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Effect of low dose *Actinobacillus actinomycetemcomitans* lipopolysaccharide pretreatment on cytokine production by human whole blood

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Background and objective: Periodontal disease is known to influence the systemic condition in various ways, and the bacteria and their products, such as lipopolysaccharides (LPS), may spread from periodontal lesions via the systemic circulation to affect distant organs. The level of LPS in plasma from such patients is reported to be very low, and this low level of LPS is suspected to have priming or desensitizing effect. Thus, we investigated the effects of low dose LPS pretreatment on LPS-dependent cytokine production by whole blood cells *ex vivo*.

Methods: Blood samples obtained from seven systemically and periodontally healthy individuals were pretreated with or without 5 pg/ml *Actinobacillus actinomycetemcomitans* LPS, followed by further stimulation with 1 ng/ml *A. actinomycetemcomitans* LPS. The concentrations of interleukin-1 beta (IL-1β), IL-6, IL-10 and tumor necrosis factor-alpha (TNF-α) in the culture supernatants were then determined using enzyme-linked immunosorbent assay (ELISA). In addition, intracytoplasmic cytokine staining of whole blood cells was performed for flow cytometry.

Results: Pretreatment with 5 pg/ml *A. actinomycetemcomitans* LPS significantly enhanced the production of IL-1β and IL-6 from whole blood when further induced by 1 ng/ml LPS (1.72 times higher for IL-1β, 2.18 times higher for IL-6 than without pretreatment). The pretreatment did not enhance the production of either TNF-α or IL-10. Intracytoplasmic staining showed that the monocyte fraction was primarily involved in producing IL-1β and IL-6. Flow cytometric analysis revealed that pretreatment increased the number of IL-1β and IL-6 producing cells as well as mean fluorescence intensity of the stained cells.

Conclusion: A low dose of bloodstream LPS found in periodontitis patients appears to be sufficient to prime monocytes, and may be capable of affecting the systemic responses of immune and inflammatory cells.

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Recent studies in periodontal medicine suggest a mild to moderate association between periodontal disease and certain systemic disorders such as cardiovascular diseases, diabetes and preterm birth (1–3). Systemic circulation of bacteria or their products such as lipopolysaccharides (LPS) from periodontal lesions has been proposed to explain the link between oral and systemic disease. Severe, chronic periodontal disease provides a rich source of subgingival microbes and their host-response products and may exert its effect on systemic conditions over a long period (4–6).

A wealth of evidence shows that significant doses of viable gram-negative bacteria can invade periodontal connective tissues (7) and subsequently enter circulation during various periodontal treatment procedures. Scaling and root planing, tooth extraction, tooth brushing and flossing, and even mastication, can result in bacteremia (8–12).

Bacterial endotoxins or LPS are unique glycolipids present in the outer cell membrane of all gram-negative bacteria. LPS is known to initiate the pathophysiological changes that often accompany gram-negative bacterial infections in humans, including hypotensive shock, disseminated intravascular coagulation, and metabolic abnormalities. The amount of detectable LPS in sulcular exudates (13) as well as in gingival tissue (14) increases with the degree of clinical inflammation of the periodontal tissue. However, only few studies have detected LPS in the serum or plasma of periodontitis patients. Recently, even gentle mastication was shown to induce the release of bacterial LPS of oral origin into the bloodstream, and the levels of LPS positively correlated to the severity of periodontitis (15). The mean plasma level of LPS in such patients is very low and is considered to have no direct effect on host cells in terms of cytokine release *in vitro* or *ex vivo* (16). The significance of low-level endotoxemia originating from periodontal lesions is uncertain.

A substimulatory dose of LPS is well documented to have a priming or desensitization effect on various mammalian cells. Murine macrophages pretreated with a substimulatory dose of LPS manifested significant enhanced production of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) upon subsequent stimulation with LPS (17, 18). This phenomenon has been termed LPS-induced priming. On the other hand, rabbit macrophages stimulated *in vitro* and *in vivo* with LPS became refractory to subsequent LPS stimulation, as determined by assessing the cells for secondary TNF-α release (19). The above phenomenon is known as LPS-induced tolerance or desensitization. Therefore, the low levels of endotoxin in blood may modulate activation of host cells including whole blood cells and endothelial cells, epithelial cells, and smooth muscle cells (20).

The purpose of the present study was to determine whether detectable plasma levels of LPS in periodontitis patients have priming or desensitizing effect on whole blood cells *ex vivo*, in terms of production of pro-inflammatory cytokines.

