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Splenic B-cell activation in lipopolysaccharide-non-responsive C3H/HeJ mice by lipopolysaccharide of *Porphyromonas gingivalis*

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Abstract. *Porphyromonas gingivalis* 381 lipopolysaccharide (LPS) definitely exhibited mitogenic activity in purified B-cells, separated from spleens of LPS-responsive C3H/HeN mice and LPS-non-responsive C3H/HeJ mice by using a magnetic cell sorting system. The mitogenic activity induced by *P. gingivalis* LPS was incompletely inhibited by polymyxin B. *P. gingivalis* LPS also induced a higher production of interleukin-6 (IL-6) in splenic B-cells of C3H/HeN mice as compared with *Escherichia coli* LPS. Furthermore, *P. gingivalis* LPS, but not *E. coli* LPS, induced definite IL-6 production in C3H/HeJ mice. *P. gingivalis* LPS increased tyrosine, serine/threonine phosphorylation of proteins with various major induced bands in splenic B-cells of both C3H/HeN and C3H/HeJ mice. Additionally, radioiodinated *P. gingivalis* LPS, similarly to *E. coli* LPS, bound to a 73-kDa protein on C3H/HeJ as well as C3H/HeN B-cells. Thus *P. gingivalis* LPS may activate B-cells of C3H/HeJ as well as C3H/HeN mice via the LPS-specific binding protein on the cells.
Key words. *Porphyromonas gingivalis*; lipopolysaccharide (LPS); C3H/HeJ mice; B-cells; protein phosphorylation; cytokine production.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria, and it is known as a highly potent effector of immune responses for immunocompetent cells [1]. The monocytes/macrophages are important as primary targets for LPS, and the LPS-induced activation results in the production of proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1, IL-6, IL-8, and IL-10 [2]. LPS can also act as a potent mitogenic agent for B-cells, inducing antigen-independent polyclonal growth and maturation to immunoglobulin M (IgM) secretion [3].

Porphyromonas gingivalis, which is a Gram-negative, black-pigmented anaerobic rod, is suspected of being one of the major periodontopathic organisms in chronic periodontal diseases [4]. Antigen-specific antibodies to the surface components of *P. gingivalis* have been detected in the sera and crevicular fluid of patients with periodontal diseases [5], and antigen-specific antibody-secreting cells have been increased in the inflamed gingival tissues [6]. Among these components, *P. gingivalis* LPS has been presumed to be an important agent for the activation of host cells. The chemical and biological properties of *P. gingivalis* LPS molecule differ from those of the classical enterobacterial LPS, and its endotoxic ability is very low as compared with enterobacterial preparations [7].

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C3H/HeJ mice are hyporesponsive to the immunobiological effects of LPS from *Enterobacteriaceae* including *Escherichia coli*. *P. gingivalis* LPS [8], similarly to *Bacteroides fragilis* LPS [9, 10], exhibited mitogenic activities in spleen cells from LPS-non-responsive C3H/HeJ as well as LPS-responsive C3H/HeN mice. However, the mechanism of activation of immunocompetent cells including B-cells in C3H/HeJ mice by *P. gingivalis* LPS has remained obscure. The present study was designed to investigate LPS-binding and intracellular signal transduction of splenic B-cells of C3H/HeJ mice after stimulation with *P. gingivalis* LPS as compared with *E. coli* LPS.

Materials and methods

Animals. Two inbred mouse strains, C3H/HeN and C3H/HeJ mice (male, 8 weeks old) were obtained from Japan SLC Inc., Hamamatsu (Japan).

Bacteria and LPS preparation. *P. gingivalis* strain 381 was grown anaerobically in GAM-broth (Nissui, Tokyo, Japan) supplemented with hemin and menadione at 37 °C for 26 h [8]. Bacterial cells were collected by centrifugation, washed three times with pyrogen-free water and lyophilized. LPS were extracted from lyophilized cells by the hot phenol/water method [11], and the crude extract was purified by repeated ultracentrifugation (100,000 *g*, 3 h) followed by treatment with nuclease P1 (Yamasa Shoyu Co., Chosi, Japan) and finally lyophilized. *E. coli* O55:B5 LPS was

obtained from List Biological Laboratories, Campbell (CA, USA).

Cell preparation. Murine mononuclear cells (MNC) were isolated by Histopaque (Sigma Chemical Co., Chicago, IL, USA) separation from spleen cells of C3H/HeN and C3H/HeJ mice. B-cells were separated from splenic MNC by the magnetic cell sorting (MACS) system [12]. Briefly, MNC were incubated for 10 min at 4 °C with a fluorescein isothiocyanate (FITC)-labeled rat anti-mouse CD45R/B220 (Pharmingen, San Diego, CA, USA) for positive B-cell separation. After washing with pyrogen-free phosphate buffered saline (PBS; pH 7.5, Biken, Osaka, Japan), cells were incubated at 4 °C for 10 min with goat anti-rat immunoglobulin (IgG)-conjugated with superparamagnetic microbeads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). The labeled cells were applied to a MACS-steel-wool column within the magnetic field with the MACS-magnet (Miltenyi Biotech GmBH). A non-magnetic cell fraction was eluted from the column with 3 volumes of phosphate buffered saline (PBS). The labeled cells were eluted with PBS after the column had been demagnetized by removal from the magnetic field. After MACS separation, the efficiency of the separation was determined by flow cytometry using a Flow Cell Sorter (FCS-IX, Japan Spectroscopic Co., Tokyo, Japan). The eluted fraction contained 98.2 to 99.1% of CD45R/B220 positive cells. Throughout the MACS procedure, the live cells were found to comprise 92 to 97% in each cell fraction as determined by stain exclusion with 0.5% trypan blue (Wako Pure Chemical Industries, Osaka, Japan) in PBS.

