the regular check-up system

Yoshiaki Nomura[1], Akihisa Tsurumoto[1], Fusao Nishikawara[2], Mizuho Motegi[2], Nobuhiro Hanada[2] and Takashi Kumagai[3]

[1] Department of Preventive Dentistry and Public Health, Tsurumi University School of Dental Medicine
2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, JAPAN
[2] Department of Oral Health, National Institute of Public Health
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, JAPAN
[3] Hiyoshi-Dental Office
2-1-16 Hiyoshi-cho, Sakata-shi, Yamagata 998-0037, JAPAN

Abstract Regular check-ups are important for reducing the risk factors of dental caries. Through regular check-ups, clinicians empirically know that the incidence of the new dental caries was suppressed. However, the effects of the regular check-up system have not been thoroughly evaluated. Our primary concern was to evaluate the efficacy of the regular check-up system with professional preventive care for preventing dental caries. In this study, we evaluated attitudes toward regular check-ups. Five hundred and thirteen patients who visited one dental office in Japan from 1981 to 2000 and who were under 12 on the first visit were examined for dental caries, salivary mutans streptococci, and Lactobacilli to obtain baseline values and the values for the more recent visit analyzed in this study. Salivary mutans streptococci and Lactobacilli were counted using Dentocult SM and Dentocult LB. Most of the risk factors, particularly the salivary levels of the mutans streptococci, were reduced by regular check-ups in this study. There was a greater risk reduction in particular for the salivary levels of mutans streptococci in patients undertaking regular check-ups. Reduced salivary levels of Lactobacilli were also observed. However, the changes between the groups in the attitude toward regular check-ups were not statistically significant. This result indicates that most of the risk factors investigated in this study could be reduced by regular check-ups, particularly the levels of mutans streptococci, which has been suggested to be a strong etiology of dental caries.

Key words
Lactobacilli,
Mutans streptococci,
Regular check-up system,
Risk factors

Introduction

It has been suggested that regular check-ups for dental caries effectively reduce the incidence of dental caries[1,2]. The current national guidelines for preventing dental caries emphasize the importance of regular check-ups[3,4]. However, compliance and attitude toward preventing dental caries are not evaluated at these check-ups.

Received on September 29, 2003
Accepted on January 14, 2004

The risk factors of dental caries have been classified into three main categories—teeth, substrate, and oral micro flora[5]. Characteristics of micro flora, such as the oral levels of mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) and Lactobacilli, were suggested to be the most important risk factors[6,7]. These bacteria have been evaluated for the effects of the caries preventive programs. It has been suggested that salivary levels of the mutans streptococci can be reduced by using anti-microbial drugs[8]. However, in the conventional regular check-ups and treatment for preventing

dental caries, professional tooth cleaning and application of fluoride have sometimes been used and sometimes not used[9-11]. These strategies suppressed the incidence of dental caries. However, whether or not major risk factors such as salivary levels of these bacteria could be reduced was uncertain.

This study investigates the efficacy for the prevention of dental caries by regular check-ups with the professional preventive care and effect of the compliance with regular check-ups. It also investigates whether or not the salivary levels of the mutans streptococci and Lactobacilli could be reduced by the regular check-ups without using anti-microbial drugs.

Materials and methods

Subjects and evaluation method

The caries risks were evaluated for all the patients at one of the private dental office in Japan that participated, using the methods described by Petersson *et al.*[12] Two thousand one hundred and thirty-two patients under the age of 20 visit the private dental clinic for professional preventive programs since 1980 when this dental office opened. At 1981, this private dental office began to construct the database for the management of the patient's data, especially for the oral conditions and risks for the dental caries and periodontal disease.

Of these patients, 448 patients who visited the dental office from 1981 to 2000 and who were under 12 years of age at the first visit were examined for dental caries and salivary mutans streptococci and Lactobacilli to obtain baseline values and the values for the most recent visit analyzed in this study. Before the examination, informed consent was obtained to use the data of the oral conditions and the results of the saliva test for the construction of the data base and the possibility to use the data for publication. The examination of the dental caries was carried out by the dental hygienist, then checked again by the dentist and both dental hygienist and dentist had the clinical experience more than 10 years.

The Salivary mutans streptococci and Lactobacilli were counted using commercially available mutans streptococci and Lactobacilli evaluation kits, Dentocult SM and Dentocult LB (Orion Diagnostica Co. Ltd., Epsom, Finland). The results of this test were categorized according to the manufacturer's instructions.

Regular check-ups and preventive treatment

During a regular check-ups, dental plaque, one of the risk factors for dental caries[9,10], is controlled by the dentist or dental hygienist by professional tooth cleaning. Fluoride is then applied by the dentist or dental hygienist[6]. The risks of micro flora are reduced through this intervention to control dental plaque.

In a professional care program, the dentist or dental hygienist cleans the tooth surfaces by hand brushing and dental floss. 2% APF-containing paste (Fluorident gel, Stone Pharmaceuticals, Philadelphia, USA) is applied to the tooth surface by the toothbrush then indicated for the patients to bite a cotton roll for 5 min. Then, patients rinsed out one or twice and prohibited not to rinse for 30 min.

Fissure sealant or scaling to remove the dental calculus is performed if necessary. In addition, instructions regarding diet and using the fluoride containing toothpaste (950 ppm) are provided.

