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.

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

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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)

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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.

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

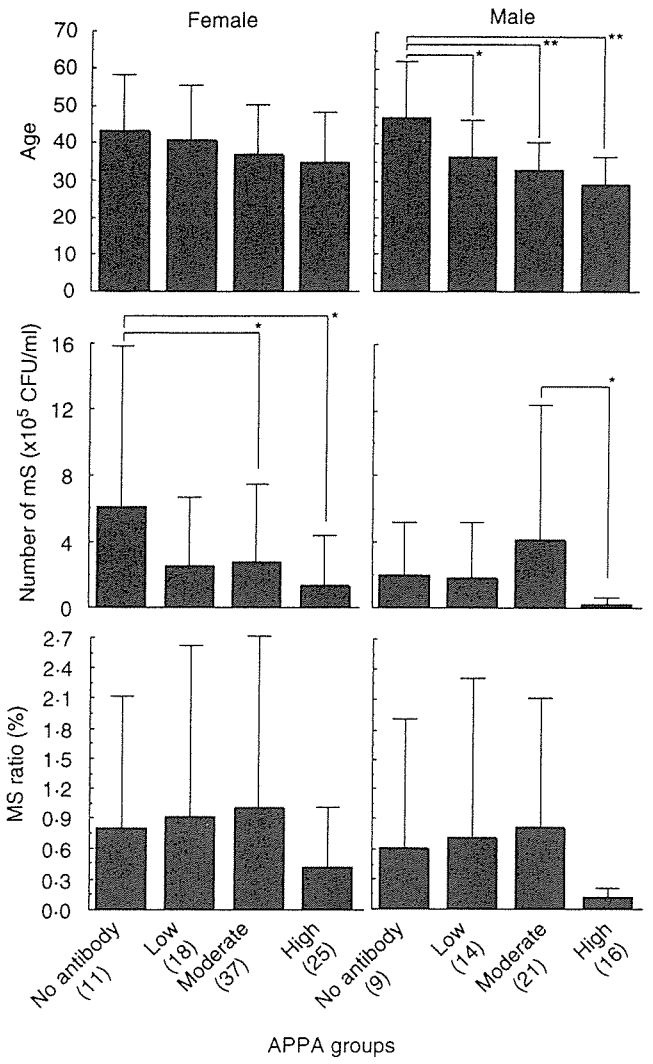

Fig. 1. Relationships between age, number of mS organisms, mS ratio, and levels of a-PPA in female and male subjects. Measurement of mS number and distribution of a-PPA titre in 4 groups are described in Materials and Methods. Results are expressed as mean ± SD of each parameter. ( ): Number of subjects in each age group. Asterisks denote significantly different relative antibody level ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$).

heterozygous *DRB1*0405, DRB1*1502, DRB1*1501,* and others. The number of tS was also lower in the mixed genotype group than *DRB1*1501, 0901, 0101,* and others. In contrast, the titre was higher in heterozygous *DRB1*1501* than the mixed genotype, *DRB1*0101,* and others, while LB and mS ratio were significantly lower than in *DRB1*0901* and others. Further, the titre as well as tS number and LB were higher in the *DRB1*0901* than the mixed genotypes and *DRB1*1501.* There were no significant differences between various DRB1 groups in Age, DMFT and mS.

Production of hu-anti-PAc (361–386) peptide IgG antibodies in mice

To establish a small animal model for production of hu-IgG antibodies to PAc (361–386) peptide, we grafted hu-CD45$^+$, -CD4$^+$ and -CD8$^+$ cells and injected the PAc peptide into NOD-SCID mice, after which we analysed production of the hu-anti-PAc (361–386) peptide IgG antibody (aPPG) in the those hu-PBMC-NOD-SCID mice. IL-4 and IL-10 are pleiotropic cytokines produced by activated Th2 cells [46,47] that have been identified as important regulators for B cell development [48]. Further, we investigated whether IL-4 or IL-10 had an effect to regulate the production of aPPG. Prior to the analysis for aPPG, significant proportions of hu-CD45$^+$, -CD4$^+$CD8$^-$, and -CD4$^-$CD8$^+$ cells in the spleen and peritoneal cells were also detected by FACS analysis to determine the grafting efficacy of the hu-cells into the mice (data not shown). The production of aPPG was up-regulated by intraperitoneal administration of the peptide (30 ng/mouse) without cytokines in 7 of 9 mice expressing different heterozygous DRB1 genotypes and 1 mouse expressing homozygous *DRB1*0405*, in contrast to the nonimmunized mice (Fig. 2b). In addition, co-administration of IL-4 with the peptide showed positive effects for increasing aPPG (Fig. 2c), whereas coadministration of IL-10 inhibited the increase in 4 of 6 mice expressing different heterozygous *DRB1* genotypes and 1 mouse expressing homozygous *DRB1*0405* (Fig. 2d).

DISCUSSION

There has been increasing interest in recent years in the establishment of a PAc peptide antigen, and studies of a candidate antigen, PAc (361–377) peptide, as well as T and B-cell epitopes that induce an inhibiting antibody to interaction with salivary components and colonization of *S. mutans* on the rat's tooth surfaces and the multiple agretope (L- - V-K- - A) that are restricted by various HLA-DR genotypes have been reported [17–19,21,23]. The spread peptide, PAc (361–386) peptide to the C-terminal of PAc (361–377) peptide, includes a multiple agretope. The PAc (361–377) peptide has been shown to induce specific antibodies to mutans streptococci (*S. mutans* and *S. sobrinus*), however, not other streptococci in mice, because the peptide possesses a high homologous amino acid sequence between *S. mutans* and *S. sobrinus* [18,20,21]. As a result, the PAc (361–386) peptide is considered to be a candidate antigen for induction of the antibody that specifically inhibits colonization of *S. mutans* and *S. sobrinus* in humans.