Mitogenicity. Splenic MNC and B-cells (each 2.5×10^5 cells) of C3H/HeN and C3H/HeJ mice were cultured with the indicated dose of test specimen in 0.2 ml of RPMI 1640 (Biken) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA) (10% FBS-RPMI) in a 96-well flat-bottom microtiter plate (Falcon 3040; Becton Dickinson and Co., Lincoln Park, NJ, USA) for 48 h at 37 °C in 5% CO_2 and 95% air. Thirty-seven KBq of a methyl-^{3}H-thymidine ([^{3}H]thymidine; 185 MBq/nmol; Amersham International, Amersham, UK) was added during the final 16 h of culture to estimate DNA synthesis, and all cultures were harvested onto glass paper filter strips. Thymidine uptake was measured in a liquid scintillation counter (model 1215; LKB-Wallec, Turku, Finland).

The effect of polymyxin B on mitogenic responses in C3H/HeN and C3H/HeJ mice was examined as follows. Splenic MNC or B-cells (each 2.5×10^5 cells) were cultured at 37 °C for 48 h in 0.2 ml of 10% FBS-RPMI with the indicated dose of polymyxin B sulphate (5×10^5 U/vial; Wako Pure Chemical Industries, Osaka, Japan) and 1 μg of a test specimen, other than 5 μg of concanavalin A (Con A; Sigma Chemical Co., Chicago, IL, USA) [13]. Cultures were pulsed for the final 16 h of incubation with 37 KBq of [^{3}H]thymidine, and the thymidine incorporation was determined as described above.

IL-6 production assay. Splenic B-cells of C3H/HeN and C3H/HeJ mice were suspended at a cell density of 2.5×10^6 cells/ml in 10% FBS-RPMI. The indicated doses of test specimens were added to cell cultures and incubated at 37 °C for 48 h to obtain culture supernatants. In some experiments, B-cells were pretreated with the indicated doses of protein kinase inhibitors such as herbimycin A (Wako Pure Chemicals Ind., Osaka, Japan), H-7, H-8 and HA1004 (Seikagaku Kogyo Co., Tokyo, Japan) at 37 °C for 4 h, and then incubated with the LPS preparation at 10 μg/well. The culture supernatants were stored at −80 °C until the assay for IL-6 production. The production of IL-6 was measured in culture supernatants by means of a commercial ELISA kit system (Endogen Inc., Cambridge, MA, USA). The assay was performed according to the manufacture's instructions, and the data were determined by using a standard curve prepared for each assay.

Western blot analysis. MACS-separated B-cells (5×10^6 cells/ml) were pretreated with or without herbimycin A at 5 μg/ml at 37 °C for 4 h and then incubated with 50 μg/ml of LPS in 10% FBS-RPMI containing 20 mM HEPES at 37 °C for 30 min. After washing with 1 mM Na_3VO_4, the cells were lysed in 80 μl of lysis buffer (1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin and 1 μg/ml of aprotinin in 20 mM Tris-HCl buffer; pH 8.0) [14]. The cell lysates were incubated for 10 min at 4 °C, and centrifuged at 10,000 *g* at 4 °C for 15 min. Western blot analysis was carried out to determine the phosphorylated proteins after stimulation. Briefly, the lysates (40 μg protein/lane) were applied to a polyacrylamide slab gel (12.5%; Daiichi Pure Chemicals, Tokyo, Japan) and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) at 15 V for 35 min with a semi-dry transfer system [15]. The membranes were blocked by Tris buffered saline (TBS; pH 7.6) containing 5% bovine serum albumin (BSA; Sigma Chemical Co., Chicago, IL, USA) for 1 h at 25 °C. The membranes were then incubated at 25 °C for 2 h with 4G10 murine antiphosphotyrosine monoclonal antibody (mAb) (Upstate Biotechnology Inc., Lake Placid, NY, USA), PSR-45 murine antiphosphoserine mAb or PTR-8 murine antiphosphothreonine mAb (BioMakor Serving Science and Medicine, Rehovot, Israel). After washing with TBS containing 1% BSA, the sheets were incubated at 25 °C for 2 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA, USA). The sheets were then immersed in 50 mM Tris-HCl (pH 7.6) containing 0.3 mg/ml of 4-chloro-1-naphthol (Tokyo

Chemical Industry, Tokyo, Japan) and 0.03% H_2O_2 to develop the color, followed by washing and drying.

LPS binding assay. LPS-binding protein on splenic B-cells of C3H/HeN and C3H/HeJ mice was analysed by using a photo-cross-linking technique [16]. The photoactivatable cross-linker, sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3′-dithiopropionate (SASD), was obtained from Pierce Chemical Co. (Rockford, IL, USA). Photoactivatable iodinated LPS from *P. gingivalis* (^{125}I-ASD-*P. gingivalis* LPS) and *E. coli* O55:B5 (^{125}I-ASD-*E. coli* LPS) derivatives with SASD were prepared [17].