Statistical analysis

Prior to the analysis, the patients were classified into groups of regular attendees, irregular attendees, and those who never attended regularly, according to their compliance with the regular check-ups. The criteria were designated as follows. Regular attendees were present for regular check-ups every three months after the caries treatment was finished, irregular attendees included patients who understood the importance of regular check-ups but who occasionally missed their regular check-ups. The remainders were patients who visited the dental office only when they were experiencing dental problems. These patients were advised to attend the dental office regularly; however they were never attended without experiencing dental problems. The data of these patients used in this study as check-ups were the data at the attended dental office when experiencing dental problems. The number of patients who developed new dental caries during the check-up periods and the mean number of incidences of new dental caries were calculated for each group. The baseline characteristics of these groups were checked by the one-way ANOVA. Logistic regression analysis was then used to evaluate the attitude for the regular check-ups, to calculate the crude odds ratios and the adjusted odds ratios associated 95% confidence intervals. The results were adjusted by factors that had co-relation

Table 1 Incidence of the new dental caries in this study

		Percentage of subjects with new dental caries	*P*-value	Number of new dental caries (mean ± SD)	*P*-value
	n	% (n)		Number of teeth	
Total	448	30.4% (136)	0.006	0.76 ± 1.54	<0.001
Regular check-ups	273	25.6% (70)		0.47 ± 0.98	
Irregular check-ups	72	30.6% (22)		0.86 ± 1.51	
No check-ups	103	42.7% (44)		1.45 ± 2.34	

Percentage of subjects with new dental caries and the mean number of new dental caries classified by their compliance with regular check-ups. *P*-values were calculated by the Chi-square test for the percentage of subjects with new dental caries and two-way ANOVA for the mean number of the new dental caries.

Table 2 Baseline characteristics of the subjects participating in this study

(A)

	Regular check-ups	Irregular check-ups	No check-ups	Total	*P*-value
n	271	69	97	437	
Mean age at first visit	8.82 ± 2.84	10.47 ± 3.75	11.36 ± 3.78	9.67 ± 3.39	<0.001
Mean treatment periods	1.19 ± 1.45	1.24 ± 1.23	1.25 ± 1.42	1.21 ± 1.42	0.931
Mean follow up periods	3.61 ± 1.98	3.03 ± 1.84	2.43 ± 1.96	3.27 ± 2.01	<0.001
dft at baseline	0.74 ± 1.74	2.08 ± 3.21	3.27 ± 4.22	1.55 ± 2.95	<0.001

(B)

	Crude odds ratio	95% CI	*P*-value	Adjusted odds ratio	95% CI	*P*-value
Age at first visit	1.056	0.993–1.123	0.084			
Follow-up periods	1.061	0.973–1.157	0.179			
dft at baseline	1.090	1.029–1.115	0.004			
Irregular check-ups	1.091	0.624–1.909	0.760	1.147	0.586–2.246	0.689
No check-ups	2.250	1.382–3.662	0.001	2.358	1.241–4.482	0.009

(A) shows the baseline characteristics for each group for the attitude of regular check-ups. *P*-values were calculated by one-way ANOVA. Statistically significant differences were observed in each group except for the treatment periods.
(B) shows the results of the crude and adjusted odds ratios from logistic regression analysis.

with the baseline characteristics in each group.

The relative risk reduction (RRR) and absolute risk reduction (ARR) were calculated. The numbers needed to treat (NNT) for the regular check-ups was then calculated using the inverse of the absolute risk reduction.

To determine the attitude for the regular check-ups and to evaluate whether salivary cariogenic bacteria were reduced or not, the methods of Friedman were used to check the difference of the salivary levels of the mutans streptococci and Lactobacilli in each group. *P*-values less than 0.05 were considered statistically significant.

Results

Sixty-five (12.9%) of the 513 patients dropped out. The main reasons were as follows: relocation 29 subjects (44.6%), and cancelled the check-ups and never come to the dental office 25 subjects (38.5%). The demographics of the patients who participated in this study were as follows. There were 297 males (45.5%) and 356 females (54.5%), the mean age at the first visit was 5.77 ± 3.10, the distribution was less than 5 years old: 224 (50%), 5–10 years old: 182 (40.6%), 11 or 12 years old 42 (9.4%) and the mean follow-up period was 4.22 ± 2.25 years.

Table 1 shows the percentage of subjects with

Table 3 NNT for the regular check-ups

	RRR	ARR	NNT
Regular check-ups vs. No check-ups	40.0	17.1	5.9
Regular check-ups vs. Irregular check-ups	16.1	12.2	8.2

Relative risk reduction, absolute risk reduction and numbers needed to treat (NNT) for the attitude of the regular check-ups. NNT was calculated by the inverse of the absolute risk reduction.

Table 4 Changes in the salivary levels of the mutans streptococci evaluated by Dentocult SM

(A)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	30	11.0	39	14.3	74	27.1	<0.001
1	41	15.0	45	16.5	51	18.7	
2	78	28.6	81	29.7	77	28.2	
3	124	45.4	108	39.6	71	26.0	

(B)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	6	8.2	5	6.5	6	7.8	0.399
1	12	16.5	14	19.4	17	23.4	
2	26	36.4	30	41.9	29	40.6	
3	28	38.9	23	32.3	20	28.1	

(C)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	8	7.8	16	15.3	17	16.0	0.038
1	16	15.6	12	11.5	17	16.0	
2	37	35.6	42	40.3	40	38.7	
3	42	41.1	34	32.7	30	29.1	

Table 4 shows the results of number and percent of subjects for the changes in salivary levels of the mutans streptococci on each visit. (A) indicates the regular attendees, (B) irregular attendees, and (C) no check-ups. Data were analyzed by Friedman Test. A statistically significant reduction of the salivary levels of mutans streptococci was observed in regular check-up patients.

new dental caries and the mean number of new dental caries, classified by their attitude toward regular check-ups. The result clearly illustrates that regular check-ups reduce the incidence of new dental caries. This result was found statistically significant by one-way ANOVA.

