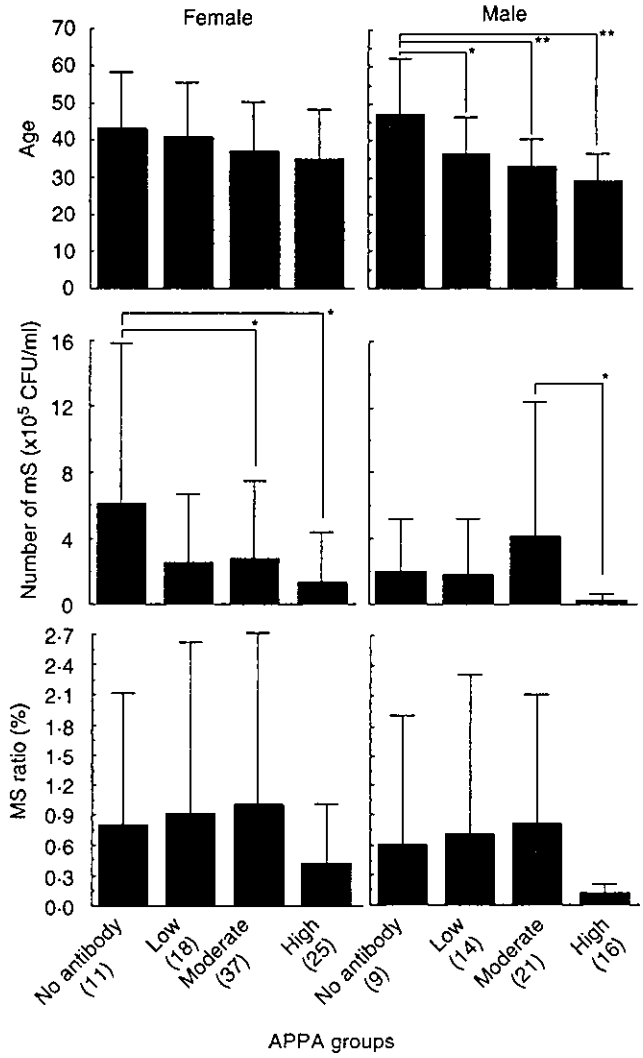

Fig. 1. Relationships between age, number of mS organisms, mS ratio, and levels of a-PPA in female and male subjects. Measurement of mS number and distribution of a-PPA titre in 4 groups are described in Materials and Methods. Results are expressed as mean ± SD of each parameter. ( ): Number of subjects in each age group. Asterisks denote significantly different relative antibody level (*$P < 0.05$, **$P < 0.01$).