B-cells (2×10^6 cells) suspended in 20 µl of RPMI 1640 were incubated with 20 µl of ^{125}I-ASD-*P. gingivalis* or -*E. coli* LPS (containing approximately 1 µg of LPS, respectively) at 37 °C for 30 min. After incubation, the reaction mixture was irradiated with short-wavelength UV light (4-W maximum emission at 254 nm) for 10 min to induce covalent cross-linking of LPS to target cells. The treated cells were washed three times with RPMI 1640 to remove excess uncross-linked ^{125}I-ASD-LPS, lysed by using 50 µl of SDS sample buffer with 2-mercaptoethanol, and heated at 100 °C for 5 min. The lysates were applied to the polyacrylamide gradient gel (4 to 20%; Daiichi Pure Chemicals, Tokyo, Japan). Molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, CA, USA). The gels were stained with 0.2% Coomassie blue R250/50% methanol/12% acetic acid and destained with 20% ethanol/10% acetic acid. The gels were dried and autoradiographed to detect LPS-binding protein bands on target cells. Kodak X-Omat XK-1 film and DuPont cassettes with two DuPont Cronex Lightning Plus intensifying screens were used for the autoradiography. The film was exposed to the gel at −70 °C for 1 to 3 days.

Statistical analysis. The significant difference between the mean results for the different groups was determined by the Student's *t*-test.

Results

Mitogenic effect of *P. gingivalis* LPS and the inhibitory effect of polymyxin B on splenic B-cells from C3H/HeN and C3H/HeJ mice. *P. gingivalis* 381 LPS as compared with *E. coli* LPS exhibited almost comparably mitogenic activities in splenic MNC and purified B-cells (higher than 98%) from LPS-responder C3H/HeN mice (fig. 1). The mitogenic response of *P. gingivalis* LPS was also observed definitely in MNC and B-cells of LPS non-responder C3H/HeJ mice. The antibiotic polymyxin B has been shown to bind to the lipid A region of LPS [18]. We further investigated the inhibitory effect of polymyxin B on mitogenic activities of *P. gingivalis* LPS on splenic MNC and B-cells. The addition of polymyxin B to C3H/HeN MNC cultures markedly inhibited the thymidine incorporation by *E. coli* LPS but not by Con A (fig. 2), whereas polymyxin B scarcely inhibited *P. gingivalis* LPS-induced mitogenic responses. In splenic MNC and B-cells of C3H/HeJ mice, polymyxin B also could not completely inhibit the thymidine incorporation of MNC and B-cells stimulated with *P. gingivalis* LPS.

IL-6 production by splenic B-cells from C3H/HeN and C3H/HeJ mice after stimulation with *P. gingivalis* LPS. *P. gingivalis* LPS induced IL-6 production in C3H/HeN B-cells, and the activity was more powerful than that of *E. coli* LPS (fig. 3). *P. gingivalis* LPS also exhibited IL-6-producing activity on C3H/HeJ B-cells, whereas the stimulation of B-cells with *E. coli* LPS resulted in scarcely any activity.

As shown in figure 4, herbimycin A, an inhibitor of tyrosine kinases, markedly inhibited *P. gingivalis* LPS- and *E. coli* LPS-induced IL-6 production in a dose-dependent manner. Furthermore, H-7, a potent protein kinase C (PKC) inhibitor, at 100 µM completely exhibited the inhibitory effect. H-8 and HA1004, inhibitors of cyclic adenosine guanosine-3′,5′-monophosphate (AMP/GMP)-dependent protein kinases (PKA/PKG) with relatively high affinity, blocked IL-6 production, while higher concentrations of HA1004 were required as compared with those of H-7 and H-8 (fig. 5).

Phosphorylation of LPS-induced protein on splenic B-cells of C3H/HeN and C3H/HeJ mice. *P. gingivalis* LPS gradually increased tyrosine phosphorylation of protein in C3H/HeJ as well as C3H/HeN mice, with major induced bands of 38 kDa and 92 kDa 30 min after the stimulation, as detected by anti-phosphotyrosine immunoblotting (fig. 6). *E. coli* LPS stimulation of B-cells also induced the same two bands in C3H/HeN mice but not in C3H/HeJ mice. The pretreatment with herbimycin A at 5 µg/ml resulted in definite attenuation of the tyrosine phosphorylation of proteins at 30 min after the stimulation (data not shown). As shown in figure 6, stimulation of splenic B-cells with *P. gingivalis* LPS also led to an increase in the serine phosphorylation of protein, observed in a 118-kDa band at 30 min in both C3H/HeN and C3H/HeJ mice. Additionally, an increase in threonine phosphoprotein was detected in 74-kDa and 92-kDa bands at 30 min after stimulation with *P. gingivalis* LPS in both murine strains (fig. 6).

Determination of the LPS-binding protein binding to *P. gingivalis* LPS on splenic B-cells of C3H/HeN and C3H/HeJ mice. Since *P. gingivalis* LPS was found to stimulate B-cells of C3H/HeJ mice, the presence of LPS-binding protein to *P. gingivalis* LPS was examined on B-cells from LPS-responder and non-responder mice. The apparent molecular mass of the LPS-binding protein on B-cells of C3H/HeN mice binding to ^{125}I-ASD-*P. gingivalis* LPS was 73 kDa, similar to that of the binding protein for ^{125}I-ASD-*E. coli* LPS (fig. 7). On C3H/HeJ B-cells, the 73-kDa protein was also found to

