Histopathological Study of the Effect of Periodontopathic Bacterial Lipopolysaccharide on Changes in Rat Periodontal Tissue after Penetration through the Gingival Sulcus

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Abstract: This study was performed to clarify the in vivo damage to periodontal tissue induced by lipopolysaccharide (LPS) from Actinobacillus actinomycetemcomitans Y 4 and Porphyromonas gingivalis 381, which penetrate the junctional epithelium. Each LPS solution (5 mg/ml) was continuously dropped into the gingival sulcus of the lower first molar of a Lewis rat for 30 min every 24 h. The animals were sacrificed 30 min after the 1st, 3rd, 7th, 10th and 15th administration and their mandibles were examined histopathologically and histomorphometrically \( (n=25) \) in each group. A gradual increase in inflammatory findings was observed in both LPS groups. However, A. actinomycetemcomitans LPS induced more marked inflammation and degeneration of periodontal tissue. Loss of connective tissue attachment and rete peg elongation were significantly greater in rats administered with A. actinomycetemcomitans LPS compared to those administered with P. gingivalis LPS. Degeneration of junctional epithelium and subepithelial connective tissue was found at the 1st and 3rd administrations. At the 7th and 10th administrations, epithelial cells and collagen fibers were detached from root surfaces. Rete pegs showed deep invasion toward alveolar bone and epithelial detachment downward migration. At the 15th administration, osteoclastic bone resorption was in some cases observed on the alveolar bone crest in the A. actinomycetemcomitans LPS group. These findings suggest that A. actinomycetemcomitans LPS had a greater effect on periodontal tissue destruction than P. gingivalis LPS.

Key words: Lipopolysaccharide, A. actinomycetemcomitans, P. gingivalis, Histopathological study
Introduction

Gram-negative bacteria and their component lipopolysaccharide (LPS) are significantly involved in the etiology and progression of periodontitis\(^1\,\,^2\). Among gram-negative periodontopathic bacteria, Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans) and Porphyromonas gingivalis (P. gingivalis) have received considerable attention and various in vitro studies focusing on differences in the immunological activities of their LPS have been performed. We recently showed that A. actinomycetemcomitans LPS induces stronger in vitro cytokine production from human neutrophils than P. gingivalis LPS\(^3\). Therefore, differences are also expected in in vivo situations. However, only a few in vivo studies examining the influences of Escherichia coli (E. coli) LPS have been published\(^4\,\,^5\). In addition, the method of LPS administration must be carefully selected in order to avoid damage to the junctional epithelium and connective tissue attachment. In the present study, we used a method that stimulates the natural invasion of LPS into periodontal tissue. Solutions of A. actinomycetemcomitans and P. gingivalis LPS were repeatedly dropped into the gingival sulcus of rats, and the histopathological and histomorphometrical changes over time for each group were then compared.

Materials and Methods

Purification of LPS

A. actinomycetemcomitans Y 4 in Todd-Hewit broth and P. gingivalis 381 in GAM broth (Nissui Seiyaku Tokyo Japan) were cultured with 1% yeast extract (Difco Detroit, MI, USA) at 37°C under anaerobic conditions (10% H\(_2\), 10% CO\(_2\), 80% N\(_2\)) for 3 days. The cells were then centrifuged, washed with pyrogen-free water 3 times and freeze-dried.

Crude LPS was purified using a slight modification of the hot phenol-water procedure\(^6\,\,^7\) and then treated with nuclease and pronase as described by Koga et al\(^8\). Briefly, lyophilized organisms were suspended in 110 ml of pyrogen-free water and 88 ml of phenol. The mixture was then stirred at 68°C for 20 min, cooled on ice and centrifuged at 7000 g for 20 min. The aqueous phase was removed, and the insoluble precipitate re-extracted twice with 110 ml of water. The combined aqueous phases were dialyzed against distilled water at 4°C, neutralized with NaOH and lyophilized.