S. mutans is a pathogen of dental caries, infecting the oral cavity of almost all humans. The PBMC from subjects used in the present study showed positive serum anti PAc(361–386) peptide antibody level in hu-PBMC-NOD-SCID mice injected with control:PBS and were thus sensitized to *S. mutans* antigens. Accordingly, it is speculated that, in the hu-PBMC-NOD-SCID mice to whom *S. mutans*-sensitized PBMC had been transplanted, the specific antibodies produced might have resulted from secondary responses to the immunization with the PAc(361–386) peptide. Consequently, PAc (361–386) peptide was confirmed as an ideal peptide antigen for induction of the antibody in humans by ELISA and the NOD-SCID mouse system. Recent studies involving immunization with synthetic peptides and fusion proteins with PAc from the catalytic and glucan-binding regions of glucosyltransferase (GTF) have shown a reduction in the level of smooth surface caries in both active and passive immunized rats following infection with *S. mutans* or *S. sobrinus* [15,49]. Several GTF and PAc peptides speculated to have high binding characteristics to MHC class II have also been studied for their immunogenicity in rats and mice [50,51], and the binding motifs of GTF to MHC class II have been reported as well [22]. However, the antigenicities of these peptides have not been investigated in a

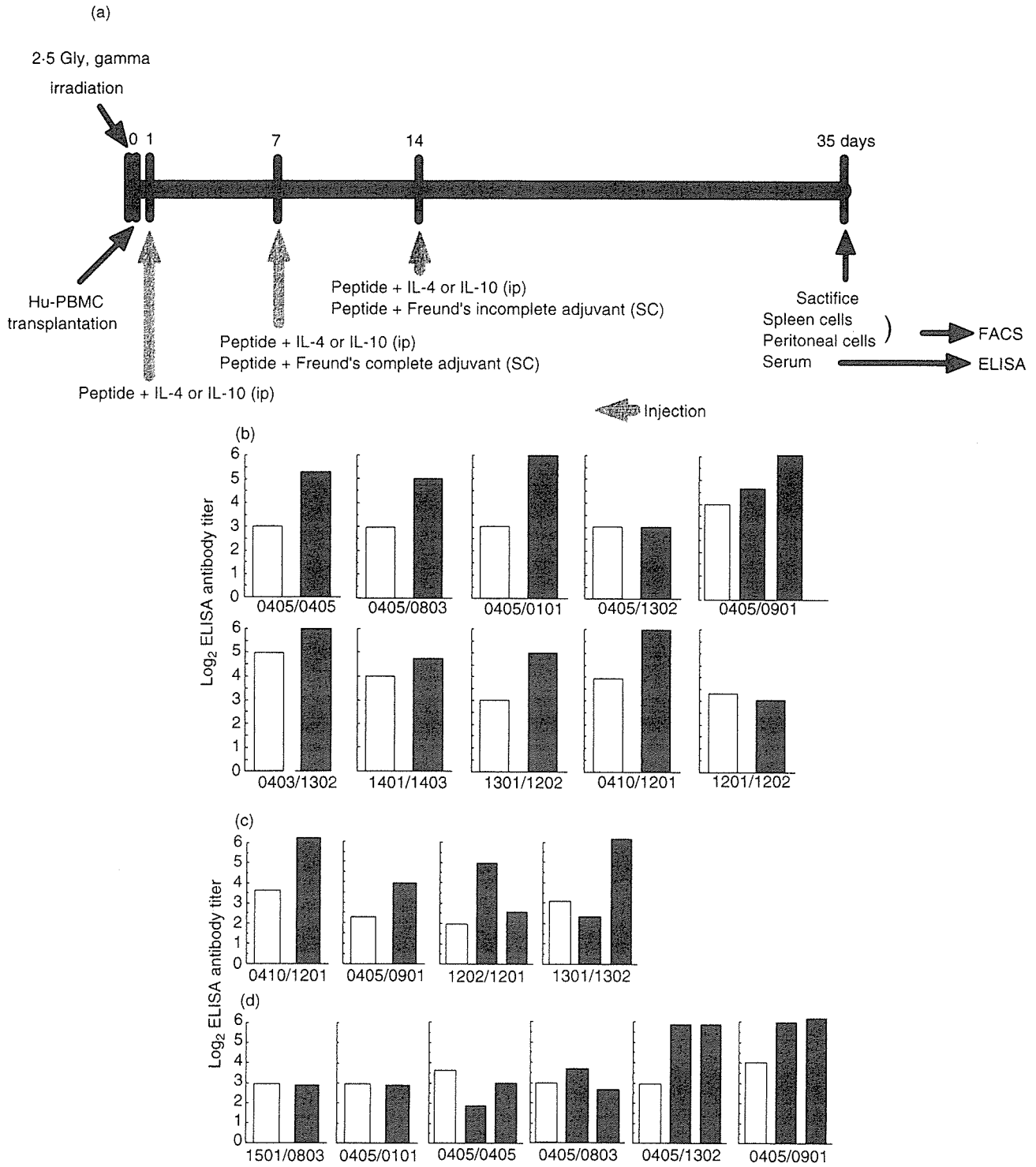

Fig. 2. Production of a-PPG in hu-PBMC-NOD-SCID mice following immunization with PAc (361–386) peptide. The immunization schedule was shown in (a). The peptide (■) in PBS (b), IL-4 (c), or IL-10 (d) was injected into 1 or 2 NOD-SCID mice grafted with hu-PBMC expressing the heterozygous or homozygous HLA-*DRB1* genotype from a single donor. The peptide immunization procedure is described in Materials and Methods. The bar graph shows the Log2 ELISA antibody titre in individual injected mouse serum samples. As a control, a PBS injection without the peptide (□) was performed and the peptide immunogenicity was compared with the control in the production of a-PPG in mice sera.

human immune system. The present study is the first to show that the PAc (361–386) peptide is a unique antigen for the recognition and induction of inhibiting antibodies to mutans streptococci in the human immune system. Our results may provide useful information for the construction of peptide-based vaccines using various epitopes in PAc and GTF to prevent dental caries.