Material and methods

Subjects

Seven periodontally healthy subjects (three females and four males, mean age 27.4 ± 3.0 years, range 24–33 years) were enrolled in the study. Informed consent was obtained from all subjects and the Ethics Committee of the Tokyo Medical and Dental University approved the research protocol.

Whole-blood stimulation

Blood samples were collected in LPS-free tubes containing 100 IU of lithium heparin, an anticoagulant. The heparinized blood was immediately diluted 1 : 1 with pyrogen-free culture medium, RPMI 1640 (Sigma Chemicals Co., St. Louis, MO, USA) and was initially stimulated for 3 h at 37°C in 5% CO_2 with or without 5 pg/ml *Actinobacillus actinomycetemcomitans* LPS. After the pretreatment, secondary stimulation was performed with 1 ng/ml of the same LPS. The blood samples were then incubated for 3, 6, 12 and 20 h intervals. The culture supernatants were collected after centrifugation and stored at −80°C until use.

Preparation of LPS

A. actinomycetemcomitans (serotype b), which was isolated from a juvenile periodontitis patient, was grown anaerobically (80% N_2, 10% H_2 and 10% CO_2) using GAM broth (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with yeast. Bacteria were harvested by centrifugation, washed three times with distilled water and lyophilized. LPS was isolated from the bacteria using the hot phenol–water method (21).

Cytokine assays

Cytokine levels were measured using an enzyme-linked immunosorbent assay (ELISA) for secreted IL-1β, IL-6, TNF-α and IL-10 (ELISA Kit, BioSource International, Camarillo, CA, USA). The assay was performed according to the manufacturer's instructions. Data were determined using a standard curve prepared for each assay.

The ratio of the amount of cytokine production with pretreatment to that of without pretreatment (R) was calculated for each patient.

Flow cytometry and intracytoplasmic staining

Flow cytometry and intracytoplasmic staining were performed to determine which cells produce IL-1β and IL-6 using Cytofix/Cytoperm Plus™ kit (PharMingen, San Diego, CA, USA). Whole blood was diluted 1 : 1 with RPMI 1640, and then incubated with 5 pg/ml *A. actinomycetemcomitans* LPS for 3 h at 37°C, after which Golgistop™ (PharMingen), containing a protein transport inhibitor, was added to promote intracytoplasmic cytokine accumulation. The diluted whole blood was then incubated for another 6 h at 37°C. Red blood cells were hemolyzed for 10 min with FACS® lysing solution (Becton Dickinson, Mountain View, CA, USA). After centrifugation at 500 *g* for 5 min, the supernatant was removed. Cells were then washed once

with RPMI 1640. After washing, cells were fixed and permeabilized with Cytofix/Cytoperm solution at room temperature. After 20 min of incubation, cells were centrifuged at 500 *g* and the supernatant was removed. Cells were incubated for another 20 min and then washed with Perm/Wash™ solution (PharMingen). Fixed/permeabilized cells were reacted with Perm/Wash solution containing anti-IL-1β or anti-IL-6-phycoerytherin (R&D Systems Inc. Minneapolis, MN, USA) in the dark. After 30 min of incubation, cells were washed with Perm/Wash solution and resuspended in phosphate-buffered saline containing 2% paraformaldehyde.

Cytometric analysis was performed using a FACScan® flow cytometer (Becton Dickinson). The data analysis was performed using the CellQuest® program (Becton Dickinson).

LPS contamination test

All serum, materials and reagents used in the experiment were checked for contamination with endotoxin by an endotoxin-specific chromogenic test (Endspec® ES test TE; Seikagaku Kogyo, Tokyo, Japan). No contamination above the detection limit (5 pg/ml) was found.

Statistical analysis

Student's *t*-test was used for intergroup comparison. Wilcoxon signed-rank test was used for comparison of the ratio (*R*). *p*-values less than 0.05 were considered significant.