Baseline characteristics of the patients in each group are shown in Table 2-A. Statistically significant differences were found in mean age of the first visit, mean follow-up periods and baseline dft between each group. Patients were divided into two groups depending on whether they had new dental caries or not, and only the baseline DMFT was correlated with the incidence of dental caries (data not shown).

We performed logistic regression analysis to investigate the odds ratios of the attitude towards

Table 5 Changes in the salivary levels of the Lactobacilli evaluated by Dentocult LB

(A)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	119	43.6	126	46.2	189	69.2	<0.001
1	55	20.1	47	17.2	36	13.2	
2	56	20.5	58	21.2	30	11.0	
3	43	15.8	42	15.4	18	6.6	

(B)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	25	34.2	30	41.9	33	46.0	0.009
1	18	24.4	17	24.1	18	25.4	
2	18	24.4	17	24.1	16	22.2	
3	12	17.1	7	9.7	5	6.4	

(C)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	48	46.5	48	46.8	55	53.8	0.006
1	13	12.7	9	8.5	20	19.4	
2	25	23.9	26	25.6	23	22.3	
3	17	17.0	20	19.2	5	4.5	

Table 5 shows the number and percent of subjects with changes in salivary levels of the Lactobacilli at each visit. (A) indicates regular attendees, (B) irregular attendees, and (C) no check-ups. Data were analyzed by Friedman Test.

regular check-ups for the incidence of new dental caries. The crude odds ratio was 0.524 for regular check-ups, 1.091 for irregular check-ups and 2.250 for no check-ups for the incidence of new dental caries. The odds ratios were then adjusted by the age of first visit and the baseline dft. The odds ratio for regular check-ups subsequently became 0.553 and that for no check-ups became 2.358. These odds ratios also were statistically significant (Table 2-B).

By using these data and the attitude toward regular check-ups, we calculated the relative risk reduction (RRR), absolute risk reduction (ARR), and number needs to treat (NNT). As shown in Table 3, the NNT for the regular attendees and no check-ups was 5.9, and that for regular attendees and irregular attendees were 8.2.

We next conducted a Friedman test to check the association of the attitude toward regular check-ups and salivary levels of cariogenic bacteria, such as mutans streptococci and Lactobacilli. As shown in Table 4, the salivary levels of mutans streptococci in regular attendees were reduced, and the difference was statistically significant. Levels were especially reduced in the check-up periods. Statistically significant reduction was found in the salivary levels of mutans streptococci in the group of no check-ups. Specifically, it was reduced between the first visit and the treatment completion. However, a slight difference was found in the check-up periods. Salivary levels of Lactobacilli tended to be reduced in all groups (Table 5).

Discussion

Recently, the prevalence and incidence of the dental caries has declined remarkably in Western countries; the same tendency has been observed in Japan. However, some populations are still affected

by dental caries. For population strategies, water fluoridation effectively suppresses dental caries. However, even in such an environment, dental caries could not be completely suppressed. In this respect, regular check-ups and professional preventive dental care based on risk assessment are still necessary. Some reports have evaluated the effects of regular check-ups[13-15]. However, the compliance or attitude was not totally evaluated in these studies, and these studies used only the increment of DMFT of tooth mobility for the outcomes of the regular check-ups. Our results also demonstrated that the increment of new dental caries has a statistically significant relation with the attitude for regular check-ups. In this study, we calculated the NNT for the attitude of the regular check-ups. Rijkom *et al.*[16] previously found that fluoride gel treatment suppressed new dental caries in 6- to 15-year-old children. The NNTs of the fluoride gel treatment were 18 in a population with a caries incidence of 0.25 DMFS per year and 3 in a population with a caries incidence = 1.5 DMFS per year (treatment duration 1 year). In our preventive programs, fluoride gel was applied regularly. Our results of the NNT were included in the 95% confidence intervals of the results of Rijkom *et al.* Furthermore, if we classified patients with the criteria described above, the NNT for the incidence of new DMFS were 0.25. However, in our preventive programs, NNT was 5.9 or 8.2. This may be because our preventive programs included not only fluoride application but professional tooth cleaning or instruction on dietary habits. This total program may thus be reflected in the results.

