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Quantitative messenger RNA expression of Toll-like receptors and interferon-α1 in gingivitis and periodontitis

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Introduction: In addition to bacteria, viruses have been reportedly implicated in periodontitis. However, the available data are confined to Toll-like receptor 2 (TLR2) and TLR4, which recognize bacterial products in periodontitis. In the present study, we investigated the expression levels of TLR5, -7, and -9 messenger RNAs (mRNAs) in addition to those of TLR2 and -4, and compared gingivitis and periodontitis. Interferon-α1 (IFN-α1), which is important for the antiviral response, was also compared.
Methods: Gene expression was analyzed using quantitative real-time polymerase chain reaction for 59 periodontitis and 27 gingivitis tissue samples together with viral serology in some patients. The presence of plasmacytoid dendritic cells (pDCs), a robust producer of IFN-α, was immunohistochemically analyzed in an additional seven periodontitis and two gingivitis specimens.
Results: The expression levels of TLR2, -4, -7, and -9 were significantly higher in periodontitis lesions than gingivitis lesions. The expression level of TLR5 was comparable to levels of TLR2 and -4; however, no significant difference was found between gingivitis and periodontitis. Although the expression of IFN-α1 mRNA was higher in periodontitis lesions compared with gingivitis lesions, the level was quite low. Only a few pDCs were found in some periodontitis specimens. No difference was found for antibody-positivity between gingivitis and periodontitis.
Conclusion: This is the first study to show that a variety of TLRs are up-regulated in periodontitis lesions compared with gingivitis lesions, suggesting that diverse microbial and possibly viral antigens are involved in the pathogenic mechanisms for periodontal diseases. However, the ligands recognized by the various TLRs in periodontal lesions remain to be determined.

Key words: gingivitis; interferon-α1; periodontitis; real-time polymerase chain reaction; Toll-like receptors

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Chronic inflammatory periodontal diseases are infectious diseases caused by a group of periodontopathic bacteria. However, it is now evident that the immune response to bacterial products, such as fimbriae, proteolytic enzymes, and lipopolysaccharide, and the subsequent production of proinflammatory cytokines and other inflammatory mediators, are of particular importance in tissue destruction of the periodontium (20).

The products of infectious agents are now designated as having pathogen-associated molecular patterns and are recognized by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), on the cells involved in innate immunity (8). TLRs can be grouped into families according to the types of ligands they recognize. Lipid-based structures are recognized by TLR2 and TLR4. For example, lipoprotein, peptidoglycan, and lipoteichoic acid are recognized by TLR2, and lipopolysaccharide is recognized by

TLR4. Viral and/or bacterial nucleic acids are recognized by TLR3, TLR7, TLR8, and TLR9; the best characterized is the recognition of the CpG motif in DNA by TLR9. The protein ligand flagellin is recognized by TLR5.

Several studies have demonstrated an association of herpesviruses with periodontal disease by showing their presence in gingival tissue, gingival crevicular fluid, and subgingival plaque, in the presence of periodontal disease (23). Members of the herpesvirus family are composed of a double-stranded DNA genome contained within a nucleocaspid surrounded by a lipid envelope. It has been suggested that periodontal disease activity is involved in the initial recruitment of herpesvirus-infected inflammatory cells, for example, by plaque accumulation and the subsequent reactivation of virus by concurrent infection or by other risk factors.

Viruses as well as bacteria may therefore have roles in periodontal disease activity. However, only limited information is available on PRR expression in periodontal diseases (13, 14) and the expression of the PRRs involved in virus recognition or of type I interferon (IFN), an important antiviral molecule, has not been reported in periodontal diseases.

The aim of the present study is to describe the expression pattern of various TLRs and IFN-α1 in gingivitis and periodontitis lesions.

Materials and Methods

Patients and biopsies

Fifty-nine patients with moderate to advanced chronic periodontitis, referred to the Periodontal Clinic of the Niigata University Medical and Dental Hospital, took part in this study. Gingival biopsies were obtained at the time of periodontal surgery or extraction of severely involved teeth. As controls, 27 gingivitis tissues showing no supporting tissue destruction were also obtained from sites requiring extraction for reasons other than periodontitis, such as orthodontic treatment or pericoronitis. The modified diagnostic criteria of Kornman et al. (9) for periodontitis and gingivitis were used:

- Periodontitis: subjects having at least one interproximal site with ≥50% bone loss and total mean bone loss of ≥16%,
- Gingivitis: subjects having no probing pocket depth, no sites with bone loss >10% and clinical signs of inflammation.