Our findings suggest that production of the anti-PAc (361–386) peptide antibody is regulated by ageing, HLA-DR genotype, and cytokines, as the antibody titre was found to have a negative correlation with ageing, with a significant correlation in males however, not in females, who might have had a menopausal disorder or pregnancy at some time (Fig. 1). Optimum level of the antibody titre was also required for the decrease of mutans streptococci in saliva. Wallengren *et al.* [52] indicated that the level of salivary antibody response differs among genetically different individuals. Some investigators have also reported a relationship between HLA and caries susceptibility [53–55], as well as associations between HLA class II genes and mS and/or LB [23,27,52,56]. Further, Acton *et al.* demonstrated that DR-4 might have a part in controlling dental caries and that *DRB1*-4 allele frequencies in African-American women were positively associated with *S. mutans* level [56]. In a recent study, Wallengren *et al.* [35] found lower salivary IgA activity in response to *S. mutans* in tests with *DRB1*0401* and *0404,* while Ozawa *et al.* showed that there was no association between DR-4 (*DRB1*0405*) and mutans streptococci or lactobacilli [57]. In the present study, homozygous *DRB1*0405* in the mixed genotypes showed a negative association with production level of aPPA and numbers of tS bacteria in saliva, while heterozygous *DRB1*0405* showed a positive association with the production level of aPPA, however, not with other bacterial parameters. Therefore, the *DRB1*-4 allele may respond to an antigen presenting molecule of *S mutans*. *DRB1*0405* showed poor reactivity to PAc (361–386) peptide in its homozygous expression and did not disturb the susceptibility of another *DRB1** allele to the peptide in an individual with 2 MHC genotypes. Previous reports as well as our studies of various subject groups have revealed unique features regarding the DR4 association, and the present findings may suggest involvement of the *DRB1*0405* allele and others in the aetiology of oral streptococci.

In addition to *DRB1*0405*, homozygous and heterozygous *DRB1*1502* in mixed genotypes showed susceptibility similar to *DRB1*0405*, as the mixed group of homozygous *DRB1*0405* and *1502*, and heterozygous *DRB1*0405/1502* were correlated with lower levels of tS therefore they may have an association with the regulation of oral flora. Our data also indicate that heterozygosity or homozygosity of *DRB1*0901* increases predisposition to a high frequency of LB in saliva, and that *DRB1*1501* positive status in individuals produces aPPA and eliminates the susceptibility conferred by other HLA-DR *DRB1* genotypes to colonization by mS and LB. A negative association with the haplotype that includes the *DRB1*1501* allele was also reported in *Helicobacter pylori* related diseases [58], while patients with the *DRB1*1501/DQB1*0602* haplotype showed significantly reduced responses and were less likely to develop severe systemic diseases caused by group A streptococcal infections [59]. These results indicate that the *DRB1*1501* allele may be involved with resistance to infectious diseases occurring in the upper alimentary region by employing mucosal immunity.

Based on our findings, we propose that host immunogenic factors involved in regulating PAc responses may have an influence on the severity of mutans streptococci colonization. Our data also suggest that this effect is mediated through differential presentation of streptococcal PAc by distinct class II alleles, resulting in significant differences in the magnitude of mutans streptococci biofilm formation. The effects of class II allelic variation on the induction of inhibiting antibodies may also be regulated by polymorphisms of other host immunogenetic factors such as cytokines. This wide scope of regulators provides an intriguing model for investigation of the role of host-biofilm formation and understanding of the underlying mechanism of these genetic associations. However, there were no significant differences between DMFT and the other parameters, except gender, in the present study. In adult individuals, it may be difficult to clarify the associations of DMFT with microbial parameters or antibody titre, as not only dental caries but also periodontal diseases have an association with missing teeth, and can reveal past dental status [60]. Therefore, a definitive discussion regarding the relationship between the antibody titre and DMFT requires further investigation in young population.

In general, oral biofilm exhibits significant resistance to antimicrobial agents and is capable of a strong physiological response to agent-caused stress. The differential agent sensitivity of biofilm and dispersed biofilm cells indicate that its physical structure enhances normal cellular systems for growth, which are dependent on the nutritional status of the organism [61]. The adherence of planktonic cells to a surface structure is inhibited by agents such as anti-PAc (361–386) peptide antibody and may not induce the appearance of biofilm, therefore, the present findings indicate that immunological elements of the host defense system operate in cooperation with each another. The protective features of this antibody may make it possible to design a multiepitope caries vaccine to be given to individuals expressing various MHC class II types. In the future, a mucosal adjuvant such as mutant cholera toxin [62] may be a powerful means to safely elevate the level of antibody in a peptide vaccination. In addition, regulators of antibody induction may also be used as indicators of dental caries risk for development of a diagnostic method.

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A longitudinal study of the relationship between periodontal disease and bone mineral density in community-dwelling older adults

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Abstract

Objective: Bone loss is a common feature of periodontitis and osteoporosis. Both diseases may share common etiologic agents which may either affect or modulate the process of both diseases. The purpose of this study was to evaluate the relationship between systemic bone mineral density (BMD) and periodontal disease among older people.