Treasure[17] reviewed the effects of the preventive programs evaluated by NNT using fluoride or anti-microbial drugs. Two studies on fluoride gel application were available. One study shows the NNT was 18, and the other that it was 2. For fluoride varnish, NNTs were 11 to 8. Our NNT results were more effective than those of other studies. This may be because the population in our study visited the private dental office for regular check-ups and preventive programs. In Japan, regular check-ups are not covered by insurance. The awareness of health promotion may thus affect the results. In our results, the salivary levels of the mutans streptococci and Lactobacilli had statistically significant differences when observed by groups for the attitude towards check-ups. For the baseline values, the difference may result from the number of decayed caries. It is well known that many mutans streptococci and Lactobacilli exist in the decayed caries lesions[18,19]. There were no statistically significant differences for mutans streptococci in the groups when the treatment was finished, and reductions of mutans streptococci were observed in each group. This may also result from effective treatment for dental caries that is the reservoir of the mutans streptococci and Lactobacilli. However, the attitude toward check-ups affected the salivary levels of these bacteria. Neither mutans streptococci nor Lactobacilli could be eradicated by the preventive programs we used since we normally don't use anti-microbial drugs. The attitude toward regular check-ups reflected health promotion and may have affected the results.

In conclusion, our results suggest that most of the risk factors investigated in this study could be reduced by regular check-ups, particularly the levels of mutans streptococci and Lactobacilli that have been suggested to be a strong etiology of dental caries.

References

1) Milen, A., Hausen, H., Paunio, I. and Heinonen, O.P.: Caries of primary teeth and regularity of dental check-ups. *Community Dent Oral Epidemiol* **9**: 266–269, 1981.
2) Whittle, J.G.: Attendance patterns and dental health of parents and children. *Community Dent Health* **10**: 235–242, 1993.
3) Scottish Intercollegiate Guideline Network. Preventing Dental Caries in Children at High Caries Risk, 2000; http://www.dundee.ac.uk/tuith/Static/info/sign47.htm.
4) Lewis, D.W. and Ismail, A.I.: Periodic health examination, 1995 update: 2. Prevention of dental caries. The Canadian Task Force on the Periodic Health Examination. *CMAJ* **152**: 836–846, 1995.
5) Keyes, P.H.: Present and future measures for dental caries control. *JADA* **79**: 1395–1404, 1969.
6) Schroder, U., Widenheim, J., Peyron, M. and Hagg, E.: Prediction of caries in 1 1/2-year-old children. *Swed Dent J* **18**: 95–104, 1994.
7) Nomura, Y., Senpuku, H., Hanada, N. and Kumagai, T.: Mutans streptococci and Lactobacillus as risk factors for dental caries in 12-year-old children. *Jpn J Infect Dis* **54**: 43–45, 2001.
8) Skold, L., Sundquist, B., Eriksson, B. and Edeland, C.: Four-year study of caries inhibition of intensive Duraphat application in 11–15-year-old children. *Community Dent Oral Epidemiol* **22**: 8–12, 1994.
9) Bratthall, D.: Caries, views and perspectives. *Scand J Dent Res* **100**: 47–51, 1992.
10) Twetman, S., Petersson, L.G. and Pakhomov, G.N.: Caries incidence in relation to salivary mutans streptococci and fluoride varnish applications in

preschool children from low- and optimal-fluoride areas. *Caries Res* **30**: 347–353, 1996.
11) Rethman, J.: Trends in preventive care: caries risk assessment and indications for sealants. *JADA* **131**: 8–12, 2000.
12) Petersson, G.H. and Bratthall, D.: Caries risk assessment: a comparison between the computer program 'Cariogram', dental hygienists and dentists. *Swed Dent J* **24**: 129–137, 2000.
13) Karkkainen, S., Seppa, L. and Hausen, H.: Dental check-up intervals and caries preventive measures received by adolescents in Finland. *Community Dent Health* **18**: 157–161, 2001.
14) Bagramian, R.A., Graves, R.C. and Srivastava, S.: A combined approach to preventing dental caries in schoolchildren: caries reductions after 3 years. *Community Dent Oral Epidemiol* **6**: 166–171, 1978.
15) Bullock, C., Boath, E., Lewis, M., Gardam, K. and Croft, P.: A case-control study of differences between regular and causal adult attenders in general dental practice. *Prim Dent Care* **8**: 35–40, 2001.
16) van Rijkom, H.M., Truin, G.J. and van't Hof, M.A.: A meta-analysis of clinical studies on the caries-inhibiting effect of fluoride gel treatment. *Caries Res* **32**: 83–92, 1998.
17) Treasure, E.T.: Methods of stopping or reversing early carious lesions fluoride: a European perspective. *J Dent Educ* **65**: 1073–1077, 2001.
18) Marchant, S., Brailsford, S.R., Twomey, A.C., Roberts, G.J. and Beighton, D.: The predominant microflora of nursing caries lesions. *Caries Res* **35**: 397–406, 2001.
19) Ozaki, K., Matsuo, T., Nakae, H., Noiri, Y., Yoshiyama, M. and Ebisu, S.: A quantitative comparison of selected bacteria in human carious dentine by microscopic counts. *Caries Res* **28**: 137–145, 1994.