Each crude LPS extract was suspended in 100 ml of pyrogen-free water and centrifuged at 100,000 g for 3 h. The pellet was suspended in 10 ml of 10 mM Tris buffer (pH 7.4) containing 0.1 mM ZnCl\(_2\) and 400 mg nuclease P 1 (Yamasa Shoyu, Choshi, Japan). This solution was incubated at 37°C for 16 h and dialyzed against distilled water at 4°C. The dialyzed solution was recentrifuged twice, and the pellet was washed with pyrogen-free water and lyophilized. The lyophilized LPS was suspended in 0.1 M borate buffer (pH 7.4) containing 2 mM CaCl\(_2\) and 1 mg pronase (Boehringer Mannheim GmbH, Mannheim, Germany). The mixture was incubated at 37°C for 24 h and then heated at 100°C for 5 min, followed by centrifugation at 5,000 g for 10 min. The supernatant was dialyzed against distilled water, neutralized with NaOH and lyophilized.

Characteristics of LPS used

Chemical components of each LPS

The chemical content of each type of LPS was determined as previously described\(^8\). Heptose and hexose were quantitated by the method of Wright and Rebers\(^9\). Hexosamine, 2-keto-3-deoxyoctonate (KDO) and phosphorus were quantitated by the methods of Davidson\(^10\), Osborn\(^11\), and Ames & Dubin\(^12\), respectively. Protein content was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA). Nucleic acid content was determined by absorption at OD 260.

Induction of IL-1 \(\beta\) by LPS

IL-1 \(\beta\) produced by macrophages treated with each LPS was examined. Peritoneal macrophages from untreated BALB/c mice were isolated by peritoneal lavage with ice-cold sterile RPMI 1640. Cells were resuspended in RPMI 1640, supplemented with 100 U of penicillin/ml, 100 \(\mu\)g of streptomycin/ml, and 10% heat-inactivated fetal calf serum and were plated, at 1 x 10^6 cell per well, in 48 well-plates (Iwaki, Funabashi, Japan). The plates
were incubated for two hours at 37°C in an atmosphere of 5% CO₂ in air. Nonadherent cells were removed by washing three times with fresh medium. Adherent macrophages were cultured with 0.01, 0.1 or 1 μg LPS/ml for 18 h at 37°C in 5% CO₂. *E. coli* 0111:B4 LPS was purchased from Sigma Chemical Co. (St Louis, Mo, USA) and prepared as previously described. Cell-free supernatants were stored at -80°C before quantification of IL-1β. Supernatants were examined for IL-1β using a quantitative enzyme-linked immunosorbent assay (ELISA) kit, Intertest-1βX (Genzyme, Cambridge, USA) according to the manufacturer’s protocol. The detection limit for IL-1β of the ELISA kit was 10 pg/ml. Supernatants from unstimulated macrophages were used as controls.

**Induction of periodontal tissue destruction**

Sixty-five 7 wk old male Lewis rats with an average weight of 170 g were used. They had been bred under SPF (specific pathogen free) conditions at the Laboratory Animal Center for Biochemical Research (Nagasaki University School of Medicine). The experimental protocols were approved by the local Institutional Animal Care and Use Committee.

Rats were divided into three groups: the animals exposed to, i) *A. actinomycetemcomitans* LPS (n=25), ii) *P. gingivalis* LPS (n=25), and iii) PBS (control group, n=15). LPS from *A. actinomycetemcomitans* or *P. gingivalis* was mixed with PBS (pyrogen-free) to a final concentration of 5 mg LPS/ml (total volume 50 μl) and was dropped into the lingual side of the gingival sulcus of the first left mandibular molar over 30 min using a micropipette. This procedure was repeated every 24 h for 15 days under general anesthesia. Five rats from each LPS group were sacrificed at 30 min after the 1st, 3rd, 7th, 10th and 15th administrations. Fifty microliters of PBS was administered to the control group using the same procedure and three rats were sacrificed at each time point. After intracardiac perfusion with periodate-lysine-parafomaldehyde (PLP) under general anesthesia (diethyl ether), a block from the mandible was removed and then fixed by immersion in PLP. It was then decalcified using 10% ethylenediaminetetraacetic acid tetrasodium salt (EDTA-4 Na) in phosphate-buffered saline for 10 days and embedded in paraffin according to the AMeX method.