Results

Cytokine production from whole blood cells pretreated with a low dose (5 pg/ml) of *A. actinomycetemcomitans* LPS

Heparinized whole blood was incubated with 1 ng/ml *A. actinomycetemcomitans* LPS. Levels of IL-1β, IL-6 IL-10 and TNF-α in the supernatant increased with time and reached peak levels at 12 h after stimulation (Figs 1A–D). Cytokine release from whole blood was minimal after incubation without stimulation. Heparinized whole blood was preincubated for 3 h with or without 5 pg/ml *A. actinomycetemcomitans* LPS and stimulated further with 1 ng/ml *A. actinomycetemcomitans* LPS. The presence of 5 pg/ml *A. actinomycetemcomitans* LPS during primary culture resulted in increased production of IL-1β and IL-6 by whole blood cells on secondary stimulation, compared to non-LPS pretreated whole blood cells after 12 h (Figs 2A and B). After 6 h, significant enhancement was observed in IL-1β and IL-6 production by the pretreatment (IL-1β; 1.72 times higher, $p < 0.05$: IL-6; 2.18 times higher, $p < 0.05$) (Table 1). The pretreatment with 5 pg/ml *A. actinomycetemcomitans* LPS slightly enhanced LPS-induced production of TNF-α and IL-10 from whole blood cells, but not to significant levels (Figs 2C and D).

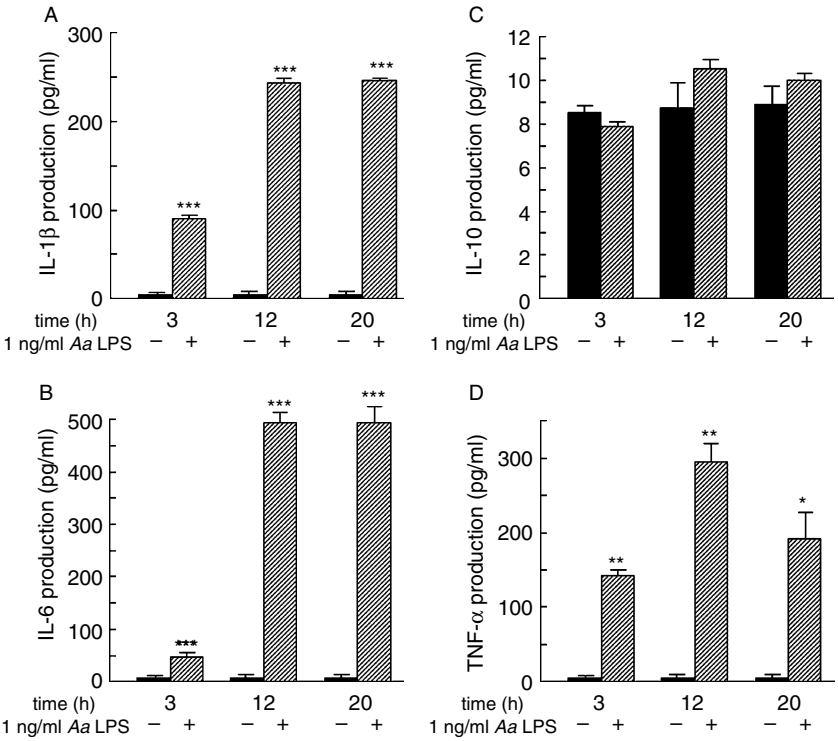

Fig. 1. The time course of (A) interleukin-1β (IL-1β) production, (B) IL-6 production, (C) IL-10 production and (D) tumor necrosis factor-α (TNF-α) production from whole blood induced by 1 ng/ml *Actinobacillus actinomycetemcomitans* lipopolysaccharide (*Aa* LPS).

Detection of cells producing IL-1β and IL-6 using flow cytometry

Intracytoplasmic staining of whole blood cells was performed to identify the cells that produced IL-1β and IL-6 on LPS-stimulation. Flow cytometry analysis revealed that the monocyte fraction in whole blood was mostly responsible for IL-1β as well as IL-6 productions (Fig. 3). The polymorphonuclear leukocyte and lymphocyte fractions contained few stained cells.

Pretreatment with 5 pg/ml *A. actinomycetemcomitans* LPS significantly increased the percentage of IL-1β and IL-6 producing cells as well as the mean fluorescence intensity of IL-1β and IL-6 stained cells after secondary stimulation with 1 ng/ml *A. actinomycetemcomitans* LPS, compared to non-LPS-pretreated whole blood cells after 6 h (Tables 2 and 3).