Materials and Methods: Among all 4542 inhabitants aged 70 years according to a registry of residents in Niigata city in Japan, 600 people were selected randomly. One hundred and eighty-four subjects who did not have diabetes mellitus, whose blood sugar was < 140 mg/dl, who had more than 20 teeth, who were non-smokers, and who did not take medication for osteoporosis, were included in the study. Four dentists performed clinical evaluations on probing attachment level (PAL). We also utilized the data on BMD of the heel, which we measured using an ultrasound bone densitometer. Follow-up clinical surveys were done by measuring PAL after 3 years. Finally, 179 subjects who could participate in both the baseline and the follow-up examinations were included in the analysis. After dividing the subjects into an osteopenia group (OG) and non-osteopenia group (NOG), we evaluated the relationship between BMD and the number of progressive sites which had $\geqslant 3$ mm additional attachment loss during 3 years after controlling the known confounding factors.

Results: The mean number of progressive sites for the OG and the NOG, respectively, were 4.65 ± 5.51 and 3.26 ± 3.01 in females and 6.88 ± 9.41 and 3.41 ± 2.79 in males. Two-way analysis of variance was performed to discriminate among effects of gender, BMD, and gender–BMD interaction. A significant effect of BMD (OG or NOG, $p = 0.043$) with a significant interaction ($p = 0.038$) was observed. Furthermore, BMD was associated with the number of progressive sites which had $\geqslant 3$ mm additional attachment loss during the 3 years ($p = 0.001$) by multiple linear regression analysis.

Conclusions: This study suggested that there was a significant relationship between periodontal disease and general BMD.

Key words: bone loss; etiology; periodontal disease

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Periodontal destruction is frequently experienced by elderly people (Slade & Spencer 1995, Brown et al. 1996) and it contributes to as much as 40% of tooth extraction (Johnson 1993). Periodontal disease is characterized by absorption of alveolar bone as well as by loss of the soft-tissue attachment to tooth. On the other hand, osteoporosis is the most common metabolic bone disease among the elderly (65 years and older), and the incidence of osteoporotic fractures obviously increases with aging. Because bone loss is a common feature of periodontitis and osteoporosis, both diseases may share common etiologic agents which may either affect or modulate the process of both diseases. Given that the final expression of periodontitis is predicated by the complex interactions occurring within an intricate mosaic of host, microbial and environmental factors, it was felt that the contribution of bone mineral density (BMD) as a risk factor might be worthy of investigation (Offenbacher 1996). The clinical consequence of these findings suggest that physicians should be encouraged to send their osteoporotic patients to dentists for a periodontal examination and dentists should be encouraged to send their patients with severe periodontal disease for a medical examination for osteoporosis.

However, the relationship between osteoporosis and periodontal disease has been suggested in a limited number of studies. The results of some previous studies have indicated a relationship between periodontal disease and osteoporosis (Von Wowern et al. 1994, Mohammad et al. 1997, Tezal et al. 2000), while others have not shown any significant relationship (Elders et al. 1992, Klemetti et al. 1994, Lundstrom et al. 2001). All of these studies used the cross-sectional study design, and examined bone loss and periodontal condition in females. Even if the loss of BMD was more significant in females than in males, the role of factors involved in the regulation of BMD in males as well as in postmenopausal females needs to be evaluated further with reference to oral bone loss and periodontal disease. In addition, it is necessary to evaluate the relationship between BMD and progression of periodontitis in longitudinal studies.

Likewise, the results may easily be confounded by other factors such as intake of medications, smoking, race and age. Many of the studies conducted to date have been plagued by relatively small sample sizes and lack of adequate control of potential confounding variables. Larger studies are needed to better define the relationship between BMD and periodontal disease.

The purpose of this study was to evaluate the relationship between systemic BMD and periodontal disease, controlling the known confounding factors.

Materials and Methods

Subjects and clinical assessment

Initially, questionnaires were sent to all 4542 inhabitants aged 70 years according to a registry of residents in Niigata City in Japan, and they were informed of the purpose of this survey. The response rate was 81.4% ($N = 3695$). Among them, after dividing into male and female groups, 600 people were selected randomly in order to have approximately the same number of each gender for the study (screened population). The subjects for the study agreed to undergo medical and dental examinations, and signed informed consent forms regarding the protocol, which was reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Niigata University. The examinations were performed at local community centers in Niigata City. Four dentists performed clinical evaluations on the following items: (1) number of teeth present, (2) probing attachment level (PAL). Mouth mirrors with a light, and pressure-sensitive plastic periodontal probes, set to give a constant probing force of 20 g and graduated at 1 mm intervals (VIVACARE TPS PROBE®, Schaan, Liechtenstein), were used. All functioning teeth, including third molars, were assessed, except for partially erupted teeth. PALs were measured at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) and rounded to the nearest whole millimeter. In cases where a restorative margin was apical to the cemento-enamel junction (CEJ), PAL was measured taking account of the anatomical features of the teeth and, if present, the CEJ of the adjacent tooth/teeth.

Seventeen volunteer patients were examined by each of the four examiners in the Faculty Hospital of Dentistry, Niigata University, and their results were compared. The percentage of agreement ranged from 70.0% to 100% for PAL. The κ ranged from 0.62 to 1.00 for PAL. The four examiners did not have any information on BMD of the subjects.