From each block, 4 μm thick serial sections were cut in order to examine the bucco-lingual section of the first molar. Fifteen groups of serial sections, each containing 10 sections, were obtained from each specimen at each time point. The first sections from each group of serial sections were stained with hematoxylin and eosin (H.E.), and the second with Azan. Sections were then examined histopathologically and histomorphometrically. Furthermore, for some sections in the *A. actinomycetemcomitans* group at the 15th administration, with tartrate resistant acid phosphatase (TRAP) staining was used.

**Histomorphometrical analysis**

The tissue sections stained with H.E were used to histometrically analyze loss of connective tissue attachment and elongation of rete pegs. The dis-
tances from the cemento-enamel junction to the coronal portion of the connective tissue attachment and to the apical portion of the rete peg were measured with a 25 μm microgrid under X400 magnification (Fig. 1). The average values from 15 sections of each specimen were calculated. Furthermore, the averages obtained from the five experimental or three control specimens at each time point were statistically analyzed. No loss of connective tissue attachment was observed in the control groups and the significant differences between experimental groups were statistically analyzed using one-factor ANOVA and Fisher's PLSD test. Significant differences in rete peg elongation among the three groups were analyzed using one-factor ANOVA and Scheffe's F test.

Results

LPS Characteristics

Table 1 shows the chemical components of each LPS used. The carbohydrate contents of A. actinomycetemcomitans and P. gingivalis LPS were 23.6 and 23.2%, respectively. Neither heptose nor KDO were detected in LPS from P. gingivalis. Table 2 shows the level of IL-1 β from macrophages treated with each LPS. Both LPS types induced IL-1 β synthesis from peritoneal macrophages, although no IL-1 β synthesis was detected in unstimulated macrophages. Induction by A. actinomycetemcomitans LPS was more marked than that by P. gingivalis LPS, although weaker than that by E. coli LPS.

Histopathological Findings

Control group

There was no histopathological change in either the junctional epithelium or the connective tissue of the control group (Fig. 2).

Table 1 Chemical components of purified LPS in percent dry weight

<table>
<thead>
<tr>
<th></th>
<th>Heptose</th>
<th>Hexose</th>
<th>Hexosamine</th>
<th>KDO</th>
<th>Phosphorus</th>
<th>Protein</th>
<th>Nucleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. a&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.3</td>
<td>1.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>P. g&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>19.5</td>
<td>3.7</td>
<td>ND</td>
<td>0.3</td>
<td>4.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Standard</td>
<td>Sedoheptulose</td>
<td>Glucose</td>
<td>Galactosamine</td>
<td>KDO</td>
<td>KH₂PO₄</td>
<td>IgG&lt;sup&gt;e&lt;/sup&gt;</td>
<td>DNA&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>: A. actinomycetemcomitans, <sup>b</sup>: P. gingivalis, <sup>c</sup>: determined as percent dry weight, <sup>d</sup>: not detectable, <sup>e</sup>: human immunoglobulin G, <sup>f</sup>: deoxyribonucleic acid from herring sperm.

