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*J Dent Res* 89(2):186-191, 2010

## ABSTRACT

In bacterial infection, Nucleotide-binding Oligomerization Domain (NOD) 1 and NOD2 induce innate immune responses by recognizing fragments of the bacterial component peptidoglycan (PGN). To determine the roles of these receptors in detection of periodontal pathogens, we stimulated human embryonic kidney cells expressing NOD1 or NOD2 with heat-killed *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* or their soluble PGNs (sPGNs). All bacteria and their sPGNs could stimulate activation of NF- $\kappa$ B. However, there were differences in NOD1- and NOD2-stimulatory activities among the species of bacteria. *P. gingivalis* showed weaker NOD1- and NOD2-stimulatory activities than did other bacteria. These differences in activities were confirmed by production of interleukin-8 from oral epithelial cells stimulated with sPGNs. These findings indicate that both NOD1 and NOD2 might be involved in the recognition of periodontal pathogens, and that the weak NOD-stimulatory property of *P. gingivalis* might be helpful for survival in the periodontal pocket.

**KEY WORDS:** NOD, peptidoglycan, periodontal pathogens.

DOI: 10.1177/0022034509354843

Received September 25, 2008; Last revision June 16, 2009; Accepted August 26, 2009

# NOD1 and NOD2 Mediate Sensing of Periodontal Pathogens

## INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by periodontopathic bacteria and is characterized by inflammation of supportive tissue surrounding teeth. Gram-negative bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* have been reported to be associated with periodontitis (Slots *et al.*, 1980; Socransky *et al.*, 1998; Nishihara and Koseki, 2004).

Peptidoglycan (PGN) is a constituent of the cell membrane of bacteria and is one of the endotoxins that induce inflammatory responses such as the production of cytokines and inflammatory mediators (Stewart-Tull, 1980; Yoshimura *et al.*, 1999). PGN is a heteropolymer built of glycan strands cross-linked through peptide chains. The peptide chains are made of two kinds of short peptides, stem peptides that are linked to the glycan chain, and a cross-bridge that links the stem peptides. The glycan of PGN consists of repeating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), but there is a diversity in the composition and sequence of both the stem peptides and the cross-bridge in PGN in bacterial species (Schleifer and Kandler, 1972). A notable difference is that the third amino acid of the stem peptide is *meso*-diaminopimelic acid (*meso*-DAP) in PGN of Gram-negative bacteria, in contrast to L-Lys in that of Gram-positive bacteria. The number of amino acids of stem peptides ranges from 2 to 5 (mostly tetrapeptides).

Recently, Nucleotide-binding Oligomerization Domain (NOD) 1 and NOD2 were identified as cytosolic sensors for fragments of PGN. Nod1 recognizes gamma-D-glutamyl-*meso*-DAP, which is preferentially found in Gram-negative bacteria (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a), whereas Nod2 recognizes muramyl dipeptide (MDP), which is found in PGN of virtually all bacteria (Girardin *et al.*, 2003b). NOD1 and NOD2 have been reported to be involved in the recognition of infectious bacteria, the induction of immune responses, and the elimination of bacteria through activation of NF- $\kappa$ B (Viala *et al.*, 2004; Kim *et al.*, 2008).

NOD1 and NOD2 are expressed in oral and periodontal pocket epithelium (Sugawara *et al.*, 2006). Because of increased expression of NOD1 and NOD2 at the inflammatory gingival site, NOD1 and NOD2 are thought to play a role in the development of periodontitis. However, it has been reported that some periodontal pathogens, such as *P. gingivalis* and *F. nucleatum*, have L,L-DAP and *meso*-Lanthionine in PGN, respectively, instead of *meso*-DAP, which is essential for recognition by NOD1 (Vasstrand *et al.*, 1979; Barnard and Holt, 1985). Since NOD1- and NOD2-stimulatory activities of mucopeptides are greatly affected by the kind of third amino acid, as well as by the number of amino acids of stem peptides (Girardin *et al.*, 2003c; Magalhães *et al.*, 2005), it is not clear whether a

periodontal pathogen or its PGN is potent to stimulate NOD1 and NOD2. The aim of this study was to determine the roles of NOD1 and NOD2 in detection of periodontal pathogens.