The subjects' height, weight and grip power were measured to the nearest 1 mm or 0.1 kg, respectively, to calculate the body mass index (kg/m^2, BMI) or grip power/body weight (kg/kg). We also utilized the data on BMD of the heel, which we measured using an ultrasound bone densitometer (Lunar Achilles™, GE Medical Systems, Madison, WI, USA). The ultrasound signal is sent to os calcis. Ultrasound densitometry enables the measurement of the physical properties of bone, specifically BMD. The ultrasound measurement contains two criteria, the velocity (speed of sound (s); SOS) and frequency attenuation (broadband ultrasound attenuation (dB/MHz); BUA) of sound wave as it travels through bone (Langton et al. 1984, Rossman et al. 1989). The stiffness is a clinical index combining SOS and BUA, which is calculated by the spread speed of supersonic waves. The formula is $(BUA - 50) \times 0.67 + (SOS - 1380) \times 0.28$. This charts the SOS and BUA into biologically relevant ranges. Stiffness is indicated in the monitor of the bone densitometer as the percentage for the value of the normal younger generation. Osteopenia was defined as a stiffness $\leqslant 85$ for 70-year-old males, and $\leqslant 69$ for females (Lunar Corporation 1991). Furthermore, a personal interview was performed to obtain the bulk of information regarding smoking habits, diabetes mellitus, and the intake of medications for osteoporosis. To monitor the general health condition, serum or plasma levels of disease markers were also investigated. These disease markers were immunoglobulins (serum IgG concentration), nutritional factors (serum albumin concentration and serum total cholesterol concentration), and blood sugar. Among the screened population, 184 subjects who did not have diabetes mellitus, whose blood sugar was < 140 mg/dl, who had more than 20 teeth, who were non-smokers, and who did not take medication for osteoporosis were included in the study.

Follow-up clinical surveys were done by measuring PAL after 3 years. As at the baseline examination, 97.3% of the subjects received the follow-up examination by the same four dentists.

Finally, 179 subjects who could participate in both the baseline and the follow-up examinations were included in the analysis.

Statistical analyses

Mean and standard deviation (SD) were used to characterize the continuous variables. Following Brown et al. (1994), a change in the attachment level of 3 mm or more was set as a conservative estimate of actual change taking place. Using the *t*-test, we compared stiffness, BMI, serum albumin concentration, serum total cholesterol concentration, grip power/body weight, serum IgG concentration, PAL at baseline and the number of sites with $\geqslant 3$ mm additional attachment loss during the 3 years between males and females.

Furthermore, we evaluated the relationship between stiffness at the baseline and the number of sites with $\geqslant 3$ mm additional attachment loss during the 3 years by two-way analysis of variance (ANOVA) for discriminating among the effects of gender, stiffness and gender–stiffness interaction. After controlling for serum albumin concentration, serum total cholesterol concentration, grip power/body weight, serum IgG concentration, gender, BMI and PAL at baseline, a multiple linear regression analysis was performed to assess the relationship between stiffness at the baseline and the number of sites with $\geqslant 3$ mm additional attachment loss during the 3 years. The level of significance was set at $p < 0.05$ for these tests.

Results

The mean number of teeth present was 25.37 ± 2.91. The average PAL was 2.61 ± 0.76. Table 1 shows the stiffness, BMI, serum albumin concentration, serum total cholesterol concentration, grip power/body weight, serum IgG concentration, PAL and the number of sites with $\geqslant 3$ mm additional attachment loss during the 3 years between males and females. The stiffness was 74.19 ± 10.65 for males and 59.42 ± 8.87 for females. A significantly greater loss of stiffness was found in females ($p < 0.001$). The serum total cholesterol concentration was significantly lower, and grip power/body weight and PAL were significantly higher in males.

After dividing the subjects into the osteopenia group (stiffness $\leqslant 69$ for females, $\leqslant 85$ for males, OG) and the non-osteopenia group (NOG), we evaluated the number of progressive sites which had $\geqslant 3$ mm additional attachment loss during the 3 years. The mean number of progressive sites for the OG and the NOG, respectively, were 4.65 ± 5.51 and 3.26 ± 3.01 in females, 6.88 ± 9.41 and 3.41 ± 2.79 in males (Fig. 1). Two-way ANOVA was performed to discriminate among effects of gender, stiffness and gender–stiffness interaction. As shown by the data in Table 2, significant effects of stiffness (OG/NOG, $p = 0.043$) with a significant interaction ($p = 0.038$) were observed. The number of progressive sites was significantly higher in the OG. Furthermore, we evaluated the mean number of teeth present at baseline and tooth loss during the 3 years. The mean number of teeth present at baseline for the OG and the NOG, respectively, were 24.91 ± 2.71 and 25.05 ± 3.10 in females, 25.80 ± 2.96 and 25.95 ± 3.15 in males. There was no significance between the OG and the NOG in females and in males. The mean number of teeth lost during the 3 years for the OG and the NOG, respectively, were 0.84 ± 2.32 and 0.74 ± 1.41 in females and 0.52 ± 1.17 and 0.73 ± 0.83 in males. There was no significance between the OG and the NOG in females and in males as well.

The results of multiple linear regression analysis are presented in Table 3. Stiffness and gender were associated with the number of progressive sites which had $\geqslant 3$ mm additional attachment loss during the 3 years (stiffness: correlation coefficient = −0.199 ($p = 0.001$), gender: correlation coefficient = −4.412 ($p = 0.020$)).

Table 1. Comparison of stiffness, body mass index (BMI), biochemical values, grip power/body weight, probing attachment level (PAL) and additional attachment loss between males and females

Variables	Subjects ($n = 179$)		*p*-value
	males	females	
stiffness (%, mean ± SD)*	74.19 ± 10.65	59.42 ± 8.87	<0.001
BMI (kg/m^2, mean ± SD)*	22.56 ± 2.59	22.69 ± 2.78	0.752
albumin (g/dl, mean ± SD)*	4.30 ± 0.28	4.33 ± 0.24	0.500
total cholesterol (mg/dl, mean ± SD)*	194.42 ± 26.90	213.37 ± 29.12	<0.001
grip power/body weight (kg/kg, mean ± SD)*	0.67 ± 0.10	0.48 ± 0.08	<0.001
IgG (mg/dl, mean ± SD)*	1515.61 ± 262.88	1566.19 ± 336.75	0.269
PAL (mean ± SD)*	2.77 ± 0.80	2.46 ± 0.68	0.005
number of sites with ⩾3 mm additional attachment loss (mean ± SD)†	5.99 ± 8.36	4.37 ± 5.11	0.116

IgG, immunoglobulin G.
*At baseline.
†During the 3 years.