Table 2 Levels of IL-1 β (pg/ml) from macrophages stimulated with each type of LPS

<table>
<thead>
<tr>
<th>LPS (μg/ml)</th>
<th>A. actinomycetemcomitans</th>
<th>P. gingivalis</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>132.8</td>
<td>57.7</td>
<td>175.7</td>
</tr>
<tr>
<td>0.1</td>
<td>58.3</td>
<td>22.4</td>
<td>130.4</td>
</tr>
<tr>
<td>0.01</td>
<td>39.8</td>
<td>20.0</td>
<td>80.9</td>
</tr>
</tbody>
</table>

Fig. 2 Histopathological findings after the 15th-administration of PBS. No remarkable changes in junctional epithelium and subepithelial connective tissue were observed. Arrow head: cemento-enamel junction. H.E. staining (original magnification, × 160).
Fig. 3 Histopathological findings of the 3rd-administration of A. actinomycetemcomitans (a) and P. gingivalis (b) LPS. Junctional epithelium degenerated and intercellular spaces were enlarged. In the subepithelial connective tissue, elongated rete peg (large arrow) and slight inflammation (small arrow) was noted. Arrow head: cemento-enamel junction. H.E. staining (original magnification, ×160).

1st LPS-administration
In both the A. actinomycetemcomitans and P. gingivalis groups, neutrophils infiltrated into the junctional epithelium and subepithelial connective tissue. The epithelial cells facing the gingival sulcus showed slight vacuolar degeneration, and the intercellular space was slightly enlarged.

3rd LPS-administration
Infiltration of neutrophils was observed in the junctional epithelium and subepithelial connective tissue in both groups and rete pegs showed elongation into subepithelial connective tissue (Fig. 3a and b). Histopathological changes were slightly more marked with A. actinomycetemcomitans LPS than with the P. gingivalis group.

7th LPS-administration
Degeneration of junctional epithelium and elongation of rete pegs in the A. actinomycetemcomitans group was more pronounced at the 7th than at the 3rd administration. Connective tissue in the free gingiva and near the junctional epithelium included amorphous structures with inflammatory cell infiltration. The degeneration was observed to be expanding toward the alveolar bone crest, which was surrounded by inflammatory cells (Fig. 4a). In the P. gingivalis group, there was more neutrophil infiltration and degeneration of junctional epithelium at the 7th than at the 3rd administration. Although elongation of rete pegs and inflammatory cell infiltration were observed, the degree of these changes was milder than in the A. actinomycetemcomitans group. Degeneration of the connective tissue was not yet apparent (Fig. 4b).

10th LPS-administration
In the A. actinomycetemcomitans group, marked degeneration of collagen fibers was observed in the area between the free gingiva and the alveolar bone crest (Fig. 5a). Rete pegs further elongated apically into necrotic connective tissue. Junctional epithelium and collagen fibers attached to the root surface appeared necrotic (Figs. 6a and b). In some sections, the bottom of the epithelial attachment had migrated more apically. Inflammatory cells had infiltrated around the necrotic connective tissue. Although there was degeneration of the junctional epithelium in the P. gingivalis group, these changes...
Fig. 4 Histopathological findings of the 7th-administration of *A. actinomycetemcomitans* (a) and *P. gingivalis* (b) LPS. Degeneration of epithelial cells (small arrow) and subepithelial connective tissue increased. In the *A. actinomycetemcomitans* LPS group, inflammatory cells circumferentially infiltrated around connective tissue near the root surface (a, large arrow). Arrow head: cemento-enamel junction. H.E. staining (original magnification, ×100).

Fig. 5 Histopathological findings of the 10th-administration of *A. actinomycetemcomitans* (a) and *P. gingivalis* (b) LPS. Marked degeneration of the junctional epithelium was noted (a and b). In the *A. actinomycetemcomitans* group, the bottom of the junctional epithelium migrated apically (large arrow) and degenerated cells seemed to be torn off from the root surface (a, small arrow). These was slight degeneration of junctional epithelium in the *P. gingivalis* group (b, small arrow). Arrow head: cemento-enamel junction. H.E. staining (original magnification, ×100).
Fig. 6 Histopathological findings from another specimen of the 10th administration of *A. actinomycetemcomitans* LPS. Rete pegs invaded into the cleft of markedly degenerated connective tissue (a, small arrow). Collagen fibers on the coronal root surface revealed no apparent structure (a, b, large arrow). Arrow head: cemento-enamel junction. H.E. staining (original magnification, ×144).