Clin Exp Immunol 2004; 137:393–401 doi:10.1111/j.1365-2249.2004.02548.x

Role of peptide antigen for induction of inhibitory antibodies to *Streptococcus mutans* in the human oral cavity

Y. TSUHA*,¶, N. HANADA†, T. ASANO‡, T. ABEI§, S. YAMAGUCHI§, M. A. SALAM¶, R. NAKAO¶, H. TAKEUCHI†, N. KUROSAKI* & H. SENPUKU¶ *Department of Comprehensive Oral Health Care, Graduate School, Tokyo Medical and Dental University, †Department of Oral Health, National Institute of Public Health, §Division of Experimental Animal Research, National Institute of Infectious Diseases ‡Pacific Dental Clinic and ¶Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan

(Accepted for publication 20 May 2004)

SUMMARY

The alanine-rich repeating region (A-region) in the surface protein antigen (PAc) of *Streptococcus mutans* has received much attention as an antigenic component for vaccines against dental caries. The PAc (residue 361–386) peptide in the A-region possesses a multiple binding motif (L- -V-K- -A) to various HLA-DR molecules and a B-cell core epitope (- Y- - -L- -Y- - - -) that recognizes the inhibiting antibody to *S. mutans*. In the present study, we investigated the immunogenicity of the PAc (361–386) peptide in humans and regulators of induction of the anti-PAc (361–386) peptide IgA antibody (aPPA) in saliva. The PAc (361–386) peptide was confirmed as an ideal peptide antigen for induction of the inhibiting antibody to *S. mutans* in 151 healthy human subjects (36·6 ± 12·6 years old) by quantitative analyses of oral bacteria and ELISA, as the aPPA titre in human saliva decreased significantly in an age-dependent manner. Homozygous *DRB1*0405* and *1502*, and heterozygous *DRB1*0405/1502* showed a negative association with production of aPPA and tended to reduce the number of total streptococci in saliva. In contrast, the *DRB1*1501* allele was significantly correlated with a high level of induction of the antibodies, and also tended to reduce lactobacilli and mutans streptococci. Further, peptide immunogenicity was confirmed in NOD-SCID mice grafted with human peripheral blood mononuclear cells. Our results indicate that the interplay between regulators such as age, *DRB1* genotype, cytokines, and peptide immunogenicity may provide a potential means for developing a vaccine useful for the prevention of dental caries as well as their diagnosis.

Keywords NOD-SCID mice peptide *DRB1* genotype *Streptococcus mutans* dental caries

INTRODUCTION

Streptococcus mutans has been suggested to have an association with dental caries [1,2], and epidemiological surveys have shown that greater numbers of *S. mutans* in children are associated with a higher incidence of decayed, missing, and filled teeth (DMFT), i.e. fragment caries experiences [3–5]. The cell surface protein antigens of *S. mutans*, PAc [6], Ag I/II [7], PI [8], and B [9], function essentially for colonization of the bacterium on tooth surfaces and interact with the salivary pellicle that coats the dental enamel [10–12]. The alanine-rich repeating region (residue 219–464, A-region) of the PAc molecule is important for the interaction of *S mutans* with salivary film [13–15] with a strong immunogenicity in humans [16], and may be a candidate antigen for inducing the production of inhibiting antibodies against the adherence of *S. mutans* to tooth surfaces.

The A-region is composed of 3 long and 2 incomplete repeating sequences [6]. Each repeating sequence contains sequences homozygous to the amino acid sequence, ^{365}TYEAALKQYEADL^{377}, while PAc (365–377), an important region for the adherence of *S. mutans* to tooth surfaces [17,18], as well as T- and B-cell epitopes overlap [17,19]. Further, the epitope (YEA-L-QY) of the surface protein antigen (PAg) of *S. sobrinus* [20] and its core B-cell epitope (- Y- - -L- -Y- - - -) are essential sequences in the antigenic epitopes of the PAc protein that are recognized specifically by the antibody [21]. The antibodies reacting with the core B cell epitope inhibit competitively interaction of *S. mutans* to salivary components [17,18,21]. The overlapped PAc (370–386) peptide to PAc (361–377) peptide includes a multiple binding motif (L- -V-K- -A) that reacts with HLA-*DRB1*0802, *1101, *1401,* and **1405* [22,23], and is also recognized in the A-region. Therefore, the coupled PAc (361–386)

Correspondence: Hidenobu Senpuku, Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
E-mail: hsenpuku@nih.go.jp

© 2004 Blackwell Publishing Ltd

peptide from residues 361–377 and 370–386 may be a minimum antigen of PAc that induces the inhibiting antibodies for adherence of *S. mutans* to the tooth surfaces coated by salivary components in humans.

Salivary immunoglobulin A (IgA) reacts with oral streptococci and other bacteria, and is considered an important factor for host defense against infection [24]. These important functions of IgA have focused interest on the development of mucosal vaccines [25,26], as well as its possible therapeutic use in treatment of infection [27–29]. In addition, saliva levels of the IgA antibody are associated with caries protection, because negative correlations between the IgA antibody and caries formations have been found [30–32], and salivary IgA antibodies have been reported to play an important role against *S. mutans* for the prevention of dental caries through bacteriostasis [30,31]. The human leucocyte antigen (HLA) is coded by the major histocompatibility complex (MHC) and also plays an important role in controlling the production of antibodies in saliva [33,34], as the production of salivary IgA antibodies is influenced by HLA molecules on the immune cells [33–35]. In addition, the association between the HLA allele and susceptibility to colonization by *S. mutans* or production of the salivary IgA antibody has attracted extensive interest in regards to the development of a dental caries vaccine. To investigate whether the PAc (361–386) peptide has a function as an effective antigen regarding the induction of human antibodies influenced by the HLA class II polymorphism in human saliva, we examined anti-PAc (361–386) peptide antibody titres in human subjects, and analysed the relationship between those levels and HLA-DR genotypes or pathogenic bacteria levels using human saliva.