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

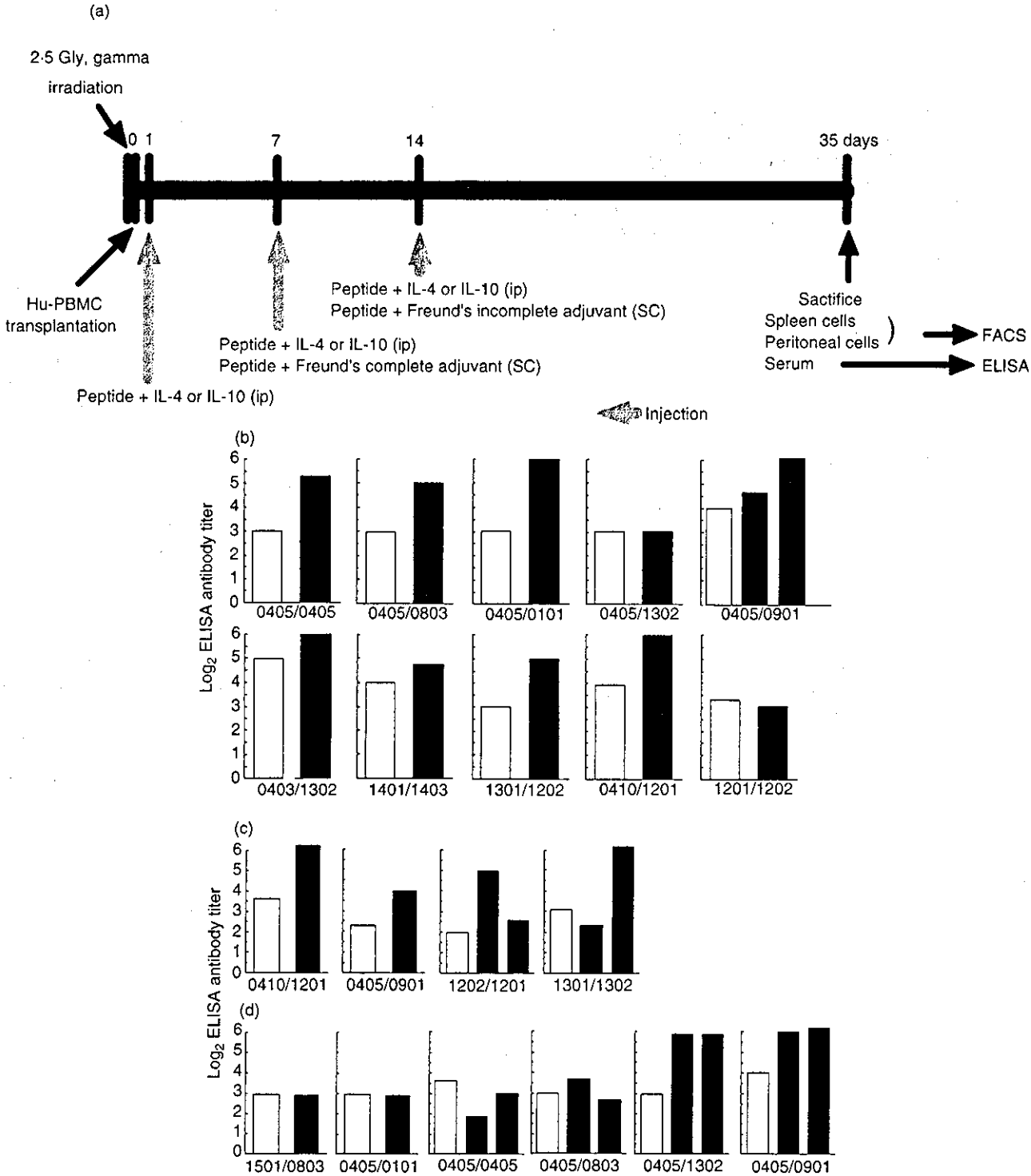

Fig. 2. Production of a-PPG in hu-PBMC-NOD-SCID mice following immunization with PAc (361–386) peptide. The immunization schedule was shown in (a). The peptide (■) in PBS (b), IL-4 (c), or IL-10 (d) was injected into 1 or 2 NOD-SCID mice grafted with hu-PBMC expressing the heterozygous or homozygous 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.

ACKNOWLEDGEMENTS

This work was supported in part by a grant-in aid for Development Scientific Research (15390571) from the Ministry of Education, Science, and Culture of Japan, and by a grant from the Japan Health Science Foundation to H.S.

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J Clin Periodontol 2004; 31: 680–684 doi: 10.1111/j.1600-051X.2004.00548.x
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Journal of Clinical Periodontology

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

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Yoshihara A, Seida Y, Hanada N, Miyazaki H: A longitudinal study of the relationship between periodontal disease and bone mineral density in community-dwelling older adults. J Clin Periodontol 2004; 31: 680–684. doi: 10.1111/j.1600-051X.2004.00548.x.

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

Accepted for publication 12 November 2003

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 ⩾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 ⩾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 ⩾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 ⩾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 ⩽69 for females, ⩽85 for males, OG) and the non-osteopenia group (NOG), we evaluated the number of progressive sites which had ⩾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 ⩾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 ⩾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.

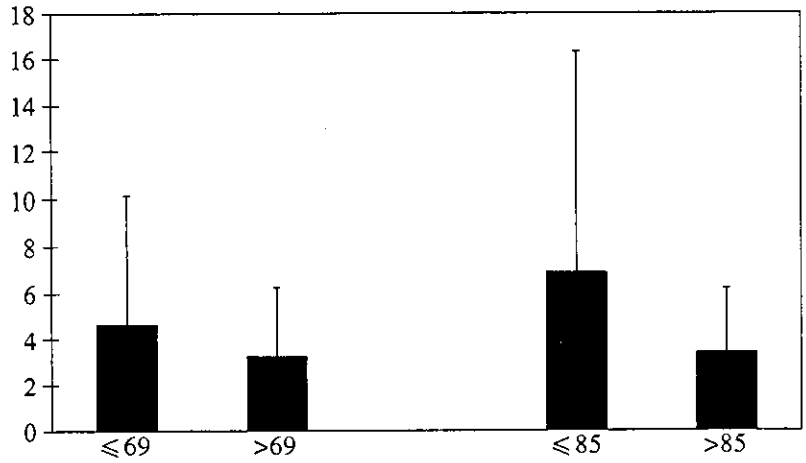

Fig. 1. Relationship between the number of progressive sites with ≥3 mm additional attachment loss and stiffness by gender. The number of subjects: stiffness ≤69 ($n = 74$) and >69 ($n = 19$) for females, ≤85 ($n = 64$) and >85 ($n = 22$) for males. a, stiffness (%)

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.

Acknowledgment

This work was supported by a grant-in-aid from the Ministry of Health and Welfare of Japan (H10-Iryo-001).

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Public Health (2004) 118, 104-109

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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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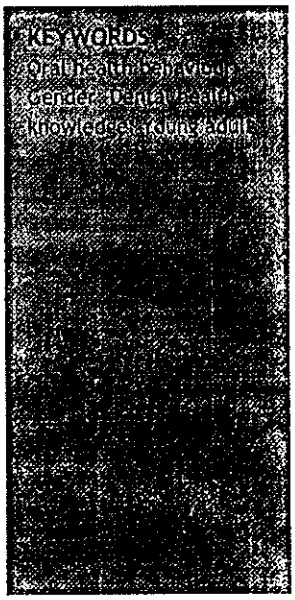
Received 28 November 2002; received in revised form 13 May 2003; accepted 20 May 2003



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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doi:10.1016/j.puhe.2003.05.007

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

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.

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%).