Discussion

The results showed that the subjects in the OG had a higher number of progressive sites with $\geqslant 3$ mm additional attachment loss during the 3 years than the subjects in the NOG. This 3-year longitudinal study clearly demonstrated that BMD is a risk predictor for periodontal disease progression in an older population.

Some systemic factors which contribute to loss of bone mass and periodontal progression have been identified (Cummings et al. 1985, Genco & Löe 1993). There were some common factors such as smoking, nutritional deficiencies, age, intake of medications and immune dysfunction (Wactawski-Wende et al. 1996). Considering these facts, it is reasonable that this study showed a significant relationship between BMD and periodontal disease progression. Maybe, systemic factors of bone remodeling also modify local tissue response to periodontal disease.

The relationship between BMD and progression of periodontitis is difficult to establish because there were many potential confounding variables, including local factors. In our previous study of an older population, we found that the subjects who had more than 20 remaining teeth were less susceptible to periodontal disease (Hirotomi et al. 2002). The results of that study prompted us to evaluate the relationship between systemic BMD and periodontal progression after controlling for teeth present, in addition to other factors, such as gender, diabetes mellitus, smoking habits and intake of medications in this study. Likewise, we restricted the age of subjects to 70 years to eliminate the influence of age on periodontal disease progression.

Various researchers have proposed several plausible findings. Kribbs et al.

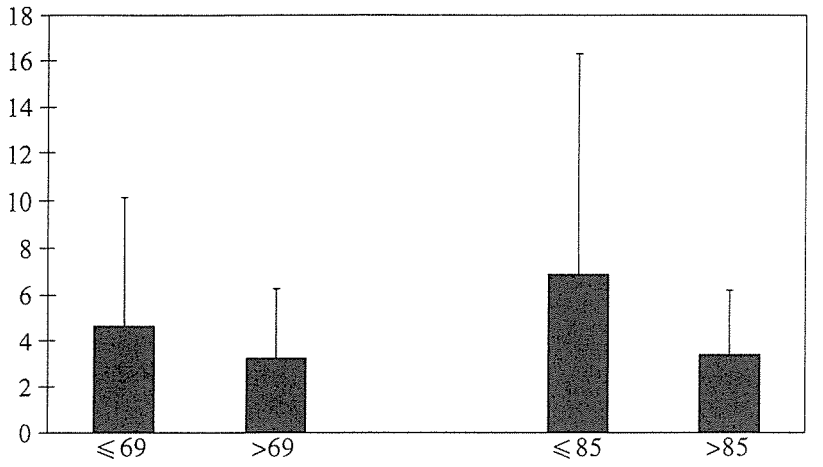

Fig. 1. Relationship between the number of progressive sites with ≥3 mm additional attachment loss and stiffness by gender. The number of subjects: stiffness ≤69 ($n = 74$) and >69 ($n = 19$) for females, ≤85 ($n = 64$) and >85 ($n = 22$) for males. a, stiffness (%)

Table 2. The results of analysis of variance for the evaluation between additional attachment loss and bone mineral density and gender

Variables	Sum of squares	df	Mean square	*F*	*p*-value
stiffness (osteopenia/non-osteopenia)	191.67	1	191.67	4.140	0.043
gender (males/females)	136.30	1	136.30	2.940	0.088
stiffness × gender	309.34	2	154.67	3.340	0.038
residual	8148.89	176	46.30		
total	8458.22	178	47.52		

Table 3. Multiple linear regression and associated *p*-values

Independent variables*	Dependent variable				
	number of sites with ≥3 mm additional attachment loss†				
	Coef.	Std. Err.	*p*-value	[95% CFI]	
stiffness (%)	−0.199	0.060	0.001	−0.317	−0.080
albumin (g/dl)	−4.286	2.200	0.053	−8.633	0.061
total cholesterol (mg/dl)	0.003	0.021	0.899	−0.039	0.044
grip power/body weight (kg/kg)	0.001	0.204	0.763	−0.341	0.464
IgG (mg/dl)	0.001	0.002	0.494	−0.002	0.005
gender (1: males, 2: females)	−4.412	1.881	0.020	−8.129	−0.695
BMI (kg/m^2)	0.195	0.231	0.401	−0.262	0.651
PAL‡	0.153	0.801	0.849	−1.431	1.736
_cons	35.687	12.544	0.005	10.896	60.479

$p = 0.033$, $R^2 = 0.106$. Coeff., coefficient; std. err., standard error; CFI, confidence interval; BMI, body mass index; IgG, immunoglobulin G.
*At baseline.
†During the 3 years.
‡Mean value of probing attachment level (PAL) at baseline.

(1990) observed a significant correlation between several skeletal bone mass measurements and the number of remaining teeth in 85 osteoporotic women between 50 and 80 years of age. Some other reports showed that mandibular bone mass was significantly correlated with skeletal bone mass as well (Klemetti et al. 1993, Von Wowern et al. 1994). Furthermore, the BMD of the mandible is affected by the mineral status of skeleton and also by general disease that causes generalized bone loss (Klemetti et al. 1993). On the contrary, Mohajery & Brooks (1992) found there was no correlation between skeletal and mandibular bone measurements. The results of these studies should be interpreted with caution since the number of subjects might be small, the age of subjects might have not been restricted, and the oral or skeletal bone loss might have been measured only in females.