## MATERIALS & METHODS

### Bacteria

The bacteria used in this study were *P. gingivalis* strains ATCC 33277, W83, TDC60, TDC117, TDC275, SU63, and GAI7802, *A. actinomycetemcomitans* Y4, *F. nucleatum* ATCC 10953, *Escherichia coli* MC4100, and *Aerococcus viridans* ATCC 10400. The cells were washed with sterilized distilled water and lyophilized.

### Peptidoglycan Purification

PGN purification was performed according to a previous report (de Jonge *et al.*, 1992). Purified Gram-negative and Gram-positive insoluble PGNs were re-suspended in PBS (2 mg/mL) and treated with mutanolysin (80 U/mL, Sigma, St. Louis, MO, USA) for 24 hrs at 37°C for solubilization. Mutanolysin cleaves the bond between MurNAc and GlcNAc. After digestion, each sample was boiled for 10 min and centrifuged, and the supernatant was used as soluble PGN (sPGN) stock solution.

### Measurement of NF- $\kappa$ B Activation

Human embryo kidney (HEK) 293T cells were kindly provided by D. Golenbock (University of Massachusetts Medical School). The cells were seeded into 96-well plates and incubated in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS). Then the cells were transfected for 24 hrs with pUNO/vector, pUNO/hNod1 or pUNO/hNod2 plasmid (Invivogen, San Diego, CA, USA), plus NF- $\kappa$ B-*firefly luciferase* and *renilla luciferase* reporter plasmid, with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activities were measured at 12 hrs after stimulation with bacterial samples. The activity of *firefly luciferase* was normalized to that of *renilla luciferase*. Each value was divided by the value for unstimulated cells that were transfected with pUNO/vector and represented as an NF- $\kappa$ B fold activation. A-iE-DAP (AnaSpec, San Jose, CA, USA) and MDP (Bachem AG, Bubendorf, Switzerland) were used as ligands for NOD1 and NOD2, respectively. *Drosophila* S2\* cells stably transfected with *dipterocin-luciferase* reporter plasmids were maintained in Schneider's *drosophila* medium (Gibco) supplemented with 10% FBS and 800  $\mu$ g/mL of G418 (Kaneko *et al.*, 2005). After differentiation into macrophage-like cells by incubation with 1  $\mu$ M 20-hydroxyecdysone for 24 hrs, the cells were stimulated with bacterial samples for 4 hrs, and luciferase activities were measured.

### Measurement of Interleukin-8 Production

Human oral epithelial cell line HSC-2 was provided by the RIKEN CELL BANK (Tsukuba, Japan), and the cells were

maintained in MEM (Gibco) supplemented with 10% FBS. After treatment with 1000 units/mL of interferon (IFN)- $\gamma$  (Sigma) for 3 days, the cells were stimulated with sPGN samples in a serum-starved condition (no FBS) to increase the uptake of bacterial samples into the cells. The concentration of FBS in the medium was increased to 10% by the addition of FBS at 2 hrs after stimulation, and the cells were further incubated for 22 hrs. The interleukin-8 (IL-8) levels in culture medium were measured by an enzyme-linked immunosorbent assay (DuoSet Kit, R&D Systems, Minneapolis, MN, USA).