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

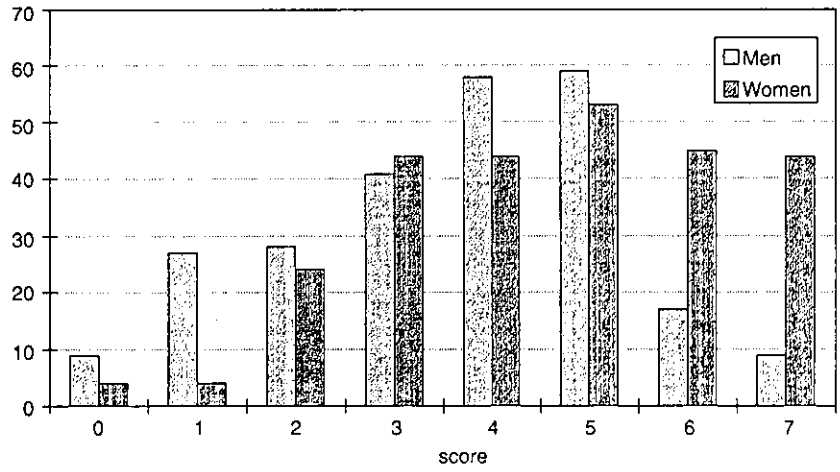

Figure 1 Number of subjects in subgroups of dental health knowledge score.

significant in women. Women with full-time employments had a significantly higher rate of using dental floss than unemployed women. Age classification was a contributing factor to both using dental floss in men and dental check-ups in women. In men, no variable was significantly associated with toothbrushing frequency and dental check-ups.

Discussion

The state of family composition of the subjects was roughly equal to that of adults aged 20-29 in Chiba City (Men: live alone 27.9%, live with family 72.1%, Women: live alone 14.0%, live with family 86.0%). Similarly, the rate of full-time workers among the subjects was roughly equal to that of adults aged 20-29 in Chiba City (Men: 71.1%, Women: 58.9%). From these aspects, the subjects in this study are thought to be representative.

Subjects of this study were young, so few were managers or professionals. We hypothesized that being employed or unemployed and, if employed, whether full-time or part-time, would be related to oral health behaviour. Therefore, as a socio-demographic variable, we used employment status (full-time, part-time, unemployed). A similar classification was used in other study.[9] Recently, the decline of health behaviour of young people who live alone is commonly pointed out in Japan. Therefore, we hypothesized that a difference would be seen between those who live alone or who live with family. For these reasons, employment status and family composition are thought to be proper as socio-demographic factors in Japanese young adults.

Women exhibited higher dental knowledge scores than men which is consistent with other reports.[15,16] Also, women exhibited better oral health behaviour than men. This finding coincides with findings of other reports.[7,8,11] It is thought that improvement of oral health knowledge and of oral health behaviour is required for men aged 20-29. Most of these subjects are salaried worker in Japan. Health education and instruction in health promotion have scarcely been provided to workers in Japan. Health promotion for workers may increase awareness of health, particularly in men.

In men, a significant relationship was seen only between using dental floss and age. However, for women, each oral health item had associated factors. Dental health knowledge was associated with using dental floss in women. Dental floss is easily acquired on the market in Japan. It is suggested that women are more ready to exhibit good health behaviour than men by acquiring health knowledge, if such behaviour is easy to carry out. In addition to dental health knowledge, employment status was significantly correlated with using dental floss in women. The social activity of being employed full-time may influence oral health behaviour in women.

Table 4 State of oral health behaviour.

	Men	Women	p
Toothbrushing frequency (per day)			
Two or more	154 (63.4)	249 (88.3)	0.001
Less than 2	89 (36.6)	33 (11.7)	
Dental floss			
Use	74 (30.7)	108 (38.3)	0.042
Not use	167 (69.3)	174 (61.7)	
Dental check-up (per year)			
One or more	11 (4.5)	48 (17.2)	0.001
Less than 1	231 (95.5)	231 (82.8)	

Note: missing data were not included in calculation.

Table 5. Odds ratios from multiple logistic regression analyses of oral health behaviour.

	Odds ratio (95% CI)		
	Toothbrushing frequency	Dental floss	Dental check-ups
Men			
Age group			
20-24	1.0	1.0	1.0
25-29	1.06 (0.60-1.88)	2.00 (1.09-3.68)*	2.32 (0.55-9.71)
Employment status			
Unemployed	1.0	1.0	1.0
Part-time	0.35 (0.03-4.12)	a	a
Full-time	1.25 (0.66-2.37)	1.22 (0.59-2.52)	2.51 (0.29-22.10)
Family composition			
Live alone	1.0	1.0	1.0
Live with family	1.06 (0.59-1.90)	0.75 (0.41-1.37)	1.43 (0.36-5.70)
Dental health knowledge			
Lower	1.0	1.0	1.0
Higher	1.37 (0.80-2.34)	1.33 (0.75-2.37)	1.88 (0.48-7.36)
Women			
Age group			
20-24	1.0	1.0	1.0
25-29	0.69 (0.32-1.50)	1.04 (0.63-1.71)	2.16 (1.11-4.21)*
Employment status			
Unemployed	1.0	1.0	1.0
Part-time	0.58 (0.17-2.04)	1.01 (0.38-2.66)*	0.22 (0.03-1.91)
Full-time	1.54 (0.64-3.74)	1.93 (1.06-3.51)	2.15 (0.93-5.01)
Family composition			
Live alone	1.0	1.0	1.0
Live with family	2.71 (1.10-6.68)*	1.32 (0.63-1.71)	0.78 (0.35-1.74)
Dental health knowledge			
Lower	1.0	1.0	1.0
Higher	1.48 (0.67-3.27)	1.87 (1.08-3.24)*	1.93 (0.91-4.07)

CI = confidence interval; $p < 0.05$

[a] OR and 95% CI cannot be calculated for dental floss and dental check-up because no part-time worker had good oral health behaviour in these categories.