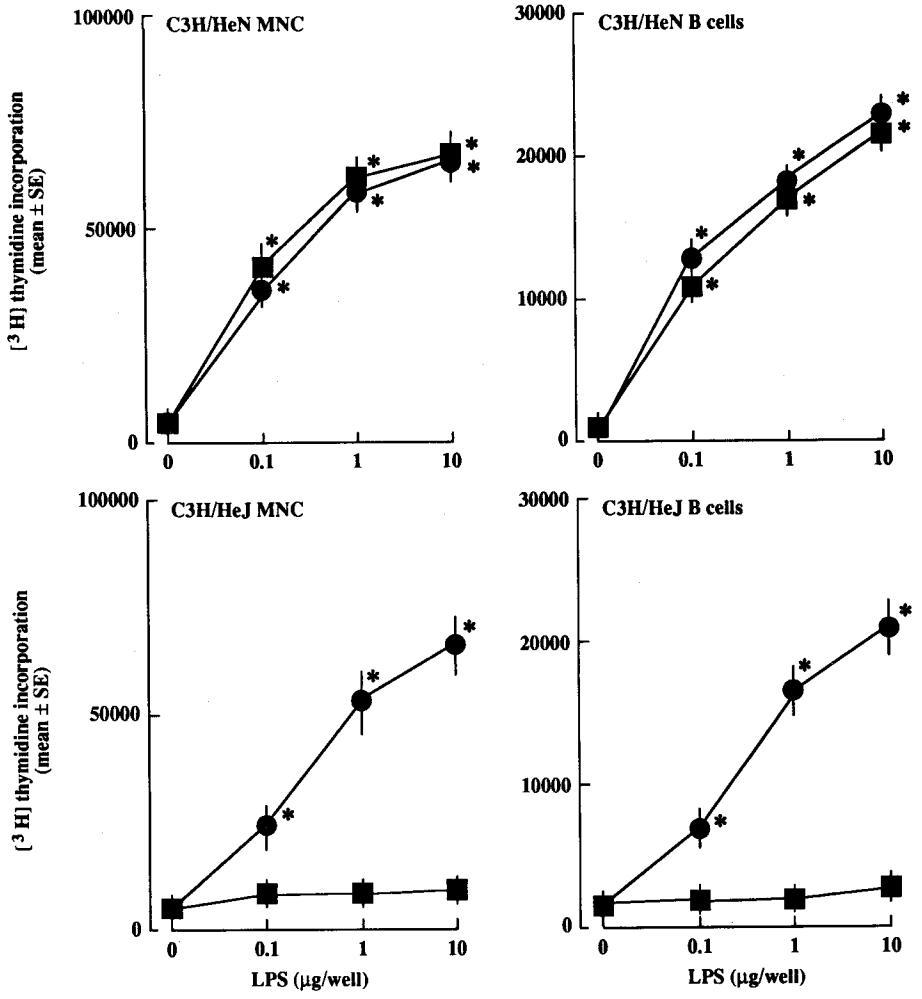

Figure 1. Mitogenic effect of *P. gingivalis* LPS on splenic MNC and B-cells of C3H/HeN and C3H/HeJ mice. Cells (2.5×10^5 cells) were cultured for 48 h with or without the indicated dose of test specimen as follows: *P. gingivalis* 381 LPS (●), *E. coli* (■). The results were expressed as counts per minute (cpm). Experiments were done at least three times, and representative results are presented. Each assay was done in triplicate; the data were expressed as the mean ± standard error of the mean (SEM) of results. The difference between the groups with and without the test specimen was significant by Student's t-test (*$p < 0.01$).

be an LPS-binding protein, binding to both *P. gingivalis* and *E. coli* LPSs. The binding of ^{125}I-ASD-*P. gingivalis* LPS to the 73-kDa protein was inhibited by both homologous *P. gingivalis* LPS and heterogeneous *E. coli* LPS (data not shown).

Discussion

P. gingivalis LPS definitely exhibited mitogenic activity in splenic B-cells of LPS-non-responder C3H/HeJ mice as well as LPS-responder C3H/HeJ mice (fig. 1). It was previously shown that *P. gingivalis* LPS was mitogenic for spleen cells from both C3H/HeN and C3H/HeJ mice [8]. Furthermore, it was reported that the unique and characteristic structure of the lipid A molecule of *P. gingivalis* was distinct from those of the lipid A from enterobacteria, and the other lipid As. This structural feature may be related to the induction of mitogenic responses in B-cells of C3H/HeJ as well as C3H/HeN mice. *B. fragilis* LPS was also a potent activator of spleen cells from both LPS-responder and non-responder mice [9, 10]. An increased thymidine incorporation was seen in splenic MNC containing B-cells, T-cells and macrophages as compared with purified B-cells stimulated with *P. gingivalis* LPS (fig. 1). Several studies have addressed the roles that macrophage- and/or T-cell-derived cytokines play in LPS-induced mitogenesis of B-cells [19, 20].

Polymyxin B, a polycationic antibiotic, is known as an inhibitor of LPS-mediated activation of immunocompetent cells, and it has been shown to bind the lipid A region of LPS [18]. The addition of polymyxin B resulted in incomplete inhibition of thymidine incorporation in *P. gingivalis* LPS-stimulated B-cells of C3H/HeN and C3H/HeJ mice (fig. 2). Two possible reasons are considered to explain the weak effect of polymyxin B on B-cell mitogenesis by *P. gingivalis* LPS: polymyxin B could not bind enough to the lipid A region on account