NOD/LtSz-scid (nonobese diabetic – severe combine immunodeficiency, NOD-SCID) mice grafted with human peripheral blood mononuclear cells (hu-PBMC) have been used as *in vivo* models for studying human lymphoid cells responses to human specific antigens [36–38]. This mouse strain supports levels of human cell grafting that are 5 to 10-fold greater than those obtained in C.B-17-Scid mice [36]. As a result, the hu-PBMC-NOD-SCID mouse model is employed for long-term *in vivo* analysis of immunoregulatary interactions between human lymphocyte activation and antigen. We also investigated immunogenicity of PAc (361–386) peptide using the hu-PBMC-NOD-SCID mouse model to clarify direct evidence for induction of the specific antibody in human immune systems. Our results may provide useful information for the prevention of dental caries as well as diagnosis of their potential risk in humans.

MATERIALS AND METHODS

Mice

NOD-SCID mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the National Institute of Infectious Diseases (NIID). Female mice at the age of 6–9 weeks were used in the present study. All experiments were performed in accordance with our institutional guidelines.

Human subjects

One hundred and fifty-one patients (60 males, age 37.6 ± 13·8; 91 females, age 35·0 ± 10·4; Overall age 36·6 ± 12·6 years old) of the Pacific Dental Clinic, Japan, participated in this study. Prior to the survey, the aim and details of the experiments were explained and consent was obtained from all subjects. 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 [39], decayed teeth (DT), missing teeth (MT), and filled teeth (FT) (DMFT) scores were recorded along with findings of dental caries. Genetic (phenotypic) typing for HLA-*DRB1* was determined using a PCR-restriction fragment length polymorphism method by the Tissue Typing Department (BML, Tokyo, Japan) with samples from 96 of the subjects.

PAc peptide synthesis

The sequences of PAc (361–386) (NAKATYEAALKQYEAD LAAVKKANAA) and PAc (346–364) (AALTAENTAIK QRNENAKA) were derived from the sequence of the PAc gene from *S. mutans* MT8148, which corresponds to a portion of the A repeat, as described by Okahashi *et al.* [40]. The PAc (residue 361–386) peptide in the A-region possesses a multiple binding motif (L- -V-K- -A) to various HLA-DR molecules and the B-cell core epitope (- Y- - -L- -Y- - - -), which is used for recognizing the inhibiting antibody to *S. mutans*. The peptide was synthesized by a stepwise solid phase procedure at Asahi Techno Glass Co. Inc. (Tokyo, Japan). The synthesized peptide samples were subsequently purified by reversed-phase high-performance liquid chromatography (HPLC) on a TSK-GEL column (1 × 30 cm) (TOSO, Tokyo, Japan) with a 10% to 45% acetonitrile gradient in 0·1% TFA, and developed over 50 min 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 analysed using a System 7300 Amino Acid Analyser (Beckman, NJ) and a Model 477 A Protein Sequencer (Applied Biosystems, Foster city, CA, USA).

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 min, and clarified by centrifugation at 10 000× g for 10 min 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.

Bacteria counting

All bacteria counting was performed by the Laboratory of Bacteriology (BML). Saliva samples were gently shaken and inoculated onto Mitis-Salivarius agar (MTS, Nippon Becton Dickinson Co. Ltd, Tokyo, Japan) and Rogosa SL agar (Nippon Becton Dickinson Co. Ltd) using an EDDY JET spiral plating system (IUL, S.A., Torrent, Spain), to count total streptococci (tS) and lactobacilli (LB) organisms. 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. The MMTSB contained 20% sucrose (Wako, Tokyo, Japan), 2 μg/ml of gramicidin (Sigma), 10 μg/ml of nalidixic acid (Wako), 10 μg/ml of colistin sulphate (Wako), and 2 mg/ml of yeast extract (Becton Dickinson Sparks, MD), and is known to be extremely precise for the counting of mS colonies [41–44]. Following anaerobic inoculation for 48 h 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 × 100.

© 2004 Blackwell Publishing Ltd,

Injection of PAc (361–386) peptide to humanized mice

The immunization schedule was shown in Fig. 2a. Transplantation of hu-PBMC into NOD-SCID mice was performed using procedures and conditions described previously [45]. hu-PBMC were isolated from 400 ml of peripheral blood taken from a normal healthy volunteer by separation using Ficoll-Conrey (Immuno-Biological Laboratories, Gunma, Japan) density gradient centrifugation. The cells were washed 3 times in Hanks Balanced Salt Solution (HBSS) (Gibco Laboratories, Life Technologies, Paisley, UK) and adjusted to a concentration of $4{\cdot}0–8{\cdot}0 \times 10^7$/ml in HBSS. hu-PBMC suspensions were then administered intraperitoneally at 0·5 ml per mouse. Groups of 3–5 female mice from a single litter were grafted with PBMC from the donor and used in the experiments. Mice were irradiated (gamma irradiation, 2·5 Gy) from a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd, Kanata, Canada) 0–1 days before human cell transfer. On 1, 7 and 14 days after hu-PBMC transplantation, some of the hu-PBMC-NOD-SCID mice were administrated intraperitoneally with a mixture of 0·0 or 250·0 ng of hu-IL-4 (204-IL, R & D system Inc, Minneapolis, MN, USA) or IL-10 (MC/9, BioSource, Camarillo, CA, USA), with 0·0 and 30·0 ng of PAc (361–386) peptide in 300 μl of phosphate-buffered saline (PBS), pH 7·4. Seven days after hu-PBMC transplantation, the mice were immunized subcutaneously with 30·0 ng of PAc (361–386) peptide emulsified in Freund's complete adjuvant (Difco Laboratory, Detroit, MI, USA). One week later, the mice were boosted by a subcutaneous injection with and without the immunizing antigen at the same dose emulsified in Freund's incomplete adjuvant (Difco). Control mice without the immunizing antigen were injected consistently with 300 μl of PBS alone. One week after the last injection, sera and spleens were extracted for testing. Genotyping for HLA-*DRB1* in the spleen cells from hu-PBMC-NOD-SCID mice injected or not injected with the peptide was performed by the Tissue Typing Department of BML.