Women aged 25-29 showed a significantly higher rate of having dental check-ups than women aged 20-24. Woolfolk et al.[17] reported that factors associated with dental health check-up frequency were gender, income level, having a usual place for dental care and anxiety about receiving dental care. In the present study, age was shown to be associated with having dental check-ups. In Japan, the rate of occurrence of periodontitis increases at about the age of 30. For oral health promotion, an increase in the number of persons who are concerned with professional care in the 25-29 age group is desirable.

Toothbrushing frequency was significantly associated with family composition in women. One study reports the relationship between family composition and health behaviour in young adults.[18] It is thought that living alone may affect the frequency of toothbrushing in young women. These findings suggest that the oral health behaviour of women is more readily influenced by various factors than that of men. Women were reported to have lower self-assessment of oral health,[15,16,19] greater dental anxiety,[20-23] and better dental attendance.[13,24] Women, harbouring these characteristics, may be ready to begin good oral health behaviour as they age or acquire dental health knowledge. Therefore, it is expected that the effect of health promotion is greater in young women than in young men. We previously reported that the relationship between caries treatment and smoking was significant in young women but in young men.[25] Health behaviour of men is more elusive than that of women. However, men may have some determinants other than the factors used in this study. Improvement of oral health behaviour of young men needs some

motivation. Motivation to improve oral health behaviour in young men is important. It is necessary to investigate factors other than those examined here are associated with oral health behaviour in young men.

In this study, sexual differences were seen in factors associated with oral health behaviour. Factors associated with oral health behaviour may differ in adults in other age groups. Similar analysis on that point by gender will yield useful findings for oral health promotion.

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CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 2004, p. 379–386 Vol. 11, No. 2
1071-412X/04/$08.00+0 DOI: 10.1128/CDLI.11.2.379–386.2004

Establishment of an Animal Model Using Recombinant NOD.*B10.D2* Mice To Study Initial Adhesion of Oral Streptococci

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Received 4 September 2003/Returned for modification 19 October 2003/Accepted 28 December 2003

An oral biofilm is a community of surface-attached microorganisms that coats the oral cavity, including the teeth, and provides a protective reservoir for oral microbial pathogens, which are the primary cause of persistent and chronic infectious diseases in patients with dry mouth or Sjögren's syndrome (SS). The purpose of this study was to establish an animal model for studying the initial adhesion of oral streptococci that cause biofilm formation in patients with dry mouth and SS in an attempt to decrease the influence of cariogenic organisms and their substrates. In nonobese diabetogenic (NOD) mice that spontaneously develop insulin-dependent diabetes mellitus (IDDM) and SS, we replaced major histocompatibility complex (MHC) class II (A^{g7} E^{g7}) and class I D^b with MHC class II (A^d E^d) and class I D^d from nondiabetic B10.D2 mice to produce an animal model that inhibited IDDM without affecting SS. The adhesion of oral streptococci, including *Streptococcus mutans*, onto tooth surfaces was then investigated and quantified in homologous recombinant N5 (NOD.*B10.D2*) and N9 (NOD.*B10.D2*) mice. We found that a higher number of oral streptococci adhered to the tooth surfaces of N5 (NOD.*B10.D2*) and N9 (NOD.*B10.D2*) mice than to those of the control C57BL/6 and B10.D2 mice. On the basis of our observation, we concluded that these mouse models might be useful as animal models of dry mouth and SS for in vivo biological studies of oral biofilm formation on the tooth surfaces.

Oral streptococci are present in large numbers in dental plaque, and several types interact with the enamel salivary pellicle to form a biofilm on tooth surfaces (9, 16, 17, 21, 29). Streptococci account for approximately 20% of the total number of salivary bacteria (24), with *Streptococcus salivarius* being the primary organism. Further, the densities of *Streptococcus mutans* and *Streptococcus sanguis* in saliva are more than 1×10^5 cells per ml. *S. mutans* is a pioneering organism that plays an important role in biofilm formation on tooth surfaces and is a primary causative agent of dental caries (9, 16, 21). The mechanical forces of salivary flow and tongue movement tend to dislodge and expel bacteria from tooth surfaces and the oral cavity (3, 5, 6), and their importance in controlling microbial colonization in the oral cavity has been well demonstrated in individuals with diabetes mellitus, Sjögren's syndrome (SS), and dry mouth, who suffer from a rapid overgrowth of biofilm and rampant caries, making them highly susceptible to oral infections (1–2, 6). Thus, attempts to investigate the initial adhesion by oral streptococci, including *S. mutans*, in mouse models are likely to aid in the understanding and prevention of oral infectious diseases caused by the components of oral biofilm.