Discussion

In the present study, we used a low dose (5 pg/ml) of LPS for pretreatment stimulation, which was based on data from a previous study. To date, only one study has reported the plasma level of LPS in patients with periodontitis. In this study, Geerts *et al.* examined the influence of gentle mastication on the occurrence of endotoxemia in

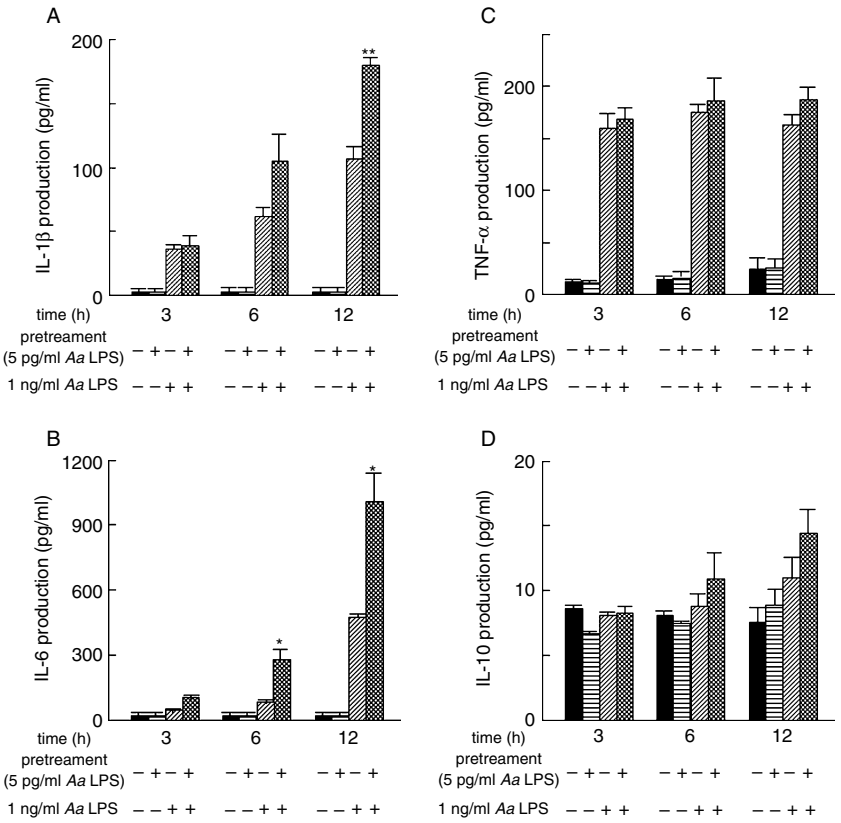

Fig. 2. Effect of low dose of *A. actinomycetemcomitans* lipopolysaccharide (*Aa* LPS) pretreatment on (A) interleukin-1β (IL-1β) production, (B) IL-6 production, (C) tumor necrosis factor-α production and (D) IL-10 production from whole blood induced by 1 ng/ml LPS.
*Significantly different between with and without pretreatment ($p < 0.05$).
**Significantly different between with and without pretreatment ($p < 0.01$).

Table 1. Effects of low dose *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) pretreatment on cytokine production from whole blood

	R
IL-1β	1.72 ± 0.14*
IL-6	2.18 ± 0.42*
IL-10	1.35 ± 0.32
TNF-α	1.15 ± 0.12

Pretreatment with low dose *A. actinomycetemcomitans* LPS significantly enhanced the production of interleukin-1β (IL-1β) and IL-6 from whole blood induced by 1 ng/ml of the same LPS. No significant differences were observed in tumor necrosis factor-α (TNF-α) and IL-10 production.
Data represent mean ± SD. Wilcoxon signed-rank test was used for statistical analysis ($n = 7$).
R, ratio of the amount of cytokine production with pretreatment to that of without pretreatment.
*Significantly different between with and without pretreatment ($p < 0.05$).

patients with or without periodontitis (15). They used the *limulus* amoebocyte lysate assay, which has a lower detection limit of 5 pg/ml, for determination of LPS level in plasma. They found that 7% of the plasma samples from periodontitis patients were LPS positive with a mean value of 1.05 pg/ml, before gentle mastication. Five to 10 min after mastication, the incidence of endotoxemia increased to 29% and the mean value increased to 3.72 pg/ml. The dose we used for pretreatment in this study is comparable to physiological plasma LPS levels detected in periodontitis patients.

In the present study, we used an *ex vivo* whole-blood system (22). Use of the whole-blood model reduces the confounding factors that may be associated with the isolation procedures, such as activation of isolated cells and the risk of contamination with biological stimulants such as LPS. Moreover, whole blood represents a more physiologic environment for examining cytokine production in response to LPS, since the cellular interactions are preserved and the presence of various plasma components (lipoproteins, sCD14, lipopolysaccharide-binding protein, bactericidal/permeability-increasing protein, albumin and transferrin) is maintained.