ELISA

For an enzyme-linked immunosorbent assay (ELISA), 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μl of PAc (361–386) peptide (concentration 20 μg/ml) or skim milk (as a control) in coating buffer at pH 9·6 for enumeration of the IgG specific to *S. mutans* [17]. The plates were washed with PBS containing 0·1% (v/v) Tween 20 (PBST) and blocked with 1% (wt/vol) skim milk in PBST for 1 h at 37°C . Excess skim milk was removed by washing 3 times with PBST, and then a 100 μl aliquot of a twofold serial dilution of saliva or sera from the inoculated hu-PBMC-NOD-SCID mice was added to the wells and the mixtures were incubated for 1 h at 37°C. The wells were then washed 5 times with PBST and further incubated for 1 h at 37°C with 100 μl of alkaline phosphatase-conjugated goat antihuman immunoglobulin A or G (both heavy and light chains) antibodies (Zymed Laboratories, South San Francisco, CA, USA). After 5 washes with PBST, bound antibodies were detected after the addition of 100 μl of 3 mg/ml para-nitrophenyl phosphate as a substrate and incubation for 90 min at 37°C. Absorbance at 405 nm (A_{405}) was measured with a microplate reader (Multiskan Bichromatic; Laboratory Japan, Tokyo, Japan). The ELISA antibody titre 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 h of incubation with the substrate.

Dot blotting

To confirm the specificity of the anti-PAc(361–386) peptide antibody in human saliva, dot blot analysis was performed using bovine serum albumin (BSA) with BSA-conjugated PAc (361–386) peptide blotted onto the nitrocellulose. The nitrocellulose blots were incubated in human saliva and alkaline phosphate-conjugated goat polyclonal antibodies raised against the human IgA antibodies, and then exposed to the substrate.

Flow cytometry

Single cell suspensions of spleen cells were prepared by gently homogenizing the cells with ice-cold HBSS. Single cell suspensions of peritoneal cells were collected by washing the peritoneal cavity with an HBSS solution. All cell suspensions were washed once in ice-cold HBSS as described below. Spleen or peritoneal cells were stained with FITC- or PE-conjugated antihuman marker mAbs in PBS/1% BSA and washed with HBSS medium. At least $10^4–10^5$ live spleen cells, including mouse and human lymphoid cells, were acquired in each run. For each mouse analysed, cells were also stained with mouse IgG conjugated to FITC and PE as an isotype control. Spleen or peritoneal cells from a non-transplanted NOD-SCID mouse were stained in parallel as an additional negative control. Fluorescence levels that excluded greater than 98% of the cells in the negative controls were considered to be positive and specific for human staining. The cells were fixed in a 3% formalin/HBSS solution and stored at 4°C until flow cytometric analysis. Samples gated on the forward light scatter (FSC) and side light scatter (SSC) were used to identify viable lymphocytes. Proportions of the major subsets were determined by single and quadrant analyses. Single cell suspensions were stained with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated antimouse CD45 (30-F11), antihu-CD45 (H130), antihu-CD4 (RPA-T4), and phycoerythrin (PE)-conjugated antihu-CD8 (RPA-T8), each purchased from BD PharMingen (San Diego, CA, USA). The percentages of FITC and PE-positive cells were measured using a FACS with the CELLquest program (Beckton Dickinson, San Jose, CA, USA).

Statistical analysis

Allele frequencies in the human subjects were calculated by direct counting. Group comparisons of the levels of parameters were analysed by ANOVA. *P*-values of ≤0·05 were considered to be statistically significant.

RESULTS

Correlations between anti-PAc (361–386) peptide antibody titre and various parameters

The differences between female and male subjects for age, anti-PAc (361–386) peptide IgA (aPPA) titre in saliva, DMFT, LB, mS number, mS ratio, and tS number were investigated. DMFT ($15{\cdot}1 \pm 7{\cdot}0$) in females was significantly higher than in males ($12{\cdot}4 \pm 4{\cdot}8$, $P < 0{\cdot}05$), whereas there were no significant differences between the other parameters. The human subjects were divided into 4 groups: the no antibody group (anti-PAc (361–386) peptide antibody titre (a) ≤0·1), low group (>0·1 but ≤1), moderate group (>1 but ≤3) and high group (≥3), and the various parameters were compared within each (Table 1). Reactions to the peptide were determined by ELISA, and also confirmed by dot blot analysis using BSA-conjugated PAc (361–386) peptide and the control (BSA) (data not shown). Mean age

Table 1. Relationship between anti-Pac (361–386) peptide anribodies in saliva and various parameters