Previous studies of *S. mutans* infections in the oral cavities of mice have been performed by feeding the animals diets containing sucrose in the presence of glucans (13, 15, 30, 43). Since the adherence of *S. mutans* to the tooth surface may depend on the balance between physical adherence and synthesis of in-

TABLE 1. Linkage markers analyzed for homozygosity to NOD-derived *Idd* loci in NOD.*B10.D2* congenic mice

Idd locus/ chromosome	Linkage marker homozygous to NOD allele	Relative microsatellite size[a]
Idd1/17	D17Mit 198	B10.D2 > NOD
	D17Mit 195	B10.D2 > NOD
	D17Mit 194	B10.D2 = NOD
	D17Mit 173	B10.D2 = NOD
	D17Mit 145	B10.D2 = NOD
	D17Mit 82	B10.D2 > NOD
	D17Mit 34	B10.D2 > NOD
	D17Mit 28	B10.D2 > NOD
	D17Mit 59	B10.D2 = NOD
	D17Mit 62	B10.D2 = NOD
Idd2/9	D9Mit 25	B10.D2 = NOD
Idd3/3	D3Mit 95	B10.D2 < NOD
	D3Mit 103	B10.D2 = NOD
	D3Mit 206	B10.D2 = NOD
Idd4/11	D11Mit 115	B10.D2 < NOD
	D11Mit 320	B10.D2 < NOD
Idd5/1	D1Mit 46	B10.D2 < NOD
Idd6/6	D6Mit 15	B10.D2 > NOD
	D6Mit 339	B10.D2 < NOD
	D6Mit 52	B10.D2 > NOD
Idd7/7	D7Mit 20	B10.D2 > NOD
Idd8, Idd12/14	D14Mit 222	B10.D2 > NOD
	D14Mit 110	B10.D2 = NOD
Idd9, Idd11/14	D4Mit 59	B10.D2 < NOD
Idd10/3	D3Mit 103	B10.D2 = NOD
Idd13/2	D2Mit 257	B10.D2 < NOD
	D2Mit 17	B10.D2 = NOD
Idd14/13	D13Mit 61	B10.D2 < NOD
Idd15/15	D5Mit 48	B10.D2 > NOD

[a] Microsatellite markers with the indicated allelic size variants were typed in backcross mice used for the intercross.

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soluble glucans in a natural environment, that infection method may be inappropriate for investigation of natural biofilm formation associated with streptococci, including *S. mutans* (18, 39).

The nonobese diabetogenic (NOD) mouse strain is currently the best available model for the study of insulin-dependent type 1 diabetes mellitus (IDDM) and SS (11, 31), both of which develop spontaneously and are characterized by lymphatic infiltration of the pancreas and salivary glands. Oral changes are prominent features of these diseases, which are manifested by dry mouth and hyposalivation (6, 7, 37). NOD mice are also used as an animal model for the study of oral infectious diseases associated with systemic diseases such as diabetes and SS or dry mouth.

The unique major histocompatibility complex (MHC) class II genes ($I\text{-}A^{g7}$, no expression of *I-E*) represent dominant susceptibility factors and mediate activated T cells during the development of diabetes in NOD mice (11, 22, 25, 36, 41, 42). In the NOD model of SS, histopathological analyses of the salivary glands in MHC-congenic strains of NOD mice have indicated that the $I\text{-}A^{g7}$ region is not required for lymphocytic infiltration (26, 31). Further, replacement of the NOD MHC