Fig. 7 Histopathological findings of the 15th-administration of *A. actinomycetemcomitans* (a) and *P. gingivalis* (b) LPS. The bottom of the junctional epithelium showed marked degeneration migrated apically (small arrow). In the *A. actinomycetemcomitans* group, junctional epithelium showed remarkable degeneration and osteoclastic bone resorption by multinucleated TRAP-positive cells was observed on alveolar bone crest (Figure inserted in a). Arrow head: cemento-enamel junction. H.E. staining and TRAP staining (original magnification, ×100 and ×110 in inserted figure).
were less severe than in the *A. actinomycetemcomitans* group (Fig. 5b).

15th LPS-administration

In the *A. actinomycetemcomitans* group, collagen fibers near the root surface had disappeared and rete pegs showed deeper migration. In addition, osteoblasts in the periosteum had disappeared and the alveolar bone surface was partially denuded. Osteoclastic bone resorption was noted at the alveolar bone crest in some sections (Fig. 7a). In the *P. gingivalis* group changes in connective tissue and inflammatory cell infiltration also had become more pronounced than in the earlier administration groups, although these changes clealy were milder than the changes in the *A. actinomycetemcomitans* group (Fig. 7b).

**Histomorphometry**

The *A. actinomycetemcomitans* group showed more extensive loss of connective tissue attachment than the *P. gingivalis* group (Table 3). Furthermore, this group also showed greater rete peg elongation than *P. gingivalis* and control groups (Table 4).

**Discussion**

In the present study, both LPS types induced degeneration of the junctional epithelium facing the gingival sulcus and subepithelial connective tissue. Previous studies have demonstrated the cytotoxicity of sonic extracts of *A. actinomycetemcomitans* or culture supernatants of *P. gingivalis* on human epithelial cells and gingival fibroblasts. Another possible path leading to tissue destruction is the altered cellular metabolism caused by LPS. For instance, LPS from *P. gingivalis* depresses proliferation of periodontal fibroblasts. These earlier investigations underscored the need for further study to clarify the cytotoxicity of LPS types.

Comparing the histopathological and histomorphometrical findings induced by both LPS types, used herein *A. actinomycetemcomitans* LPS induced stronger degeneration and inflammation than that of *P. gingivalis* (Table 3 and 4). This difference may have been a result of their different immunological activities. *A. actinomycetemcomitans* LPS also showed stronger induction of some inflammatory cytokines than did *P. gingivalis* LPS in our previous report. This tendency has also been described by other investigators.

In addition, the appearance of bone resorption differed between the two LPS groups. LPS has been shown to induce osteoclastic bone resorption and some cytokines, such as IL-1β and prostaglandin, which act as osteoclast activating factors. Some in vivo studies have demonstrated that injection of *E.
coli LPS into the gingiva of rat or mouse causes alveolar bone resorption even in the early phases\(^{23-25}\). In the present study, however, as LPS diffused rapidly through the gingiva, it took a comparatively longer time for bone resorption to appear than in the previous report in which LPS was injected into gingival tissue. Abe et al.\(^{26}\) using horseradish peroxidase (HRP) as a tracer, observed immunoelectronmicroscopically that HRP penetrated the junctional epithelium after 30 min.

Clinically the continuous effect of low concentrations of LPS should be detectable. In an in vitro study\(^{18}\), a low concentration of LPS (25-200 µg/ml) affected human gingival fibroblasts. However, due to the risk of repeated general anesthesia on the animals long term experimentation was not feasible, such that the LPS concentration described in previous reports was comparatively high\(^{4,5}\). Furthermore, it was essential that epithelial and connective tissue attachments were not mechanically damaged, in order to compare the effect of each LPS on the junctional epithelium and connective tissue. Thus, LPS was innocuously administered into the gingival sulcus using a micropipette, as in the control experiment using PBS, such that gingival inflammation did not occur even at the 15th administration.

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