### Statistical Analysis

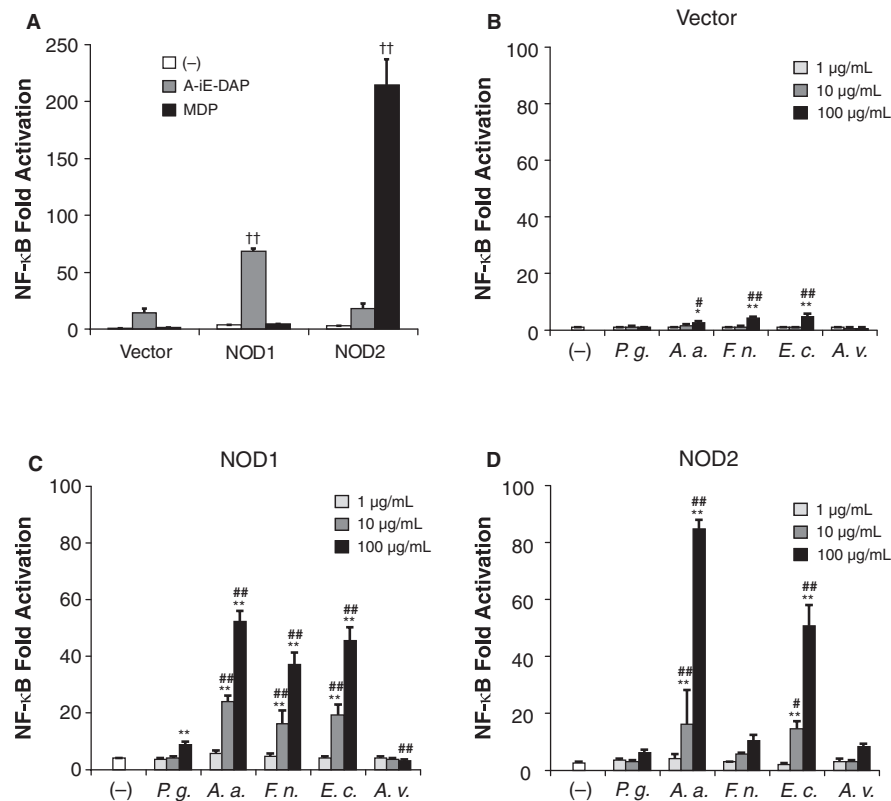
The data were analyzed with one-factor ANOVA and Fisher's PLSD test with StatView software (HULINKS, Tokyo, Japan). A probability level of  $P < 0.05$  was considered to be significant.

## RESULTS

We performed overexpression assays in HEK293T cells to explore functions of NODs, because these cells are highly transfectable and lack most of the endogenous Toll-like receptors (TLRs) that mediate bacterial recognition and activation of NF- $\kappa$ B. MDP could not stimulate NF- $\kappa$ B activation in HEK/vector cells, but A-iE-DAP weakly stimulated the cells due to the expression of endogenous NOD1 (Fig. 1A and data not shown). However, strong NF- $\kappa$ B activation was observed in HEK/NOD1 and HEK/NOD2 cells after stimulation with A-iE-DAP and MDP, respectively. These results indicated that expressed NOD1 and NOD2 functioned in the HEK293T cells, and that this overexpression system is useful for monitoring cell response specific for the activation of NOD1 or NOD2.

Next, the cells were stimulated with heat-killed periodontopathic bacteria. *A. actinomycetemcomitans*, *F. nucleatum*, and *E. coli* weakly, but significantly, induced activation of NF- $\kappa$ B in HEK/vector cells, while *P. gingivalis* and *A. viridans* failed to stimulate the cells (Fig. 1B). *A. actinomycetemcomitans*, *F. nucleatum*, and *E. coli* strongly stimulated HEK/NOD1 cells in a dose-dependent manner, and significant activations were observed at concentrations of 10-100  $\mu$ g/mL (Fig. 1C). *P. gingivalis* also stimulated HEK/NOD1 cells, but its activity was much weaker than the activities of the other 3 Gram-negative bacteria. Gram-positive *A. viridans* could not activate NOD1. In the case of HEK/NOD2 cells, *A. actinomycetemcomitans* and *E. coli* activated NOD2 in a dose-dependent manner, and those activities were statistically significant at concentrations of 10-100  $\mu$ g/mL. In contrast, *P. gingivalis*, *F. nucleatum*, and *A. viridans* could not activate NOD2 significantly, even at the maximum concentration of 100  $\mu$ g/mL (Fig. 1D).