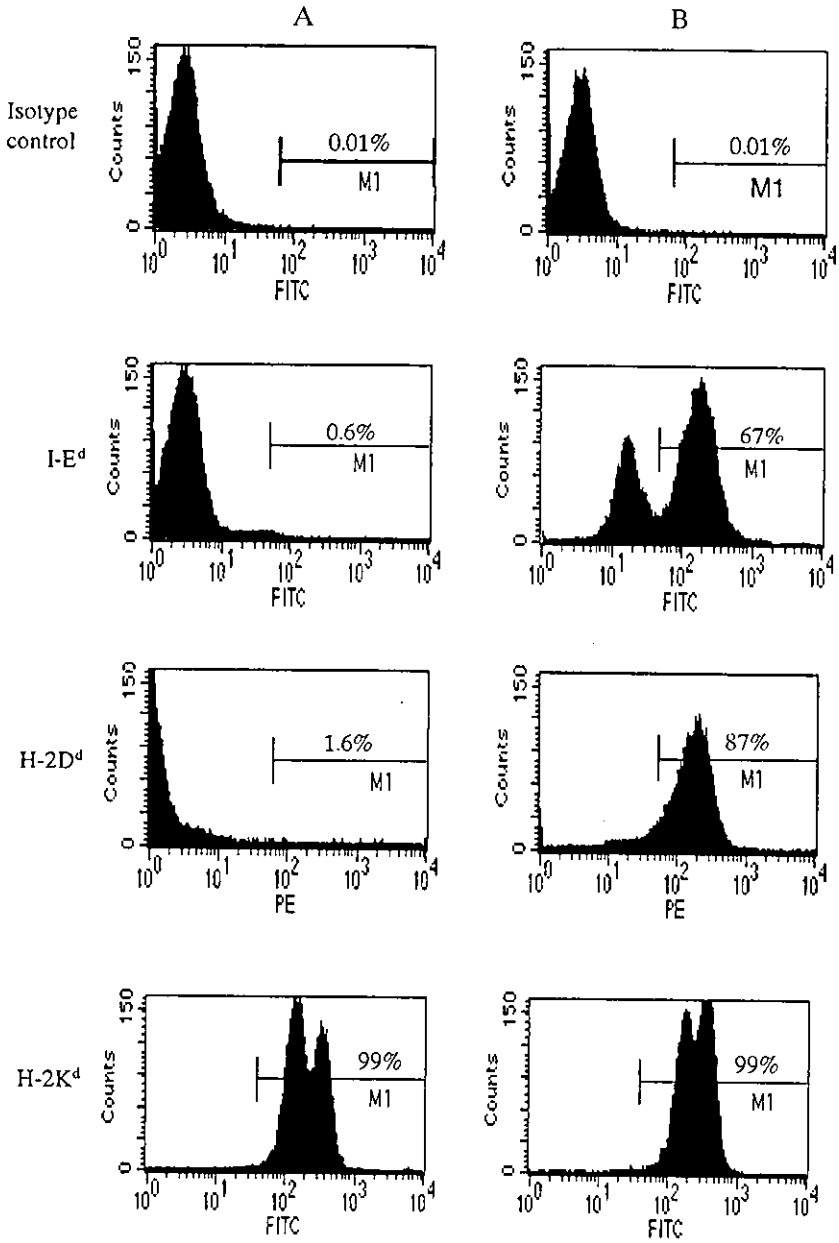

FIG. 1. Serological typing of lymphocytes. Spleen cells from NOD (A) and N5 (NOD.*B10.D2*) (B) female mice at 4 months of age were examined for the expression of MHC class I $H\text{-}2K^d$, MHC class II $I\text{-}E^d$, and $H\text{-}2D^d$ by using FACS analysis. NOD, MHC class II $I\text{-}E^d$, and $H\text{-}2D^d$ antigens were expressed in the NOD mice. Each histogram shows the percentage of total spleen cells from NOD and N5 (NOD.*B10.D2*) mice. Histograms are representative of the results of three independent experiments with 10 mice in each group, with similar results obtained in each experiment. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

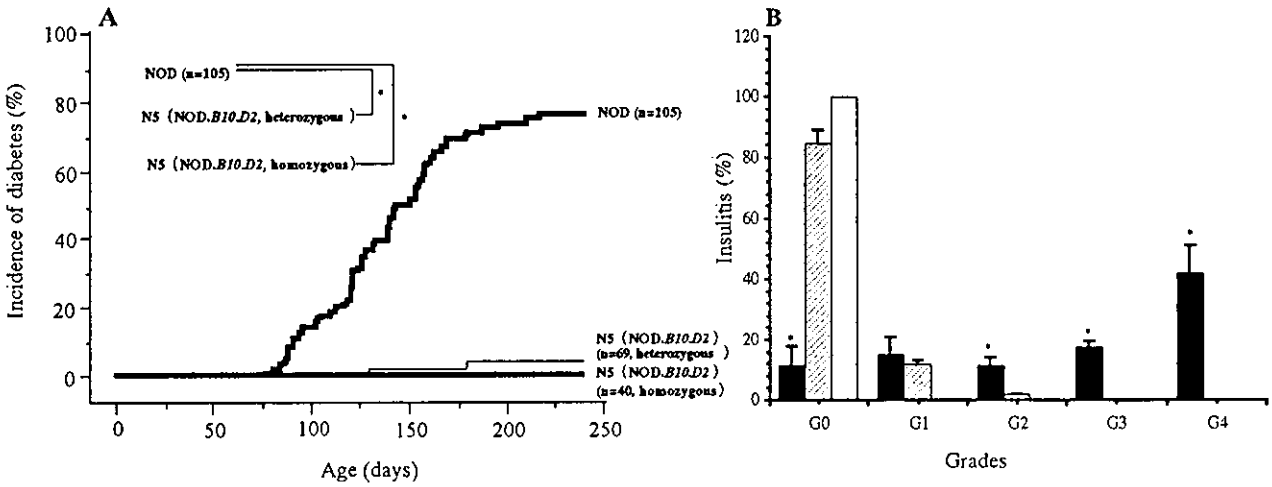

FIG. 2. Cumulative incidence of diabetes and insulitis in NOD and new N5 (NOD.*B10.D2*) mice. (A) Mice homozygous for B10.D2 MHC avoided diabetes in comparison with the heterozygous and NOD types. (B) Insulitis scores were calculated by using hematoxylin and eosin staining of pancreas sections from F_1 (□), N5 (▨), and NOD (■) female mice at 4 months of age. The degree of lymphocyte infiltration was graded as follows: G0, no infiltrating cells in the islets; G1, infiltrating cells adjacent to the islets; G2, infiltrating cells occupying less than 25% of the islets; G3, infiltrating cells occupying 25 to 50% of the islets; G4, infiltrating cells occupying more than 50% of the islets. For example, the percentage of G1 insulitis = (number of G1)/(G0 + G1 + G2 + G3 + G4 + G4) × 100. Results are expressed as the means ± standard deviations (SDs) of the results for 12 mice per strain. *, $P < 0.001$ for the results for NOD mice versus those for N5 and F_1 mice.

class I K^d region with another haplotype, MHC class I K^{wm7}, as well as replacement of the MHC class II A^{g7} E^{g7} and class I D^d regions with the corresponding region from the other MHC haplotype, has been shown to prevent diabetes (12). However, replacement with MHC class I *K* does not completely prevent development of insulitis. In another report, NOD mice pretreated nasally by using peptides restricted with MHC class I K^d showed a delayed onset of spontaneous IDDM, though insulitis could not be prevented by the induction of tolerance (23).