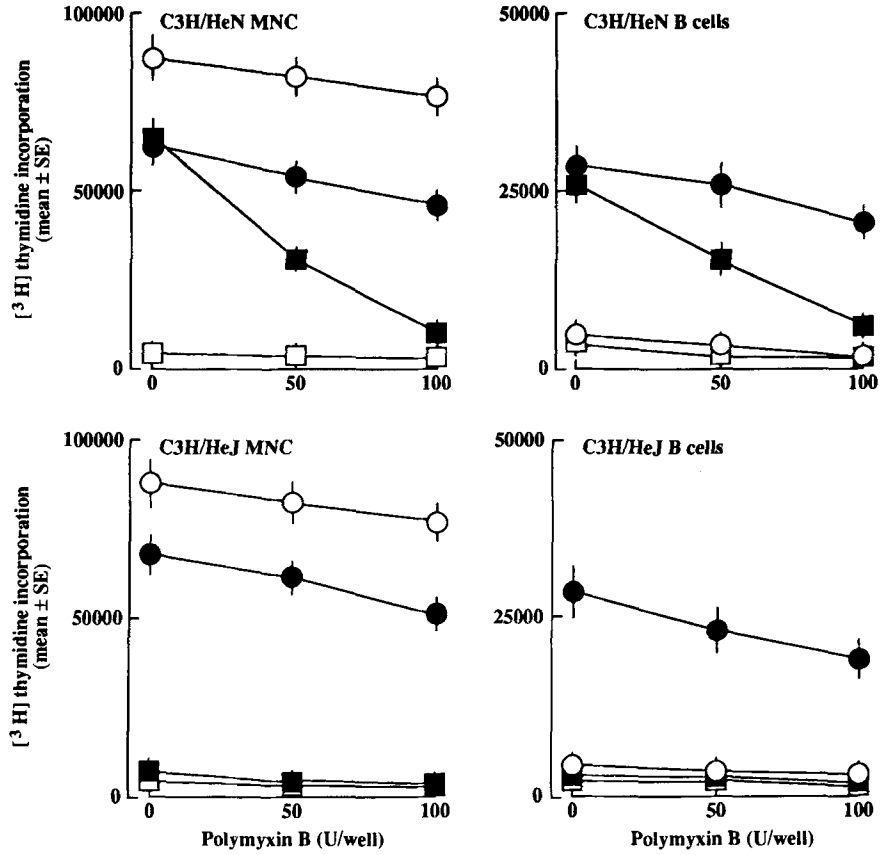

Figure 2. Effect of polymyxin B on mitogenic responses of splenic MNC and B-cells of C3H/HeN and C3H/HeJ mice to *P. gingivalis* LPS. Cells (2.5×10^5) were cultured for 48 h with 1 μg/well of *P. gingivalis* LPS (●), *E. coli* LPS (■), 5 μg/well of Con A (○) or RPMI 1640 medium alone (□) containing the indicated dose of polymyxin B. The results were expressed as counts per minute (cpm). Experiments were done at least three times, and representative results are presented. Each assay was done in triplicate; the data were expressed as mean ± SEM of results.

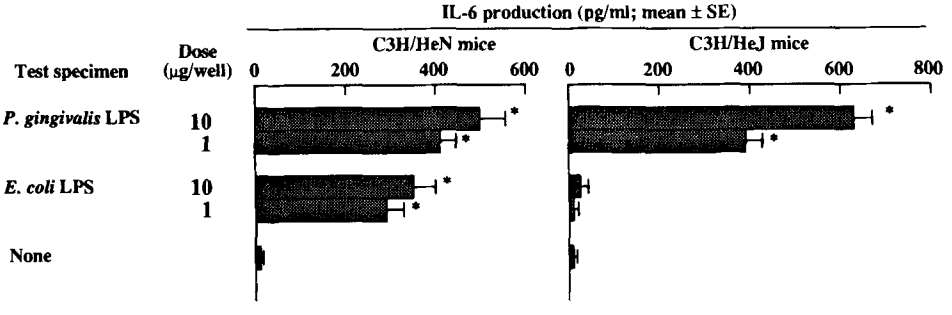

Figure 3. IL-6 production in splenic C3H/HeN and C3H/HeJ B-cell cultures stimulated with *P. gingivalis* LPS. Cells (2.5×10^5 cells) were cultured for 48 h with or without the indicated dose of test specimen. After incubation, supernatants were collected, and cytokine produced was determined by ELISA. Experiments were done at least three times, and representative results are presented. Each assay was done in triplicate; the data were expressed as the mean ± SEM of results. The difference between the groups with and without the test specimens was significant by Student's t-test ($^*p < 0.01$).

of steric hindrance to *P. gingivalis* LPS, and the presence of active components of *P. gingivalis* LPS other than the lipid A molecule, which might be associated with the B-cell activation. In the latter concept, *P. gingivalis* lipid A seems to be an active center of LPS because it was shown that the lipid A of *P. gingivalis* markedly induced the mitogenic responses in C3H/HeN and C3H/HeJ mice [8]. The steric feature of *P. gingivalis* LPS may abrogate the inhibitory effect of polymyxin B in B-cell mitogensis.