In our study with adequate control of confounding variables, a weak relationship between BMD and periodontal disease progression existed although it was statistically significant. General BMD might not influence the alveolar bone loss directly in some cases. The skeleton is heterogenic, and bone density, bone turnover rate and bone remolding ability differ in some parts of the skeleton, suggesting that those regions, although related to each other, have some degree of independence. In addition, some bias such as local oral factors for alveolar bone loss might blur a clear relationship between systemic BMD and periodontal progression.

As our study was aimed at older subjects aged 70 years who had more than 20 teeth present, the subjects whom we examined might have been periodontitis-resistant. Therefore, it was difficult for PAL to contribute to inter-individual difference in resistance to periodontitis. This might be a reason for not having a significant relationship between periodontal disease condition such as PAL at baseline and additional attachment loss during the 3 years in this study. In addition, there was no significance in the number of teeth present at baseline, and tooth loss during the 3 years between the OG and the NOG in males and females. Therefore, the selection bias by the number of teeth present might be eliminated.

Likewise, ultrasonic bone density measurement was performed to evaluate BMD of the heel in this study. The ultrasound methods assess both bone volume and bone quality accurately and safely (Heaney et al. 1989). Some researchers have evaluated BMD by ultrasonic bone density measurement (Heaney et al. 1989, Resch et al. 1990). Ultrasound densitometry of the os calcis is highly reproducible and has a high correlation with BMD measured by dual-energy X-ray absorptiometry (DEXA) in different parts of the skeleton such as the spine or femur (Yamazaki et al. 1994).

In conclusion, this study suggested that there was a significant relationship between periodontal disease and general BMD in the present study.

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Sexual differences in oral health behaviour and factors associated with oral health behaviour in Japanese young adults

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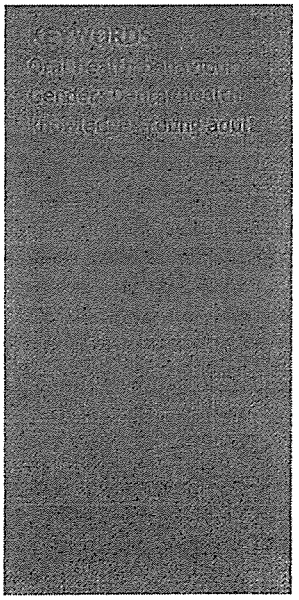

Summary The aim of this study is to compare the state of oral health behaviour between genders and to analyse factors associated with oral health behaviour by gender in young adults. Anonymous questionnaire data were collected from 527 adults (245 men and 282 women) aged 20-29 years who consulted dentists in Chiba City. The Chi-square test, Mann-Whitney analysis, and logistic regression analysis were used to examine the differences in oral health behaviour and determinants of oral health behaviour between young men and young women. The rate of good behaviour among women were significantly higher than those among men in each oral health behaviour item (toothbrushing frequency $p < 0.001$; using dental floss $p = 0.042$; dental check-ups $p < 0.001$). In women, factors associated with each oral health behaviour were as follows: toothbrushing frequency (family composition $p = 0.030$); using dental floss (dental health knowledge $p = 0.025$, employment status $p = 0.031$), and dental check-ups (age group $p = 0.024$). In men, a significant relationship was seen only between using dental floss and age group ($p = 0.025$).

This study indicated that young women had better oral health behaviour and that more factors were associated with their oral health behaviour in comparison with young men.

Introduction

In Japan, the percentage of adults with periodontitis increases strikingly after the age of 30.[1] The oral health behaviour of adults aged 20-29 was worse than that of other adult age groups,[2] which is thought to be one of the causes of oral health deterioration after age 30. The maintenance and improvement in health status are dependent on good health behaviour.[3] Good oral health behaviour is known to yield a good oral health status.[4-7] Therefore, oral health behaviour must be improved, especially in young adults.

Oral health behaviour in adults is known to be associated with various factors, such as socio-economic status,[8-13] race,[9] and urbanization.[10,12] Sexual differences are also seen in oral health behaviour.[9,10,13,14] Lifestyles of men differ from

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those of women. Furthermore, young women have their own biological characteristics, which include pregnancy, delivery and child-rearing. Therefore, that there are gender differences in the factors associated with oral health behaviour can be expected. However, there has been no analysis comparing of factors associated with oral health behaviour between genders. Such factors also differ according to country and era. In order to promote oral health effectively, it is necessary to investigate factors associated with oral health behaviour of the targeted populations.

In the present study, we examined a sample of adults aged 20-29 living in Chiba City. The purpose of this report is to describe their oral health behaviour and compare determinants of each oral health behaviour item (toothbrushing frequency, using dental floss, and having dental check-ups) between genders.

Subjects and methods

Setting

The targeted population for this study was adults aged 20-29. We asked the Chiba City Dental Association, which has 390 registered dentists (membership rate: 84%), to recruit dentists willing to cooperate with the study. Selected to participate were 150 dental clinics distributed proportionally in each area of Chiba City according to population. The purpose and procedures of this study were explained to the cooperating dentists and they were asked to obtain information from patients by means of a questionnaire (Table 1). We instructed each dentist to select four subjects as follows: a man aged 20-24; a man aged 25-29; a woman aged 20-24; and a woman aged 25-29. These subjects were selected randomly from each category of gender and age.