In the present study, we attempted to establish an animal model for oral infectious diseases such as dental caries by focusing on replacement of the MHC class II and class I *D* region but not the class I *K* region in nondiabetic NOD mice by outcrossing B10.D2 mice (K^d, $I\text{-}A^d$, and D^d) with NOD mice (K^d, $I\text{-}A^{g7}$, and D^b) because the MHC class I *K* region in B10.D2 mice is identical with that in NOD mice (12). The present backcrossed and intercrossed NOD mice with the MHC class II and MHC class I *D* region replaced with that from B10.D2 mice developed SS, however, not diabetes. We then attempted to determine whether these mice would be useful as animal models for a sucrose-free study of the initial adhesion of oral streptococci on tooth surfaces in humans.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Streptococcus* strains used in this study were *S. mutans* MT8148, *S. sanguis* ATCC 10556, *S. sobrinus* 6715, *S. salivarius* ATCC 9759, and *S. mitis* ATCC 6249. All bacteria were grown in an atmosphere of H_2 and CO_2 (GasPack; Becton Dickinson and Co., Franklin Lakes, N.J.) in brain heart infusion broth (Difco Laboratory, Detroit, Mich.) at 37°C overnight and were then harvested and washed twice with sterile phosphate-buffered saline (PBS).

Animals and assessment of diabetes and saliva. NOD/LtJ and B10.D2 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and Japan SLC (Shizuoka, Japan), respectively, and maintained in accordance with the guidelines of the National Institute of Infectious Diseases. Clinical onset of diabetes in NOD mice was determined by the presence of glucose in urine and blood. Urine was tested weekly using Uristix reagent strips (Bayer Medical Ltd., Newbury, United Kingdom) and confirmed to be positive by blood glucose measurements. After being anesthetized, the mice were injected with a cocktail of isoproterenol (0.20 μg/100 gm of body weight) and pilocarpine (0. 05 μg/100 gm) (Sigma Chemical, St. Louis, Mo.) in PBS as a secretagogue. Following the intraperitoneal injection, saliva was collected from each mouse by using a micropipette for 15 min and was stored at −80°C.

Bacterial sampling and CFU counting. All oral streptococci were cultured in brain heart infusion broth overnight and were then washed twice with sterile PBS. Chlorhexidine (0.2%)-soaked sterile cotton swabs were used for disinfecting the oral cavities of the mice, including the lower incisor teeth, which were immediately washed with sterile PBS. Oral streptococci were introduced into the oral cavities at a final concentration of 7×10^9 CFU in 250 μl of PBS in all females at 4 or 8 months of age for 2.5 min, after which the mice were not provided food or drinking water. Following inoculation, samples were collected from the labial surfaces of the lower incisor teeth with a sterile cotton ball and then dipped into 2 ml of PBS. The samples in PBS were sonicated by ultrasonic dispersion (power output, 60 W) for 10 seconds and were then poured onto Mitis-Salivarius agar plates containing 0.02 M bacitracin (MSB) by using an EDDY JET spiral system (Gunze Sangyo, Inc., Tokyo, Japan). CFU were determined after 48 h of anaerobic incubation at 37°C.

Generation of backcross mice. To generate the backcross generation, NOD mice were mated with B10.D2 mice to produce (NOD × B10.D2)F_1 mice, and then heterozygous F_1 mice were mated with NOD mice to produce first-generation backcross (BC1) mice. BC1, BC2, BC3, and BC7 heterozygous mice were then mated with NOD mice to produce BC2, BC3, BC4, and BC8 mice, respectively, and BC4 and BC8 heterozygous mice were intercrossed to produce N5 and N9 MHC-recombinant NOD mice. After an outcross of the NOD strain to the B10.D2 strain, repetitive backcrossing with NOD mice was performed, with breeder selections based on genomic PCRs and/or simple sequence length polymorphism (SSLP) analysis with microsatellite markers (MapPairs; Research Genetics, Huntsville, Ala.), as shown in Table 1. The PCRs (25 μl) were done using a PTC-200 (MJ Research, Watertown, Mass.) for 40 cycles (94°C, 15 seconds; 58°C, 45 seconds; 72°C, 5 min, after an initial denaturation at 94°C for 3 min) and were then analyzed on a 3% agarose gel. An SSLP analysis of the *B10.D2* allele from the backcross generation identified the mice as homozygous for allelic variants characteristic of NOD mice with replacements of the MHC region with that from B10.D2 mice at all of the *Idd17* linkage markers (Table 1). Typing of these markers without *Idd17* confirmed the homozygous presence of the NOD-derived background genome at the identified *Idd* loci in the backcross generation (Table 1).