Most endotoxic effects are known to be mediated through the activation of host immune and inflammatory cells, particularly mononuclear phagocytes, and these cells are thought to play a central role in endotoxic reactions through the production of numerous bioactive mediators, including IL-1β, IL-6 and TNF-α. Polymorphonuclear leukocytes (PMNs) are also reported to produce these cytokines in response to an LPS challenge. Hsi and Remick examined the cellular source of IL-1β in an *A. actinomycetemcomitans* LPS-stimulated human whole-blood *ex vivo* model using immunohistochemistry (23). They showed that monocytes were the major producers of IL-1β, and PMNs produce very low levels of IL-1β, whereas lymphocytes produce no detectable IL-1β. These results are consistent with our findings. In our study, the flow cytometry analysis revealed that most IL-1β and IL-6 positive cells were in the monocyte fraction, with a few observed in the PMNs fraction, and none in the lymphocyte fraction.

Pretreatment of macrophages/monocytes with substimulatory doses of LPS has been reported to initiate tolerance to subsequent LPS-dependent response (24). On the other hand, several investigators have described a priming effect due to pretreatment with LPS (17, 18). In the present study, we have shown that pretreatment of whole blood cells with 5 pg/ml *A. actinomycetemcomitans* LPS causes enhancement of the subsequent

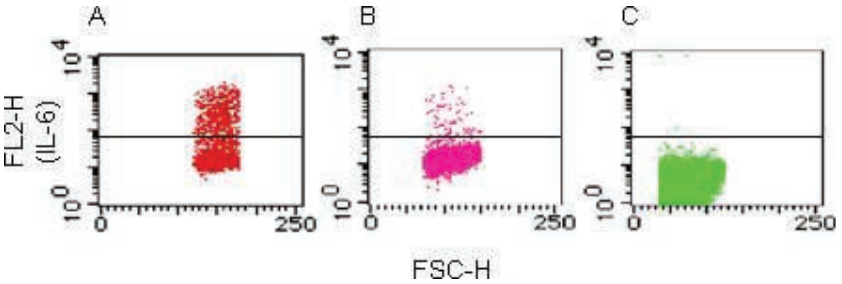

Fig. 3. Intracytoplasmic interleukin-6 (IL-6) staining patterns of different fractions in gated whole blood, 6 h after stimulation with 1 ng/ml *Actinobacillus actinomycetemcomitans* lipopolysaccharide. Most IL-6 producing cells were present in the monocyte traction (A). A few IL-6 producing cells were evident in the polymorphonuclear leukocytes fraction (B), with no positive cells in the lymphocyte fraction (C).

Table 2. Effects of low dose *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) pretreatment on intracytoplasmic interleukin-1β (IL-1β) staining of whole white blood cells

	Percentage of IL-1 producing cells	Mean fluorescence intensity
Without pretreatment	8.70 ± 0.44**	1080.50 ± 82.38*
With pretreatment	11.00 ± 0.40**	1242.27 ± 12.53*

Pretreatment with low dose *A. actinomycetemcomitans* LPS significantly increased the percentage of IL-1β producing cells in whole white blood cells as well as the mean fluorescence intensity 6 h after secondary stimulation with 1 ng/ml *A. actinomycetemcomitans* LPS.
Data represent mean ± SE. Student's *t*-test was used for inter-group comparison.
*Significantly different between with and without pretreatment ($p < 0.01$).
**Significantly different between with and without pretreatment ($p < 0.001$).

Table 3. Effects of low dose *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) pretreatment on intracytoplasmic interleukin-6 (IL-6) staining of whole white blood cells

	Percentage of IL-6 producing cells	Mean fluorescence intensity
Without pretreatment	10.78 ± 0.83*	84.70 ± 3.19*
With pretreatment	12.62 ± 0.66*	161.33 ± 10.55*

Pretreatment with low dose *A. actinomycetemcomitans* LPS significantly increased the percentage of IL-6 producing cells in whole white blood cells as well as the mean fluorescence intensity 6 h after secondary stimulation with 1 ng/ml *A. actinomycetemcomitans* LPS.
*Significantly different between with and without pretreatment ($p < 0.001$).