We then stimulated HEK/NOD1 and HEK/NOD2 cells with sPGNs. Before the experiment, we confirmed the biological activities of purified sPGNs by measuring the expression of antimicrobial peptide gene *dipterocin* in *Drosophila*



**Figure 1.** Activation of NOD1 and NOD2 by stimulation with periodontopathic bacteria samples. HEK293T cells were transfected for 24 hrs with pUNO/vector, pUNO/hNod1 or pUNO/hNod2 plasmid, together with NF- $\kappa$ B-firefly luciferase and renilla luciferase reporter plasmid. After 24 hrs, the cells were stimulated with 1  $\mu$ g/mL of A-iE-DAP, or 1  $\mu$ g/mL of MDP or heat-killed bacteria. Luciferase activities were measured 12 hrs after stimulation and expressed as means  $\pm$  SD of triplicate experiments. The results are representative of 3 different experiments. *P.g.*, *P. gingivalis* ATCC 33277; *A.a.*, *A. actinomycetemcomitans*; *F.n.*, *F. nucleatum*; *E.c.*, *E. coli*; *A.v.*, *A. viridans*. †† $P$  < 0.01 vs. control vector; \* $P$  < 0.05, \*\* $P$  < 0.01 vs. (-); # $P$  < 0.05, ## $P$  < 0.01 vs. *P.g.*

S2\* cells by a luciferase reporter assay. *Diptericin* is induced only by Gram-negative bacterial PGN stimulation in S2\* cells (Kaneko *et al.*, 2004). All stock solutions of sPGNs obtained from Gram-negative bacteria showed almost the same activity against S2\* cells at the dilution of 1/1000 (Fig. 2A). *A. viridans* sPGN could not induce the expression of *diptericin*. As was found in experiments with heat-killed bacteria, HEK/NOD1 cells were strongly activated by stimulation with sPGNs from *A. actinomycetemcomitans*, *F. nucleatum*, and *E. coli* (Fig. 2B). The sPGN from *P. gingivalis* could activate NOD1 significantly, but its activity level was 10- to 100-times lower than those of sPGNs from other Gram-negative bacteria. The sPGN from *A. viridans* could not activate NOD1. In contrast, all sPGNs activated NOD2 (Fig. 2C). *A. actinomycetemcomitans* and *A. viridans* showed the strongest NOD2-stimulatory activities, followed by *F. nucleatum* and *E. coli*, and *P. gingivalis* showed the weakest activity. The sPGNs from *F. nucleatum* and *P. gingivalis* could activate NOD2 only at the dilution of 1/100.