Stimulation of C3H/HeN B-cells with *P. gingivalis* LPS resulted in a higher level of IL-6 production as compared with that of *E. coli* LPS (fig. 3). Furthermore, *P. gingivalis* LPS induced a definite IL-6-producing activ-

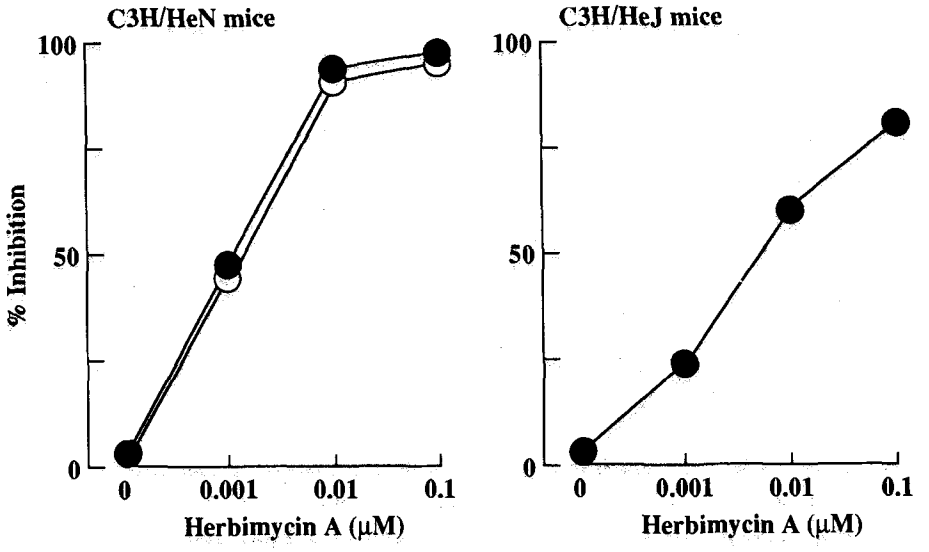

Figure 4. Effects of herbimycin A on IL-6 production induced by *P. gingivalis* LPS in splenic C3H/HeN and C3H/HeJ B-cells. Cells (2.5×10^5 cells) were pretreated with or without herbimycin A prior to stimulation with *P. gingivalis* LPS (●) or *E. coli* LPS (○) at 10 μg/well. After incubation, supernatants were collected, and the amount of IL-6 was determined by ELISA. Experiments were done at least three times, and representative results are presented. Each assay was done in triplicate, and the data were expressed as the mean ± SEM of results.

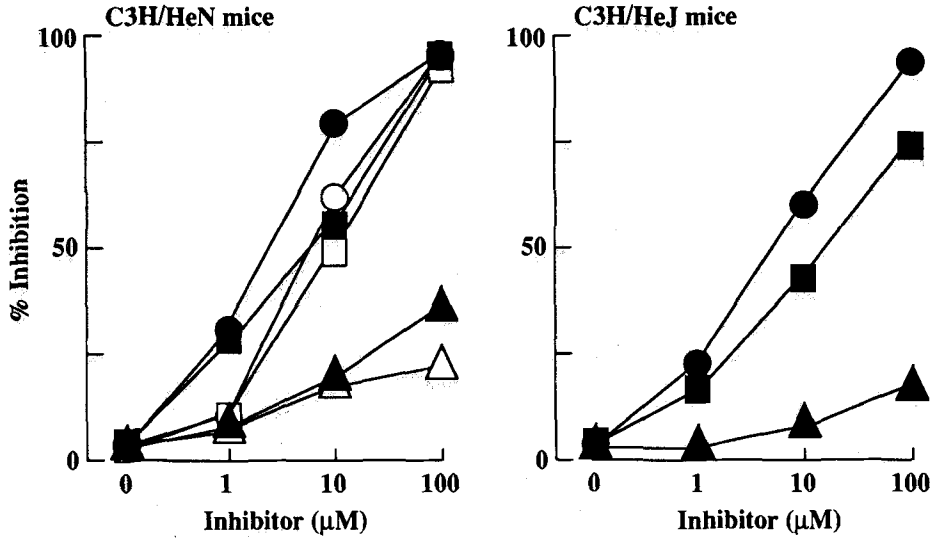

Figure 5. Effect of various protein kinase inhibitors on IL-6 production induced *P. gingivalis* LPS in splenic C3H/HeN and C3H/HeJ B-cells. Cells (2.5×10^5 cells) were pretreated with or without the following inhibitors, H-7 (●, ○), H-8 (■, □) or HA1004 (▲, △), prior to stimulation with *P. gingivalis* LPS (●, ■, ▲) or *E. coli* LPS (○, □, △) at 10 μg/well. After incubation, supernatants were collected, and the amount of IL-6 was determined by ELISA. Experiments were done at least three times, and representative results are presented. Each assay was done in triplicate, and the data were expressed as the mean ± SEM of results.

ity in C3H/HeJ B-cells, whereas *E. coli* LPS-induced IL-6 production was very low. Thus *P. gingivalis* LPS activated splenic B-cells of C3H/HeJ as well as those of C3H/HeN mice. IL-6 is an important polypeptide mediator in host immune responses and elicits a variety of immunobiological activities, including the terminal maturation of activated B-cells to antibody-secreting cells [21]. The inflamed gingival tissue of human periodontitis lesions is immunologically characterized as a B-cell-enriched region with a high frequency of plasma cells [22, 23]. An increased number of *P. gingivalis*-specific antibody-secreting cells in the inflamed gingival tissues [6] and high levels of antigen-specific antibodies in sera from periodontitis patients were demonstrated previously [5]. Thus it was suggested that *P. gingivalis* LPS may induce B-cell responses in the inflamed gingival tissues.