Alveolar bone resorption was measured on the proximal surface of each tooth on a radiograph according to the established method (19). Mean alveolar bone resorption was calculated by dividing the mean bone resorption of each subject by the number of subjects. Healthy control specimens were not included in this study because a number of studies have demonstrated that not only is histological inflammation present before the accumulation of plaque, resulting in overt clinical signs of inflammation, but it is also found to be present at quite a significant extent (2, 21). Therefore, it is difficult to distinguish healthy controls from gingivitis specimens. The clinical status of the biopsy sites is shown in Table 1. Serum samples were also taken from some of the participants (21 for periodontitis and 19 for gingivitis) whose gene expression was analyzed. Serum immunoglobulin G (IgG) antibodies to herpes simplex virus (HSV), cytomegalovirus (CMV), and the nuclear antigen of Epstein–Barr virus (EBV) were determined by enzyme immunoassay (SRL, Inc. Tokyo, Japan).

Table 1. Clinical profile of gingival biopsy sites

	Periodontitis (n = 59)	Gingivitis (n = 27)	P-value
Age (years)	52.9 ± 9.2	30.7 ± 7.0	<0.0001
Male/female	25/34	15/12	
Smokers/non-smokers	13/46	4/23	
Gingival index	1.05 ± 0.63	0.07 ± 0.27	<0.0001
Probing depth (mm)	6.43 ± 2.17	2.44 ± 0.58	<0.0001
Attachment level (mm)	7.59 ± 2.53	2.56 ± 0.51	<0.0001
Tooth mobility	1.02 ± 1.08	0.00 ± 0.00	<0.0001
Bleeding on probing (% site)	55.9	14.8	
Bone resorption (%)	64.62 ± 22.27		

Data are expressed as mean ± SD.
The data were compared by unpaired t-test.

The experimental protocol was approved by the Institutional Review Board of Niigata University and informed consent was obtained from all patients before their inclusion in the study.

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from gingival tissues using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with RNase-free DNase I (Invitrogen). The RNA was then reverse-transcribed to complementary DNA using a random primer (TAKARA SHUZO Co., Ltd, Shiga, Japan) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). For real-time polymerase chain reaction (PCR), the primers and probes were all purchased from Applied Biosystems, Foster, CA. Reactions were conducted in a 25-μl reaction mixture on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using TaqMan Gene Expression Assays (Applied Biosystems). They were incubated for 10 min at 95°C, followed by 40 cycles of a two-step amplification procedure consisting of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. The ABI PRISM SDS 2.0 software (Applied Biosystems) was used to analyze the standards and to carry out the quantifications. The relative quantity of each messenger RNA (mRNA) was normalized to the relative quantity of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) mRNA.

Immunohistochemistry

To estimate the proportion of plasmacytoid dendritic cells (pDCs) in the lesion, gingival specimens were collected from a further seven periodontitis patients (mean age: 54.3 ± 10.5 years; range 40–65) and two gingivitis patients (aged 58 and 65 years), and serial cryostat sections were prepared. The mean probing depth, probing attachment level, and bone resorption of the periodontitis sites were 7.2 ± 1.9 mm (range 5–10 mm), 10.0 ± 2.4 mm (range 6–12 mm) and 93.0 ± 13.0% (range 70–100%), respectively. No apparent tissue destruction was seen in gingivitis sites. The inflammatory gingival tissues were taken to analyze the same area as for gene expression analysis. Monoclonal anti-CD303 (BDCA-2: CloneAC144; Miltenyi Biotec Inc., Auburn, CA) and control IgG1 (eBioscience, San Diego, CA) were used for single staining by the avidin–biotin–immunoperoxidase method, as previously described (28). At least six sections per specimen were stained and identification of positive cells was carried out for the entire area of the sections using an ocular grid (0.04 mm^2) at a magnification of ×400.