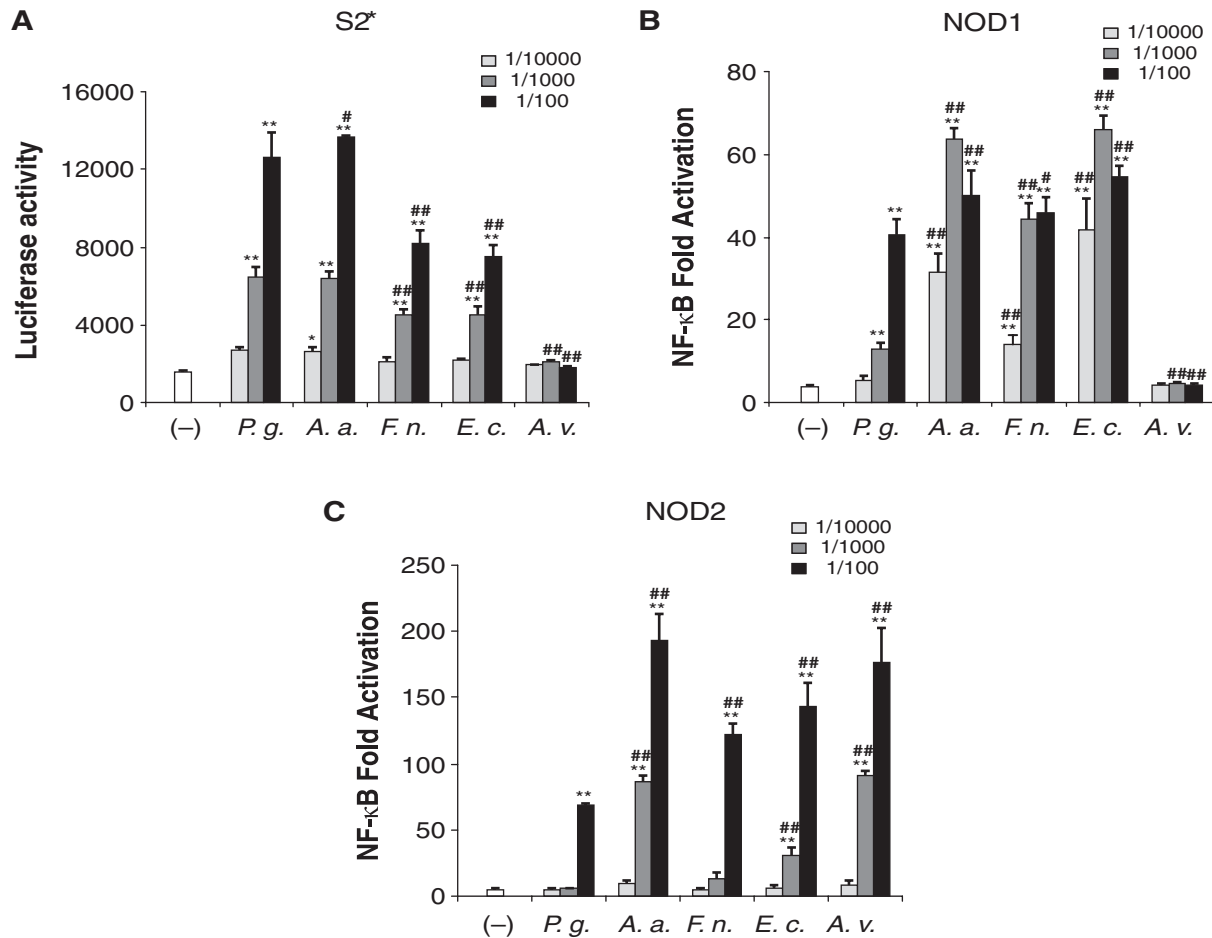
Both NOD1- and NOD2-stimulatory activities of *P. gingivalis* were weaker than those of other periodontal pathogens.

Therefore, we investigated whether these weak activities were species-specific for *P. gingivalis*. HEK/NOD1 and HEK/NOD2 cells were stimulated with heat-killed *P. gingivalis* strains W83, TDC60, TDC117, TDC275, SU63, and GAI7802, in addition to ATCC 33277. Although all *P. gingivalis* strains exhibited weaker NOD1-stimulatory activity than the activity of *E. coli*, strains W83 and GAI7802 significantly activated NOD1 at the concentration of 10  $\mu$ g/mL (Fig. 3A). Strains W83 and GAI7802 could activate NOD2 as strongly as *E. coli* could, but other strains did not show significant activity until the concentration was increased to 100  $\mu$ g/mL (Fig. 3B).

To further address the role of NODs in induction of the inflammatory cytokine IL-8 in periodontal cells, we stimulated HSC-2 cells with sPGN. Before the stimulation, HSC-2 cells were pre-treated with IFN- $\gamma$  for 3 days. IFN- $\gamma$  treatment significantly increased the response to A-iE-DAP (Fig. 4A). The sPGNs from *E. coli*, *A. actinomycetemcomitans*, and *F. nucleatum* stimulated HSC-2 cells to induce IL-8 more strongly than did those from *P. gingivalis* and *A. viridans* (Fig. 4B).

## DISCUSSION

PGN of *A. actinomycetemcomitans* has been reported to contain *meso*-DAP, which is typically observed in most Gram-negative bacteria such as *E. coli* (Schleifer and Kandler, 1972; Barnard and Holt, 1985). PGNs of *P. gingivalis* and *F. nucleatum* have been reported to contain L,L-DAP and *meso*-Lanthionine, respectively, instead of *meso*-DAP (Vasstrand *et al.*, 1979; Barnard and Holt, 1985). *A. viridans* has L-Lys-type PGN, which is typical for Gram-positive bacteria (Schleifer and Kandler, 1972). In the present study, there were differences in NOD1-stimulatory activities among the species of bacteria. *A. actinomycetemcomitans* showed the strongest activity, the same as that of *E. coli*, followed by *F. nucleatum*, and *P. gingivalis* showed the weakest activity among these Gram-negative bacteria. *A. viridans* did not activate NOD1. These results are consistent with the results of a previous study that used synthetic muramyl tripeptides (MTP) for stimulation of HEK cells expressing NOD1 (Girardin *et al.*, 2003c). In that study, it was shown that NOD1-stimulatory activity of L,L-DAP containing MTP (MTP<sub>L,L-DAP</sub>) was significantly weaker than that