IL-6 production in B-cells following stimulation with *P. gingivalis* LPS was inhibited by herbimycin A, an inhibitor of tyrosine kinases, in a dose-dependent man-

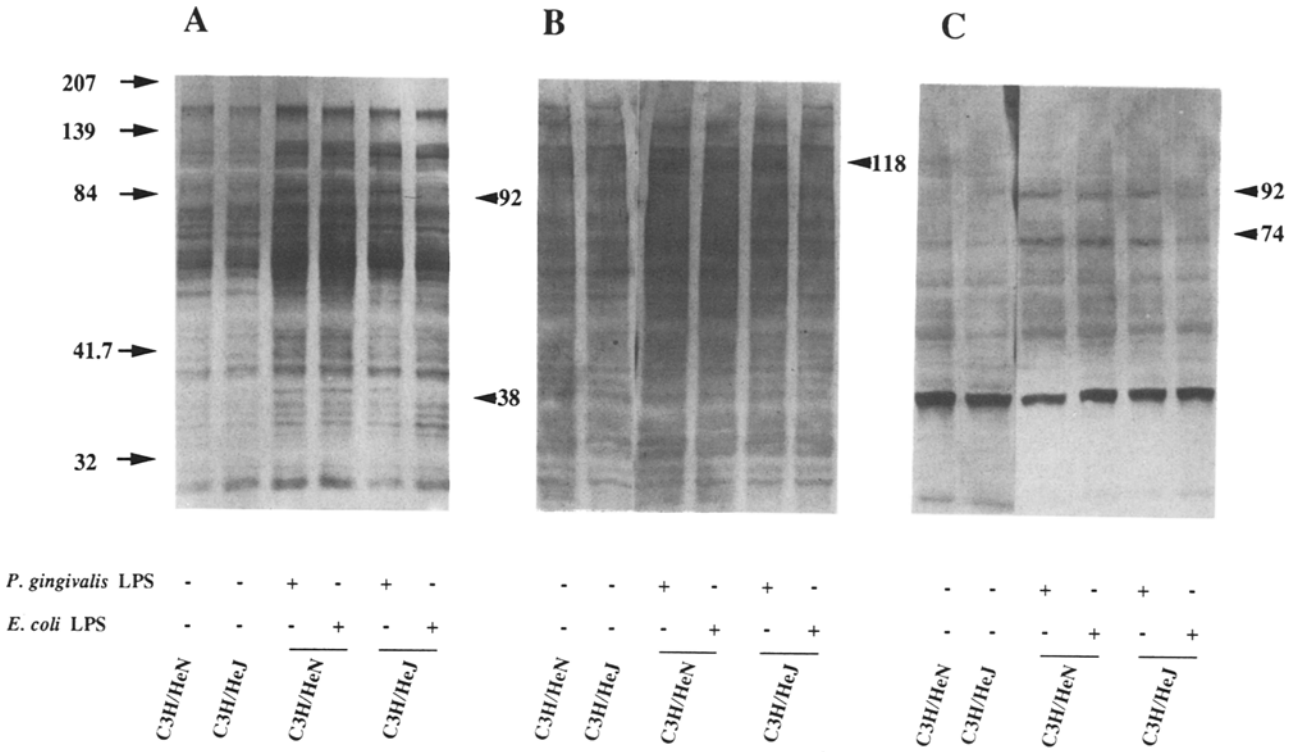

Figure 6. Effect of *P. gingivalis* LPS on tyrosine (*A*), serine (*B*) and threonine (*C*) phosphoprotein accumulation in C3H/HeN and C3H/HeJ splenic B-cells. Cells (5×10^6 cell/ml) were incubated at 37 °C for 30 min with or without the test specimen (50 μg/ml). After incubation, cell lysates were prepared, and anti-phosphoprotein immunoblot analysis was performed. Similar results were obtained in three separate experiments, and representative results are presented. The positions of the standard proteins (kDa on the left) and the phosphoproteins observed (kDa on the right) are indicated, respectively.

ner (fig. 4). Furthermore, both H-7, a potent inhibitor in blocking PKC [24], and H-8, which effectively inhibits PKA and PKG [25], exhibited the inhibitory effect (fig. 5). The inhibitory effect of HA1004, used as a control in selectively inhibiting PKC [25, 26], is weaker than that of H-7. The inhibition profiles suggest the participation of tyrosine kinase-, PKC- and PKA/PKG-mediated pathways in *P. gingivalis* LPS-induced IL-6 production.

In Western blot analysis, *P. gingivalis* LPS gradually increased tyrosine phosphorylation of protein in splenic B-cells of both C3H/HeN and C3H/HeJ mice, with major induced bands of 38 kDa and 92 kDa, 30 min after the initiation of the LPS stimulation (fig. 6). *E. coli* LPS also induced the same two bands in B-cells of C3H/HeN mice but not C3H/HeJ mice. Herbimycin A at 5 μg/ml caused a definite attenuation of the tyrosine phosphorylation of protein at 30 min after the stimulation (data not shown). Han et al. [27] indicated that LPS of *Salmonella minnesota* Re595 (Re595 LPS) induced tyrosine phosphorylation of the 38-kDa protein in murine pre-B-cell line 70Z/3 transfected with DNA encoding human CD14. LeGrand and Thieringer [28] also showed that stimulation of Re595 LPS and *E. coli*-type synthetic lipid A (compound 506) resulted in the induction of 41-kDa tyrosine phosphoprotein in 70Z/3 cells expressing CD14. Furthermore, Weinstein et al. [29] previously reported that human monocytes/macrophages responsive to compound 506 induced the major phosphoproteins of 41 kDa and 42 kDa, and that herbimycin A inhibited the induced phosphorylation response. Additionally, several tyrosine phosphoproteins, varying from 50 kDa to 110 kDa, induced by *E. coli* O26:B6 LPS, have been detected in human monocytes, and these protein bands were attenuated by treatment of herbimycin A [30]. Recently, we have observed that *P. gingivalis* lipid A induced tyrosine phosphoproteins with major bands of 31 kDa to 51 kDa in human peripheral blood MNC (T. Ogawa and H. Uchida, unpubl. results). These results indicated that *P. gingivalis* LPS may induce immunobiological activities through tyrosine protein kinases (TPK).