Statistical analysis

The difference in clinical parameters between the two groups was compared by unpaired t-test. The expression levels of TLRs and IFN-α between gingivitis and

periodontitis were compared using a Mann–Whitney *U*-test using StatView® for Windows (Version 5, SAS Institute Inc., Cary, NC). The Bonferroni correction was applied to adjust the α-level for the Mann–Whitney *U*-test for TLRs because multiple comparisons were made (with tests run for five molecules, the α-level was lowered to 0.01). The differences in the frequencies of the viral antibody-positive subjects between gingivitis and periodontitis were analyzed using the chi-squared test.

Results

TLR expression in gingivitis and periodontitis lesions

Real-time PCR investigation of the TLR profile in the tissues showed that the mRNAs for TLR2, -4, -5, -7, and -9 were all expressed in gingival tissues irrespective of their disease entities. However, the expression level of TLR7 was lower than that of TLR2, -4, and -5, and TLR9 had the lowest expression level of all the TLRs examined. The expression levels of TLR2, -4, -7, and -9 relative to GAPDH were significantly higher in periodontitis lesions than in gingivitis lesions ($P = 0.0046$ for TLR2, $P < 0.0001$ for TLR4, $P = 0.0075$ for TLR7 and $P < 0.0001$ for TLR9). Although there was no significant difference for TLR5 between gingivitis and periodontitis, the expression level was high and was comparable to that of TLR2 and -4 (Fig. 1).

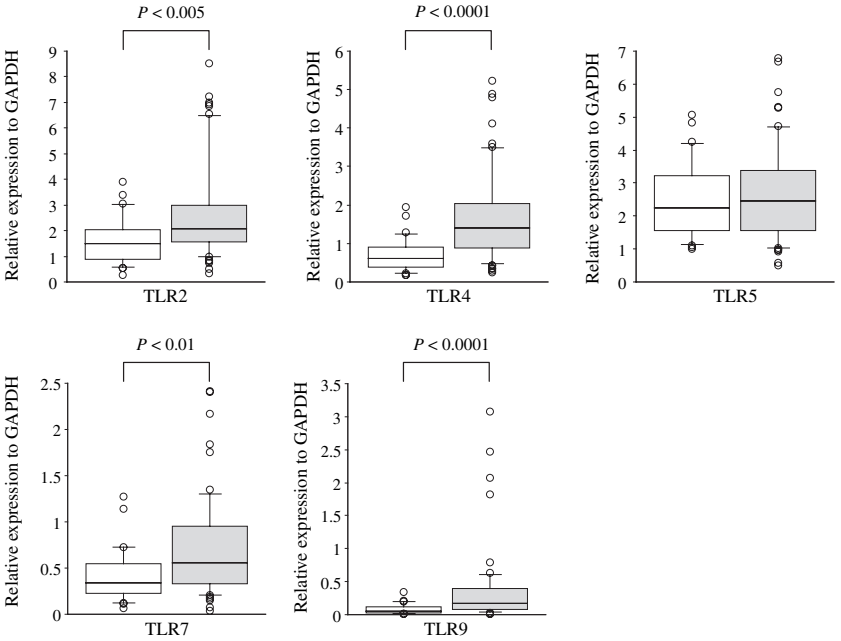

Fig. 1. Comparison of the relative gene expressions of TLRs between periodontitis ($n = 59$; solid boxes) and gingivitis ($n = 27$; open boxes) lesions. The relative quantity of mRNA was normalized to the relative quantity of GAPDH. The box plots show medians, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers. Outlying values are shown as open circles. Significant differences were observed for TLR2, -4, -7, and -9.

IFN-α1 expression in gingivitis and periodontitis

As shown in Fig. 2, IFN-α1 expression was significantly higher in the periodontitis lesions than in the gingivitis lesions ($P = 0.033$). Although one patient did exhibit a very high mRNA expression, the overall expression level was low.