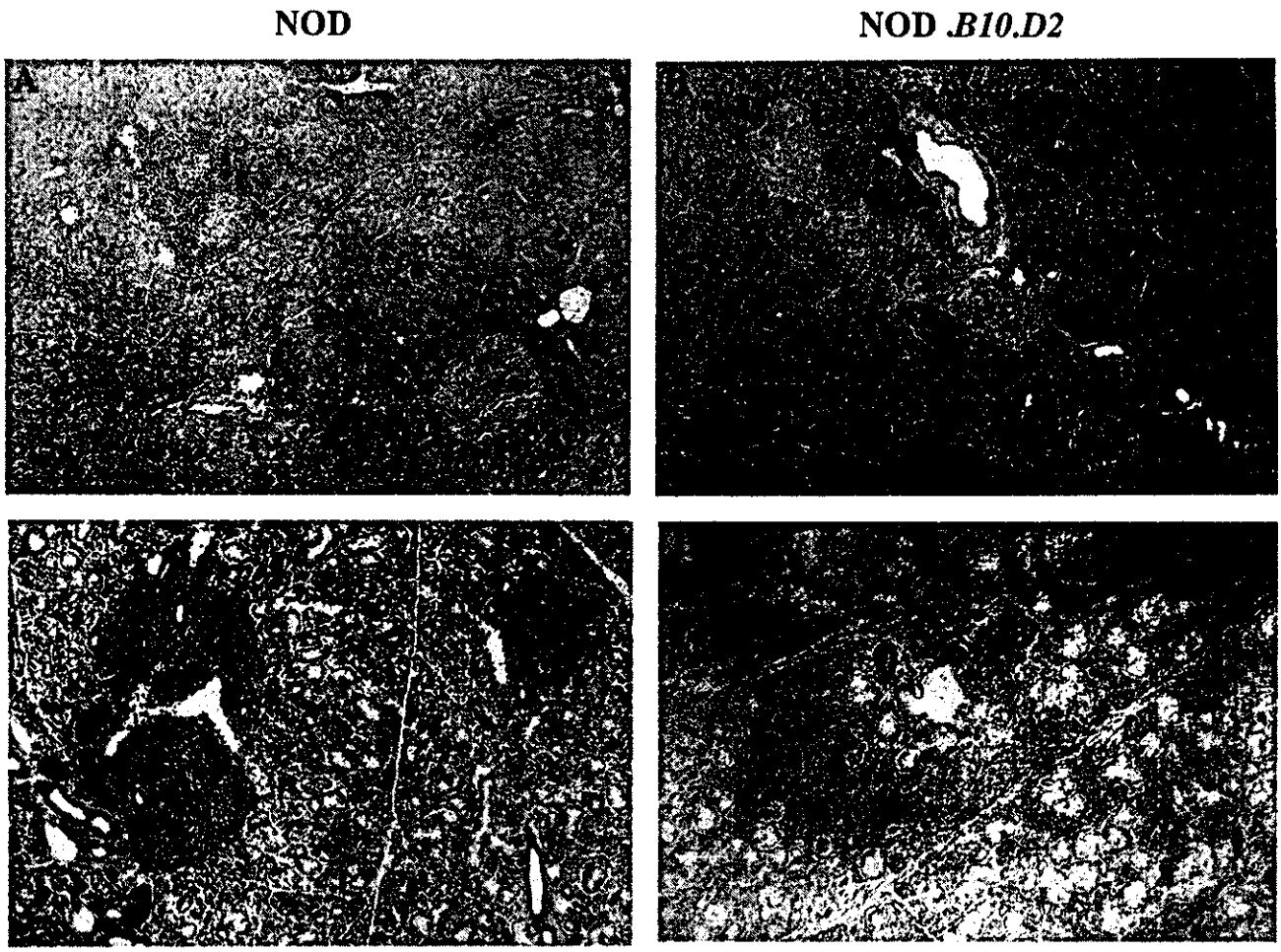

FIG. 3. Histology of exocrine glands from NOD, N5 (NOD.*B10D2*), and N9 (NOD.*B10D2*) female mice at 4 months of age. (A) Massive infiltration by mononuclear cells in the pancreas islets of NOD mice; (B) no infiltrating cells in the pancreas islets of N5 (NOD.*B10D2*) mice; (C) massive infiltration by mononuclear cells in the submandibular glands of NOD mice; and (D) moderate to massive infiltration by mononuclear cells in the submandibular glands of N5 (NOD.*B10D2*) mice; (E) massive infiltration by mononuclear cells in the submandibular glands of N9 (NOD.*B10D2*) female mice. Tissues were stained with hematoxylin and eosin. Magnification, × 100.

MHC serological typing. Spleen cells were dissociated from NOD and MHC-recombinant N5 NOD and N9 NOD mice and incubated with monoclonal antibodies (mAbs) determined to react with MHC class I and class II molecules. Fluorescein isothiocyanate- and phycoerythrin-conjugated goat anti-mouse mAb H-2K^d (SF-1.1.1) reacted in cells from both NOD and B10.D2 mice, and mAb I-E^k (14-4-4S) and mAb H-2D^d (34-5-8S) reacted in cells from B10.D2 mice. mAb I-E^k was used for detection of *I-E^d* expression because it cross-reacted with the I-E^d molecule. After being incubated with these mAbs for 45 min at 4°C, the cells were washed and then analyzed by flow cytometry (fluorescence-activated cell sorting [FACS]) (Becton Dickinson).

Histology. Pancreas specimens and submandibular glands were frozen in OCT compound. Tissue sections (5 μm) were stained with hematoxylin and eosin and were then examined for evidence of mononuclear cell inflammation. Histological observations and photomicrography were performed by using an Olympus BX50WI microscope (Olympus Inc., Tokyo, Japan).

Statistical analysis. A Kaplan-Meier cumulative survival test was used to compare the incidence of diabetes. Comparative analyses were performed by analysis of variance. A P value of < 0.05 was considered statistically significant for two-tailed comparisons. All statistical analyses were performed using StatView software for the Macintosh operating system.

RESULTS

Generation of backcross and intercross of NOD.*B10.D2* strain and assessment of diabetes and salivary flow rate. BC4 and BC8 heterozygous mice (*Mhc*: *d/d* at *K*, *d/g7* at *A* and *E*,