Table 1 Questionnaire used in this study

How often do you brush your teeth?			
Once a day			
Twice a day			
Three or more times a day			
Not every day			
How often do you floss your teeth?			
Once a week			
Twice or more a week			
Less than once a week			
Never			
How often do you have dental check-up?			
Once a year			
Twice or more a year			
Less than once a year			
Never			
Do you understand the meaning of these terms			
Dental plaque	Yes	Slightly	No
Dental calculus	Yes	Slightly	No
Periodontal disease	Yes	Slightly	No
Sealant	Yes	Slightly	No
Fluoridation	Yes	Slightly	No
Sugarless	Yes	Slightly	No
Xylitol	Yes	Slightly	No

Subjects

The subjects were 527 dental patients (245 men and 282 women) aged 20-29 years who consulted dentists in Chiba City during October 1997. They were given a questionnaire to complete which was designed to assess their dental health behaviour at first examination. The response rate was 91.3%.

Method

Oral health variables

Variables were measured using a questionnaire that assessed dental health knowledge and oral health behaviour. The seven dental health knowledge items (dental plaque, dental calculus, periodontal disease, fluoridation, sealant, sugarless, and xylitol) were selected to reveal the status of the individual's dental health knowledge. Oral health behaviour was comprised of three items, two self- care items (toothbrushing frequency, using dental floss), and a professional care item (dental check-ups). Grouping criteria are described below.

Dental health knowledge. Responses to variables measuring caries and periodontal knowledge were 'know the meaning', 'know the meaning a little' or 'don't know the meaning' and a composite variable was computed to measure the total number of 'know the meaning' responses. The composite variable was then collapsed into two categories of lower (0-3) and higher (4-7) knowledge.

Oral health behaviours. Status of each oral health behaviour was classified into good behaviour or bad behaviour. Categories of behaviour were toothbrushing frequency (times per day: 2 or more, 0 or 1), dental floss (use, not use) and dental check-ups (times per year: 1 or more, less than 1).

Socioeconomic variables

Social economic variables were recorded from answers to questions on age group (20-24 years,

Table 2 Distribution of subjects by socioeconomic variables.

	Men	Women
Age group		
20–24	125 (51.0)	139 (49.3)
25–29	120 (49.0)	143 (50.3)
Employment status		
Full-time	179 (73.7)	171 (61.1)
Part-time	3 (1.2)	27 (9.6)
Unemployed	61 (25.1)	82 (29.3)
Family composition		
Live alone	74 (30.2)	46 (16.6)
Live with family	171 (69.8)	231 (83.4)

Note: missing data were not included in the calculation.

25–29 years), employment status (full-time, part-time, unemployed), and family composition (live alone, live with family). The distribution of subjects by socioeconomic variables and age group is shown in Table 2.

Statistical analysis

Since effects of gender on oral health behaviour were expected, analyses were performed for each gender. The differences between genders with regard to the status of each dental health knowledge item and oral health behaviour were analysed using the Chi-square test. The difference between genders with regard to dental health knowledge score was analysed by Mann-Whitney analysis. Logistic regression models were used to estimate the association between oral health behaviour and socioeconomic factors or dental health knowledge. Moreover, models were constructed separately for men and women. The baseline variables included in the models were age group, employment status, family composition and dental health knowledge. Differences at the 0.05 level were considered statistically significant. SPSS for Windows (version 10.0) was used in performing all statistical analyses.

Results

The rates of persons who knew the meaning of each dental knowledge item are shown by gender in Table 3. For each dental health knowledge item, a higher percentage of women than men knew the meaning. In five items, dental calculus, periodontal disease, sealant, fluoride, and sugarless, there were significant differences between genders. More than 70% of persons knew the meaning of dental calculus (men 70.2%, women 82.9%), dental plaque (men 76.3%, women 80.4%) and sugarless (men 84.1%, women 90.0%). However, less than 20% knew the meaning of sealant (men 7.3%, women 19.3%).

Table 3 Dental knowledge by gender.

	Men	Women	*p*
Dental plaque			
Know	187 (76.3)	225 (80.4)	0.155
Don't know	58 (23.7)	55 (19.6)	
Dental calculus			
Know	172 (70.2)	232 (82.9)	0.001
Don't know	73 (29.8)	48 (17.1)	
Periodontal disease			
Know	128 (52.2)	187 (66.8)	0.001
Don't know	117 (47.8)	93 (33.2)	
Sealant			
Know	18 (7.3)	54 (19.3)	0.001
Don't know	227 (92.7)	226 (80.7)	
Fluoridation			
Know	48 (19.6)	113 (40.4)	0.001
Don't know	197 (80.4)	167 (59.6)	
Sugarless			
Know	206 (84.1)	252 (90.0)	0.027
Don't know	39 (15.9)	28 (10.0)	
Xylitol			
Know	133 (54.3)	160 (57.1)	0.284
Don't know	112 (45.7)	120 (42.9)	

Note: two women had missing information and were not included in calculations.

The distribution of the subjects in the dental health knowledge score is shown in Fig. 1. Most men had a score of 4 or 5, followed by scores of 3, 2, 1. In women, the score of 5 had the most subjects. About the same number of female subjects had scores of 3, 4, 6, and 7. Women had significantly higher scores than men ($p < 0.001$).

Oral health behaviour is shown in Table 4. In all items, women exhibited significantly higher rates of good oral health behaviour than men (*p*-value: toothbrushing frequency 0.001, using dental floss 0.042, dental check-ups 0.001). With regard to toothbrushing frequency, more than 60% of the men and more than 80% of the women exhibited good behaviour. In contrast, less than half of the subjects had good behaviour with regard to dental floss and dental check-ups. In particular, only about 5% of the men had a regular dental check-up.

To investigate the factors associated with oral health behaviour, we performed logistic regression analysis (Table 5). Family composition was associated with toothbrushing frequency in women. The associations of dental health knowledge and employment status with using dental floss were