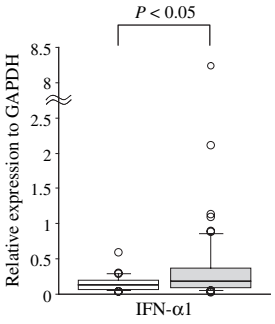

Fig. 2. Comparison of the relative gene expression of IFN-α1 between the periodontitis ($n = 59$; solid box) and gingivitis ($n = 27$; open box) lesions. The relative quantity of mRNA was normalized to the relative quantity of GAPDH. The box plots show the medians, 25th and 75th percentiles as boxes, and the 10th and 90th percentiles as whiskers. Outlying values are shown as open circles. There was a significant difference between gingivitis and periodontitis.

Immunohistological analysis of CD303 (BDCA-2)

CD303 (BDCA-2) is a characteristic marker for pDCs, which are robust producers of IFN-α. Figure 3 shows positive cells in the connective tissue subjacent to pocket epithelium in a periodontitis patient. CD303-positive cells were found in only four of the seven periodontitis specimens but were not found in the gingivitis specimens. Furthermore, as the cells were scant in number, if present at all, no quantitative analysis was carried out.

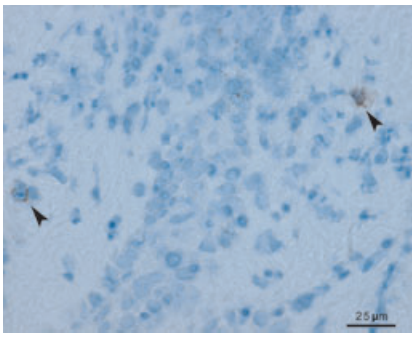

Fig. 3. Immunohistochemistry of CD303 (BDCA-2) positive cells (arrows) in the connective tissue subjacent to the pocket epithelium of a periodontitis specimen.

Viral antibody titers

Seropositivities for HSV, CMV, and EBV in both gingivitis and periodontitis are presented in Table 2. Frequencies of seropositive subjects were 65% for HSV, 85% for CMV, and 95% for EBV. Although the frequency of CMV seropositivity in periodontitis lesions tended to be higher than in gingivitis, the distribution of subjects who were antibody-positive to HSV and EBV did not differ between gingivitis and periodontitis (Table 2; anti-HSV IgG:

Table 2. Number of seropositive subjects for HSV, CMV, and EBV in each group

	Periodontitis ($n = 21$)	Gingivitis ($n = 19$)	*P*-value
HSV	16 (76.2)	10 (52.6)	0.12
CMV	20 (95.2)	14 (73.7)	0.057
EBV	20 (95.2)	18 (94.7)	0.94

Values are expressed as number (%).
Differences in the frequencies of antibody-positive subjects for each virus were analyzed by χ^2-test.

$\chi^2 = 2.43$, $P = 0.12$; anti-CMV IgG: $\chi^2 = 3.63$, $P = 0.057$; anti-EBV IgG: $\chi^2 = 0.01$, $P = 0.94$).

Discussion

This is the first evidence, based on the analysis of a relatively large number of samples, to show that TLR7 and -9 are expressed in the gingival lesions of gingivitis and periodontitis in addition to TLR2, -4, and -5. Furthermore, the expression of both TLR7 and TLR9 were significantly up-regulated in periodontitis lesions compared with gingivitis lesions, with TLR7 expression being higher than that of TLR9. Both TLR7 and TLR9 are intracellular endosomal receptors that allow pDCs to respond to single-strand RNA and DNA viruses, respectively (1), and are preferentially expressed by pDCs, whereas conventional DCs preferentially express TLR3 and -8, and low levels of TLR2 and -4 (7). Although TLR7 and -9 are also expressed in B cells, both pDCs and B cells express TLR9 more than TLR7 (6).

The viruses that have been reported to be implicated in periodontitis are herpesviruses, such as HSV types 1 and 2, EBV, and human CMV (23). Members of the herpesvirus family are composed of a double-stranded DNA genome contained within a nucleocaspid surrounded by a lipid envelope (22). DNA from herpesviruses is recognized by TLR9. Serum antibodies to HSV, CMV, and EBV were frequently detected in gingivitis as well as periodontitis and there was no significant difference in the distribution of antibody-positive subjects between the two groups. Therefore, the difference in gene expression levels of TLR9 may reflect local infections and inflammation rather than systemic infection.