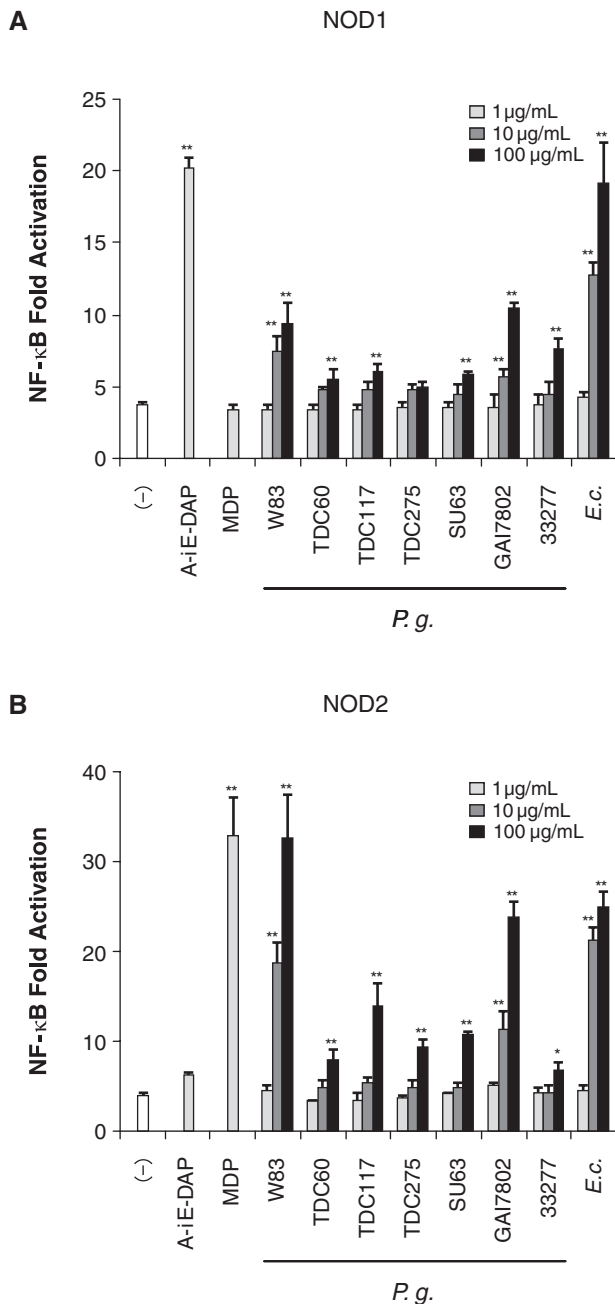


**Figure 2.** Activations of NOD1 and NOD2 by sPGNs from periodontopathic bacteria. **(A)** Dipteracin expression in *Drosophila* S2\* cells by stimulation with purified sPGNs from periodontopathic bacteria. S2\* cells stably transfected with dipteracin-luciferase reporter plasmid were seeded into 96-well plates and differentiated into macrophage-like cells by incubation with 1  $\mu$ M 20-hydroxyecdysone for 24 hrs. Then the cells were stimulated with sPGN samples, and the luciferase activities were measured 4 hrs after stimulation and expressed as means  $\pm$  SD of triplicate experiments. The results are representative of 3 different experiments. HEK293T cells were transfected for 24 hrs with pUNO/hNod1 **(B)** or pUNO/hNod2 plasmid **(C)** together with NF- $\kappa$ B-firefly luciferase and renilla luciferase reporter plasmid. After 24 hrs, the cells were stimulated with sPGN samples. Then luciferase activities were measured 12 hrs after stimulation and expressed as means  $\pm$  SD of triplicate experiments. The results are representative of 3 different experiments. *P.g.*, *P. gingivalis* ATCC 33277; *A.a.*, *A. actinomycetemcomitans*; *F.n.*, *F. nucleatum*; *E.c.*, *E. coli*; *A.v.*, *A. viridans*. \* $P < 0.05$ , \*\* $P < 0.01$  vs. (-); # $P < 0.05$ , ## $P < 0.01$  vs. *P.g.*