LPS-dependent IL-1β and IL-6 responses. Morrison and coworkers (17, 18) examined the effect of LPS pretreatment on LPS-dependent TNF-α and IL-6 production in murine macrophages, using various substimulatory concentrations (up to 0.1 ng/ml). They demonstrated that the pretreatment with substimulatory dose of *Escherichia coli* LPS significantly enhanced the LPS-dependent production of TNF-α and IL-6. These findings are partially consistent with our results reported here showing significant enhancement of IL-6 and slight enhancement of TNF-α. Shimauchi *et al.* (25) examined the effects of pretreatment with low concentration of *Porphyromonas gingivalis* LPS or *E. coli* LPS on IL-6 and IL-8 production from human peripheral blood monocytes by subsequent stimulation with a high concentration (1 μg/ml) of the same LPS. They demonstrated that the pretreatment with *P. gingivalis* LPS resulted in a significant decrease in IL-6, but not IL-8 production. In contrast, the equivalent pretreatment with *E. coli* LPS enhanced production of both IL-6 and IL-8 after re-stimulation. Our data are consistent with findings reported by Shimauchi and coworkers in terms of enhanced IL-6 production upon pretreatment with *E. coli* LPS, but not with *P. gingivalis* LPS. Chemical, structural and biological differences between *P. gingivalis* LPS and classical enterobacterial LPS, such as *E. coli* LPS and *A. actinomycetemcomitans* LPS may explain this difference in responses (26). Martin and coworkers (27) reported differential induction of endotoxin tolerance by *P. gingivalis* LPS and *E. coli* LPS. They pretreated THP-1 cells, human macrophage cell line, with LPS at concentrations from 1 to 10,000 ng/ml for 24 h, re-stimulated them with the same LPS concentration. They demonstrated that *E. coli* LPS pretreatment induced endotoxin tolerance in terms of IL-1β, IL-6 and TNF-α upon secondary stimulation. In contrast, pretreatment of THP-1 cells with *P. gingivalis* LPS resulted in a mitigation of IL-1β, but not IL-6 and TNF-α production, upon subsequent exposure to *P. gingivalis* LPS. Primary or secondary stimulation with > 100 ng/ml *P. gingivalis* LPS resulted in comparable levels of IL-6 and TNF-α, whereas stimulation of THP-1cells with > 1 μg/ml *P. gingivalis* LPS induced a significant enhancement in IL-6 and TNF-α levels upon secondary exposure. The data reported by Martin and coworkers in terms of cytokine productions were not consistent with our results or findings reported by Shimauchi *et al.* (25). The lack of consensus in the results obtained regarding effects of LPS pretreatment suggested that cellular activation and tolerance induction are closely associated events. The dose of LPS used for pretreatment, the chemical and structural properties of LPS and the culture condition, including pretreatment period, presence of serum components and isolation procedures, may affect the ability of monocytes to acquire endotoxin tolerance or cellular activation upon subsequent LPS stimulation.

Several reports have suggested the existence of primed monocytes in periodontitis patients (28, 29). McFarlane *et al.* (29) described that isolated peripheral blood monocytes from periodontitis subjects had increased spontaneous and LPS-stimulated (*A. actinomycetemcomitans* LPS; 5 μg/ml) IL-1β production compared to non-periodontitis subjects. The LPS responsiveness of monocyte in periodontitis patient must be further investigated.

Elevated levels of systemic inflammatory markers, such as C-reactive protein (CRP), CD14, IL-6, TNF-α and circulating bacterial endotoxin have been reported in patients with cardiovascular disease and diabetes (30–32). Therefore, chronic infections are considered as potential risk factors for these diseases. Kiechl *et al.* (33). examined the association between presence of chronic infections including periodontal disease and risk of carotid atherosclerosis in a random population of 826 subjects prospectively. They demonstrated that chronic infections amplified the risk of atherosclerosis development in carotid arteries. Furthermore, they demonstrated that the circulating bacterial endotoxin level in subjects with chronic infections was elevated approximately 1.5 times more than those without chronic infection, and suggested that the elevated but physiological level of LPS in bloodstream of subjects with chronic infections may be predictive of an increased risk for atherosclerosis.

Elevation of systemic inflammatory markers such as white blood cell counts, fibrinogen, CRP and sCD14 have been reported in patients with periodontal disease (34) and the levels of these markers decrease after periodontal treatment (35–37), suggesting that the infected periodontium could serve as a source of circulating endotoxin. Therefore, the low-grade endotoxemia derived from chronic periodontal infections may contribute to immune activation and be associated with the development of systemic diseases.

In summary, this study has demonstrated that the physiological plasma LPS level detected in periodontitis patients can prime monocytes and can affect responses of immune and inflammatory cells systemically. The effects of low-grade endotoxemia derived from chronic periodontal infections must be further investigated using a longitudinal intervention study with a larger sample size.

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