Stimulation of splenic B-cells in C3H/HeN mice with *P. gingivalis* LPS or *E. coli* LPS induced an increase in serine phosphorylation of a 118-kDa protein at 30 min, as shown in figure 6. Furthermore, *P. gingivalis* LPS induced serine phosphorylation of a 118-kDa protein in C3H/HeJ B-cells. The phosphorylation of serine residues indicates serine kinase activation after B-cell stimulation with the LPS. Shinomiya et al. [31] previously indicated serine phosphorylation of the 65-kDa protein in C3H/HeN, but not C3H/HeJ macrophages

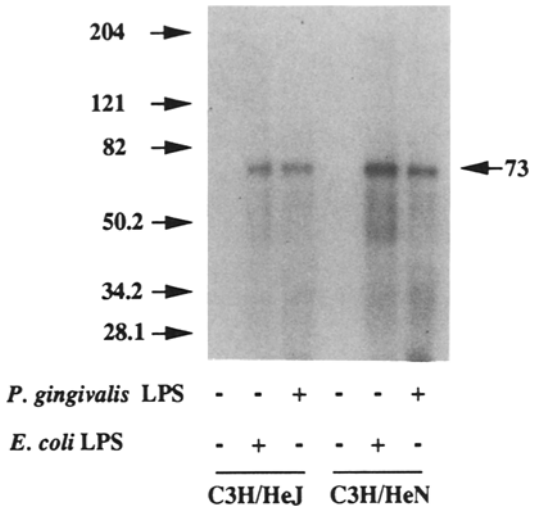

Figure 7. Determination of binding of ^{125}I-ASD-LPS from *P. gingivalis* and *E. coli* to splenic B-cells from C3H/HeN and C3H/HeJ mice. LPS-binding protein on splenic B-cells was analyzed by using a photocrosslinking technique as described in 'Materials and methods'. B-cells (2×10^6 cells) were incubated with ^{125}I-ASD-LPS at 37 °C for 30 min. After incubation, the reaction mixture was irradiated with short-wavelength UV light and lysed with SDS sample buffer. The lysates were electrophoresed on polyacrylamide gradient gel. The gels were dried and autoradiographed. The positions of the standard proteins (kDa on the left) and the binding proteins observed (kDa on the right) are indicated, respectively.

after stimulation with *Salmonella typhimurium* LPS or *S. minnesota* LPS, and they suggesed that the unresponsiveness of C3H/HeJ cells may be dependent on defects in second messenger systems. On the other hand, our results seem to show that structural features of LPS, especially bioactive lipid A, may be involved in determining the activation of the second messengers.

Threonine phosphorylation of two proteins of 74 kDa and 92 kDa was detected after *P. gingivalis* LPS stimulation in C3H/HeN and C3H/HeJ B-cells (fig. 6). Serine/threonine kinases contain PKC, PKA and Ca^{2+}/calmoduline dependent kinase (Ca^{2+}/CaM kinase) [32]. It was previously reported that the lipid moieties of *E. coli* LPS activated PKC [33], and that *P. gingivalis* lipid A and compound 506 activated serine/thronine kinases in human peripheral blood MNC (Ogawa, T. and Uchida, H., unpubl. results). *P. gingivalis* LPS induced tyrosine and threonine phosphorylation of the 92-kDa protein (fig. 6). It is undefined whether the same protein of 92 kDa is phosphorylated as with *E. coli* LPS. Further investigations are in progress to purify and characterize several phosphorylated proteins, including the 92-kDa proteins. The findings indicate that *P. gingivalis* LPS induces stimulation of both C3H/HeN and C3H/HeJ B-cells through already defined above TPK- and serine/threonine kinase-dependent pathways.

The 73-kDa protein band in B-cell fractions of C3H/HeN and C3H/HeJ mice was found to be the binding site for ^{125}I-ASD-*P. gingivalis* LPS, and the same band, bound to ^{125}I-ASD-*E. coli* LPS, was also observed in both murine strains (fig. 7). Thus these results suggested that B-cells of C3H/HeN and C3H/HeJ mice have a common binding site for LPS, and B-cell activation followed by signal transduction may be dependent on the structural features of LPS. The 73-kDa protein binding to *E. coli* LPS was characterized on murine spleen cells [16], and the binding of ^{125}I-ASD-LPS to the protein was inhibited by heterogeneous and homogeneous LPSs and lipid As [34]. The 73-kDa protein has also been identified on the surface of a wide variety of LPS-responsive cell types in mice and humans [34]. A hamster monoclonal antibody specific for the LPS-binding protein could activate macrophages [35]. Dziarski [36] previously demonstrated that bacterial cell wall peptidoglycan and LPS, particularly the $(GlcNAc)_2$ part of lipid A, bind to the same 70-kDa protein on murine spleen cells. Lei et al. [37] previously reported the presence of the 38-kDa LPS-binding protein on murine spleen cells, and the inhibition assay suggested that the 38-kDa protein specifically binds to the inner core oligosaccharide determinants within the LPS molecule. Furthermore, other investigators identified the 18-kDa, 25-kDa and 28-kDa LPS-binding proteins [38] and the 40-kDa protein [39] on murine pre-B-cell line 70Z/3. The results, taken together, indicate that *P. gingivalis* LPS, similarly to *E. coli* LPS, may activate splenic B-cells of C3H/HeJ as well as C3H/HeN mice through the LPS-binding proteins on the cells.

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