of MTP<sub>meso-DAP</sub> or MTP<sub>meso-Lanthionine</sub>. Thus, it is likely that the NOD1-stimulatory activities of periodontal pathogens are greatly influenced by the nature of the third amino acid of the stem peptide in their PGN.

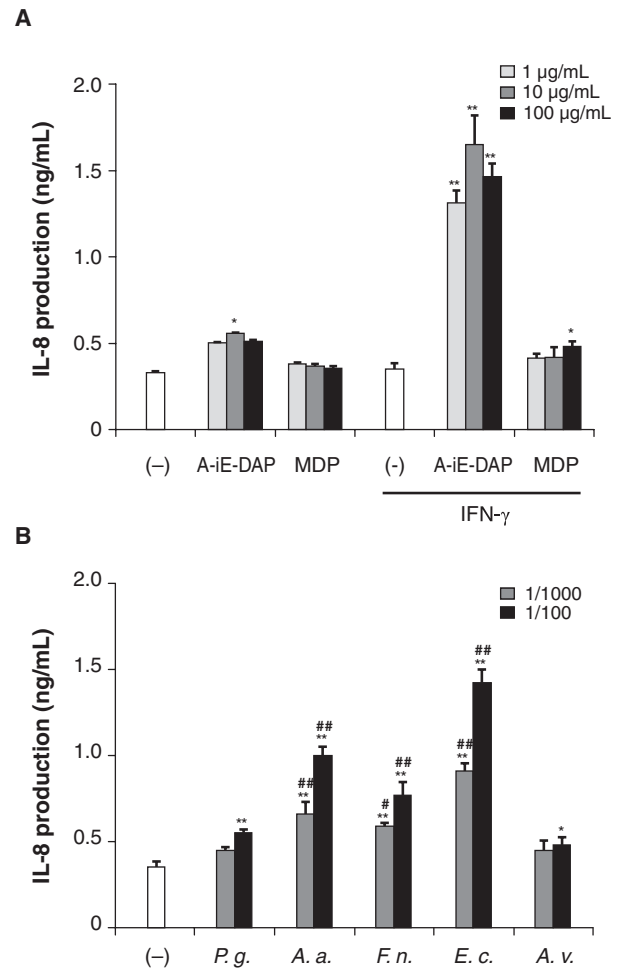
All PGNs contain an MDP structure in the mucopeptide; however, the NOD2-stimulatory activities differed among bacterial strains. *A. actinomycetemcomitans* showed the strongest activity, similar to that of *A. viridians*, followed by *E. coli* and *F. nucleatum*, and *P. gingivalis* showed the weakest activity. It has been reported that MTP<sub>L-Lys</sub> could activate HEK cells expressing NOD2 as strongly as could MDP, but MTP<sub>meso-DAP</sub>, MTP<sub>meso-Lanthionine</sub>, and MTP<sub>L-L-DAP</sub> could not activate these cells (Girardin *et al.*, 2003c). These results suggested that MDP, but not MTP, is critical for NOD2-stimulatory activities in PGNs of Gram-negative bacteria. A study on stem peptides in PGN of *E. coli*

demonstrated that most of the stem peptides were tripeptides or tetrapeptides, and that NOD2-stimulatory dipeptides accounted for only 2.1% of all stem peptides (Glauner *et al.*, 1988). The percentage of dipeptides in mucopeptides was reported to differ depending on the species or strains of bacteria (Antignac *et al.*, 2003). *E. coli* and *A. actinomycetemcomitans* might contain large amounts of dipeptides compared with *P. gingivalis*. The strong NOD2-stimulatory activities of *P. gingivalis* strains W83 and GAI7802 might be explained by this possibility. *P. gingivalis* has been classified into virulent and avirulent strains based on its ability to form necrotic abscesses in an animal model (Grenier and Mayrand, 1987). Inoculation of strain W83 in mice induced abscesses, secondary lesions, sepsis, and death of animals with higher frequency than did strain ATCC 33277 (Neiders *et al.*, 1989). In the present study, strain W83 exhibited stronger