Since TLR9 also recognizes bacterial DNA containing unmethylated CpG motifs, the results in this study are not necessarily indicative of viral involvement. In fact, it is reported that DNA from periodontopathic bacteria can stimulate TLR9-transfected HEK293 cells to produce proinflammatory cytokines (15). Although TLR9 is reported to be expressed in monocytes/macrophages, pDCs and B cells, the pDCs express higher levels of TLR9 than B cells do; both pDCs and B cells express TLR9 more than monocytes (6). The periodontitis lesion is characterized by predominant B-cell infiltration and only a few pDCs were observed in periodontitis lesions. Therefore, the higher expression of TLR9 in periodontitis compared with gingivitis could be the result of a difference in the cellular components between the two disease entities.

Quite unexpectedly, in the present study TLR5 expression was highest, and no difference was found between gingivitis and periodontitis. This finding is inconsistent with the data of Muthukuru et al., who showed that TLR5 expression was down-regulated in periodontitis tissues (14), but the precise reasons for this are not known. The TLR5 expressed in T cells, natural killer cells, and monocytes (6) is reported to recognize a protein ligand, bacterial flagellin (3). Bacterial flagellin is a structural protein that forms the main portion of flagellae, which promote bacterial chemotaxis and bacterial adhesion to and invasion of host tissues (11). Although most gram-negative as well as gram-positive bacteria express flagellae, there are only a few such reports on oral bacteria, particularly spirochetes (27). Although the entire genes coding flagellin of the oral spirochetes are not known, the *flaA-1* genes that encode the 37-kDa sheath protein from *Treponema vincentii* were detected in plaque samples from advanced periodontitis patients (18). *Treponema* species are frequently detected in periodontal health, and are increased in gingivitis and periodontitis (12). Therefore, high expression of TLR5 may reflect a high frequency of oral spirochetes in both gingivitis and periodontitis. The high expression of TLR5 in the lesions, the role of flagellin and TLR5 in the pathogenesis of gingivitis and periodontitis remain to be determined.

Accumulating evidence suggests that pDCs are the major producers of type I IFNs following infection with DNA viruses and following stimulation with certain types of CpG-oligodeoxynucleotides (CpG-ODNs) (types A and C) (10, 25). Whereas both types A and C contain a palindromic CpG sequence, type B CpG-ODNs have linear non-palindromic CpG-ODN sequences. In this respect, distinct immunostimulatory activities of these classes of CpG-ODN have been reported (26). CpG-ODNs of the B-class stimulate strong B-cell and natural killer cell activation as well as cytokine production. Only A-class CpG-ODNs stimulate pDCs to secrete IFN-α. The C-class CpG-ODNs strongly stimulate B-cell and natural killer cell activation along with IFN-α production. *Porphyromonas gingivalis* DNA has been reported to contain a palindromic CpG sequence and is capable of stimulating gingival fibroblasts (24) and epithelial cells (16) to produce proinflammatory cytokines. However, whether *P. gingivalis* DNA contains an A-class CpG-ODN or a C-class CpG-ODN has not been determined; moreover, there is no report on the effect of periodontopathic bacterial DNA on IFN-α production, a hallmark of the immune response to viral infection. Since the expression of TLR7 and -9 can be modulated by stimulation with CpG-ODN and possibly with viruses, it is hypothesized that higher TLR7 expression in gingivitis and periodontitis lesions may imply the activation of cells expressing these molecules.

IFN-α production is a characteristic feature of TLR9-mediated viral DNA signaling that utilizes the Myd88-dependent pathway resulting in the activation of IFN regulatory factor-7 (5). The expression of IFN-α was significantly higher in periodontitis compared with gingivitis, which is concordant with a previous study (17). However, the expression level was much lower compared to other cytokines (4). This could be attributable to the low number of pDCs in the lesion and it is unlikely that B cells in the periodontitis lesions are a source of IFN-α even if bacterial DNA does stimulate the cells. However, viral stimulation of pDCs via TLR9 in periodontitis remains to be determined.

In conclusion, quantitative assessment of TLR expression in different disease entities revealed the possibility that diverse microbial and possibly viral antigens could be involved in the pathogenic mechanisms of periodontal diseases. The ligands recognized by the various TLRs in periodontal lesions remain to be determined.