Groups	n	F:M	Age	DMFT	LB ($\times 10^5$/ml)	mS ($\times 10^5$/ml)	mS ratio (%)	tS ($\times 10^7$/ml)
No antibody	20	11:9	44.8 ± 14.7	13.8 ± 4.8	1.3 ± 3.4	4.2 ± 7.7	0.7 ± 1.2	4.0 ± 3.4
Low	32	18:14	38.5 ± 12.9	14.4 ± 5.5	2.7 ± 6.6	2.2 ± 3.7	0.8 ± 1.6	4.2 ± 3.5
Moderate	58	37:21	35.2 ± 11.5	13.7 ± 5.5	3.0 ± 8.3	3.0 ± 6.2	0.9 ± 1.5	3.6 ± 3.7
High	41	25:16	33.0 ± 11.1	14.3 ± 8.5	2.4 ± 4.1	1.0 ± 2.6	0.3 ± 0.5	4.3 ± 5.7
Total	151	91:60	36.6 ± 12.6	14.0 ± 6.4	2.5 ± 6.3	2.4 ± 5.3	0.7 ± 1.3	3.9 ± 4.2

Anti-Pac (361–386) peptide antibody titre in saliva: No antibody group ≤0.1; Low >0.1 and ≤1.0; Moderate >1.0 and ≤3.0; High >3.0. F, female; M, male; *n*, no. of subjects. Significant differences between each genotype $^*P < 0.05$, $^{**}P < 0.01$

(44·8 ± 14·7 years) was significantly higher in the no antibody group as compared to the moderate (35·2 ± 11·5 years) and high (33·0 ± 11·1 years) groups (P < 0·01). The number of mS (1·0 ± 2·6) and mS ratio (0·3 ± 0·5) in the high group were significantly lower than those in the no antibody (4·2 ± 7·7) and moderate (0·9 ± 1·5) groups ($P < 0·05$). There were no significant differences between DMFT, LB and tS concentration, and aPPA. The PAc (346–364) peptide contains the B cell epitope in humans [16] and was used as a control antigen. There were no observable differences between the various parameters and anti-PAc (346–364) peptide IgA antibodies in many of the saliva samples ($n = 70$) (data not shown).

Age, mS number, and mS ratio were compared between the 4 groups, and between females and males (Fig. 1). The antibody titre showed a significantly negative correlation with age in males, while mS number was significantly higher in the no antibody group as compared to the moderate and high groups among females, and higher in the moderate as compared to the high group among males ($P < 0·05$). However, there were no significant differences in mS ratio between females and males in all groups.

Correlations between DRB1 genotypes and anti-PAc (361–386) peptide antibodies

The associations between various *DRB1* genes, and the titres and bacterial parameters, as seen by ANOVA, are shown in Table 2. The aPPA titre was significantly lower in the mixed genotypes of homozygous *DRB1*0405* and *1502*, and *DRB1*0405/*1502* than

Table 2. Correlations with *DRB1*0405, 1502, 1501, 0901* or *0101* to various parameters

DRB1	n	F:M	Age	Titer	DMFT	LB ($\times 10^5$/ml)	mS ($\times 10^5$/ml)	mS ratio (%)	tS ($\times 10^7$/ml)
0405 *1502* homo *0405/1502*	9	5:4	38.4 ± 11.5	0.4 ± 0.5	12.0 ± 4.0	1.3 ± 3.2	1.2 ± 1.6	0.4 ± 0.3	1.6 ± 1.5
0405 hetero	17	13:4	38.9 ± 13.4	2.6 ± 1.3	13.2 ± 5.2	1.0 ± 3.1	1.6 ± 2.0	0.7 ± 0.9	3.7 ± 2.6
1502 hetero	18	15:3	39.9 ± 14.3	2.2 ± 1.5	13.9 ± 5.6	1.0 ± 2.6	2.9 ± 6.4	0.9 ± 1.5	3.4 ± 3.1
1501	11	5:6	37.0 ± 11.9	2.7 ± 1.0	14.2 ± 6.9	0.1 ± 0.1	0.7 ± 1.3	0.2 ± 0.0	6.4 ± 4.2
0901	25	16:9	40.4 ± 16.0	2.1 ± 1.5	14.5 ± 6.1	3.4 ± 8.5	2.8 ± 4.2	0.9 ± 1.1	4.9 ± 3.4
0101	15	8:7	34.9 ± 12.6	1.5 ± 1.1	13.7 ± 5.5	2.2 ± 5.2	1.2 ± 2.5	0.3 ± 0.4	4.0 ± 3.8
Others	21	15:6	44.7 ± 15.7	1.6 ± 2.2	13.9 ± 4.2	1.1 ± 3.0	3.4 ± 6.1	1.1 ± 1.7	3.9 ± 2.8

0405, 1502 homo, *0405/1502*: Subject group expressing HLA- *DRB1*0405/0405, 1502/1502* or *0405/1502*. *0405* hetero: Subject group expressing HLA-*DRB1*0405*/others. *1502* hetero: Subject group expressing HLA- *DRB1*1502*/others. *1501* hetero: Subject group expressing HLA- *DRB1*1501*/others. *0901*: Subject group expressing HLA- *DRB1*0901* allele. *0101*: Subject group expressing HLA- *DRB1*0101* allele. Others: Subject group expressing HLA-*DRB1*1502, 1501, 0405, 0901* and *0101* allele. Significant differences between each group $^*P < 0.05$, $^{**}P < 0.01$; numbers shown on square brackets are *P*-value without stastistical sugnificance

© 2004 Blackwell Publishing Ltd,