**Figure 3.** Activations of NOD1 and NOD2 by strains of *P. gingivalis*. HEK293T cells were transfected for 24 hrs with pUNO/hNod1 (A) or pUNO/hNod2 plasmid (B) together with NF- $\kappa$ B-firefly luciferase and renilla luciferase reporter plasmid for 24 hrs, and the cells were stimulated with heat-killed *P. gingivalis* strains: W83, TDC60, TDC117, TDC275, SU63, GAI7802, and ATCC 33277. Luciferase activities were measured 12 hrs after stimulation and expressed as means  $\pm$  SD of triplicate experiments. The results are representative of 3 different experiments. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. (-).

NOD1- and NOD2-stimulatory activities than did strain ATCC 33277. Further experiments may be necessary to demonstrate the relationship between NODs-stimulatory activities and the heterogeneity of virulence among *P. gingivalis* strains.



**Figure 4.** Production of IL-8 in oral epithelial cells. (A) HSC-2 cells were stimulated with A-iE-DAP or MDP in a serum-starved condition after treatment with or without IFN- $\gamma$ . At 2 hrs after stimulation, the cell culture medium was supplemented with 10% FBS by the addition of FBS and further incubated for 22 hrs. The levels of IL-8 in culture medium were measured. (B) IFN- $\gamma$ -primed HSC-2 cells were stimulated with sPGNs in a serum-starved condition. At 2 hrs after stimulation, the cell culture medium was supplemented with 10% FBS by the addition of FBS and further incubated for 22 hrs. The levels of IL-8 in culture medium were measured. Data are expressed as means  $\pm$  SD of triplicate experiments. The results are representative of 3 different experiments. *P. g.*, *P. gingivalis* ATCC 33277; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*; *A. v.*, *A. viridans*. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. (-); # $P$  < 0.05, ## $P$  < 0.01 vs. *P. g.*

HSC-2 cells responded well to A-iE-DAP to produce IL-8 after IFN- $\gamma$  treatment. The sPGNs from *A. actinomycetemcomitans* and *E. coli* induced IL-8 production in HSC-2 cells. *P. gingivalis* consistently induced a weaker response, but it should be noted that the differences between *P. gingivalis* and the other bacteria in IL-8 expression were not as great as the differences in NF- $\kappa$ B activation.

We carried out muramidase treatment to obtain sPGNs. Both NOD1- and NOD2-stimulatory activities of insoluble PGNs were increased after treatment with muramidase (data not

shown). Lysozyme confers muramidase activity in saliva and plays a role in bacterial killing by destroying the bacterial cell wall. Therefore, lysozyme might facilitate recognition of bacteria by NOD1 and NOD2 in addition to bactericidal functions.

*P. gingivalis*, as well as *A. actinomycetemcomitans* and *F. nucleatum*, has been reported to invade host epithelial cells (Meyer *et al.*, 1991; Lamont *et al.*, 1995; Han *et al.*, 2000; Jandik *et al.*, 2008). A previous study revealed that intracellular recognition by NOD1 and NOD2 is critical for clearance of the intracellular bacterium *Listeria monocytogenes* (Kobayashi *et al.*, 2005). In the present study, we demonstrated that NOD1- and NOD2-stimulatory activities of *P. gingivalis* were weaker than those of other periodontal pathogens. In addition, *P. gingivalis* LPS has a unique structure of lipid A, different from that of enterobacterial LPS (Ogawa, 1993), and acts as an antagonist for human lipid A receptor TLR4 (Yoshimura *et al.*, 2002). These weak immunogenic properties of *P. gingivalis* might be one of the strategies of this bacterium for escape from innate immunity and survival in the periodontal pocket (Darveau *et al.*, 1998).

## ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by grants from the Takeda Science Foundation and the Sumitomo Foundation.

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