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References

1. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006: **124**: 783–801.
2. Gemmell E, McHugh GB, Grieco DA, Seymour GJ. Costimulatory molecules in human periodontal disease tissues. J Periodontal Res 2001: **36**: 92–100.
3. Hayashi F, Smith KD, Ozinsky A et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001: **410**: 1099–1103.
4. Honda T, Domon H, Okui T, Kajita K, Amanuma R, Yamazaki K. Balance of inflammatory response in stable gingivitis and progressive periodontitis lesions. Clin Exp Immunol 2006: **144**: 35–40.

5. Honda K, Ohba Y, Yanai H et al. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature 2005: **434**: 1035–1040.
6. Hornung V, Rothenfusser S, Britsch S et al. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002: **168**: 4531–4537.
7. Kadowaki N, Ho S, Antonenko S et al. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. J Exp Med 2001: **194**: 863–870.
8. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 2006: **117**: 979–987.
9. Kornman KS, Crane A, Wang HY et al. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol 1997: **24**: 72–77.
10. Krug A, Rothenfusser S, Hornung V et al. Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. Eur J Immunol 2001: **31**: 2154–2163.
11. Macnab RM. Genetics and biogenesis of bacterial flagella. Annu Rev Genet 1992: **26**: 131–158.
12. Moore WE, Moore LV. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 66–77.
13. Mori Y, Yoshimura A, Ukai T, Lien E, Espevik T, Hara Y. Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. Oral Microbiol Immunol 2003: **18**: 54–58.
14. Muthukuru M, Jotwani R, Cutler CW. Oral mucosal endotoxin tolerance induction in chronic periodontitis. Infect Immun 2005: **73**: 687–694.
15. Nonnenmacher C, Dalpke A, Zimmermann S, Flores-De-Jacoby L, Mutters R, Heeg K. DNA from periodontopathogenic bacteria is immunostimulatory for mouse and human immune cells. Infect Immun 2003: **71**: 850–856.
16. Platz J, Beisswenger C, Dalpke A et al. Microbial DNA induces a host defense reaction of human respiratory epithelial cells. J Immunol 2004: **173**: 1219–1223.
17. Prabhu A, Michalowicz BS, Mathur A. Detection of local and systemic cytokines in adult periodontitis. J Periodontol 1996: **67**: 515–522.
18. Sato T, Kuramitsu HK. Polymerase chain reaction for the detection of *fla*A-1 genes of oral spirochaetes in human advanced periodontal pockets. Arch Oral Biol 2000: **45**: 921–925.
19. Schei O, Waerhaug J, Lovdal A, Arno A. Alveolar bone loss as related to oral hygiene and age. J Periodontol 1959: **30**: 7–16.
20. Seymour GJ, Gemmell E, Reinhardt RA, Eastcott J, Taubman MA. Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. J Periodontal Res 1993: **28**: 478–486.
21. Seymour GJ, Powell RN, Cole KL et al. Experimental gingivitis in humans: a histochemical and immunological characterization of the lymphoid cell subpopulations. J Periodontal Res 1983: **18**: 375–385.
22. Slots J. Herpesviruses, the missing link between gingivitis and periodontitis? J Int Acad Periodontol 2004: **6**: 113–119.
23. Slots J. Herpesviruses in periodontal diseases. Periodontol 2000 2005: **38**: 33–62.
24. Takeshita A, Imai K, Hanazawa S. CpG motifs in *Porphyromonas gingivalis* DNA stimulate interleukin-6 expression in human gingival fibroblasts. Infect Immun 1999: **67**: 4340–4345.
25. Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. J Immunol 2001: **166**: 2372–2377.
26. Vollmer J, Weeratna R, Payette P et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur J Immunol 2004: **34**: 251–262.
27. Wyss C, Moter A, Choi BK et al. *Treponema putidum* sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. Int J Syst Evol Microbiol 2004: **54**: 1117–1122.
28. Yamazaki K, Ohsawa Y, Yoshie H. Elevated proportion of natural killer T cells in periodontitis lesions: a common feature of chronic inflammatory diseases. Am J Pathol 2001: **158**: 1391–1398.