Effects of *Porphyromonas endodontalis* lipopolysaccharide on IL-1β, TNF-α and IL-1ra production by human polymorphonuclear leukocytes

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목적

Inflammatory cytokines으로 알려진 interleukin 1β, tumor necrosis factor α는 치수 및 치근단 질환에서 주요 역할을 하는 것으로 알려져 있다. 이들 cytokines은 주로 단백질의 대식세포화를 촉진하게 되며, PMN 또한 이런 cytokines을 합성할 수 있다는 것이 보고되었다. 또한 면역계응이 변환이나 면역반응에서 PMN의 역할이 주로 표시작용을 통해 발생된 결과가 기여하는 것이라 고할 수 있다. 반면, IL-1ra는 IL-1의 생성물을 방해하는 인자이므로, IL-1과 발병관련을 가지는 질환의 발생에 있어서 IL-1과 IL-1ra의 balance가 매우 중요한 역할을 할 것으로 생각된다. 즉, IL-1ra는 IL-1의 proinflammatory efect를 제한할 수 있는 negative feedback mechanism이라고 할 수 있다.

이 연구의 목적은 치수 및 치근단 질환의 감염에 있어서 주요 역할을 하는 *Porphyromonas endodontalis*의 LPS가 PMN의 IL-1, TNF-α, IL-1ra 생성에 미치는 영향을 알아보고 mRNA 수준에서 관찰할 것이다. 잘 알려진 nonpathogenic에 B. gingivali의 LPS를 positive control로 사용하였으며, IL-1ra가 IL-1의 생물학적 작용을 방해하는 작용을 관찰하기 위해, IL-1의 biological assay도 시행하였다.

방 법

*P. endodontalis* ATCC 33406을 정기성 조건에서 배양하고, hot phenol-water extraction의 방법으로 LPS를 추출(crude LPS)한 후, 계조화제로부터 구분한 B. gingivali의 crude LPS와 함께 경제하였다. 건강한 자원들을 대상으로 표본혈액을 계획한 후 dextran sedimentation을 거쳐, Lymphoprep로 농축하여 PMN를 분리하였다. 양이른 세포들은 RPMI 1640 (supplemented with fetal bovine serum, antibiotics)의 5×10^6 cells/ml의 세포수로 resuspend된 후 각각 다른 농도 (0.0.01. 0.1, 1 and 10μg/ml)의 LPS를 처리하여, 각각 다른 시간 (Northern blot : 1, 2, 4시간, ELISA : 2, 6, 12, 18시간)동안 37℃ 5% CO2의 조건으로 배양하였다. 상승 압력 -70°C에 보관하였고 수시에 ELISA를 이용한 단백질 농도 측정과 IL-1 biological assay에 사용하였다. 

결 과

1. 세 가지의 cytokine 모두 각각의 LPS로 처리된 PMN에서 LPS로 처리되지 않은 PMN에서보다 통계적으로 유의성 있게 높은 level의 protein 농도와 mRNA 발현을 나타냈다(p<0.05). 이는 LPS로 침입된 PMN이 염증성 cytokine을 분비할 수 있다는 것을 나타낸다.

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I. INTRODUCTION

Several different bacteria have been associated with oral infections. However, the mechanisms by which specific bacteria cause pathogenic lesions are not completely understood. The pathogens which appear to cause chronic oral infections such as periodontal disease or endodontic lesions are gram negative anaerobes\textsuperscript{10}.

Gram negative bacteria have in the outer leaflet of their outer membrane a class of macroamphilic phospholipids called lipopolysaccharide (LPS) or endotoxin which points out from the bacterial surface to its environment. The host response to LPS is an important determination of the onset and progression of pulpal and periapical diseases\textsuperscript{8}. LPS is released from the surface when bacteria die and lyse, but also when they multiply. It does not act by killing host cells or by inhibiting their functions, but rather induces the active response of host cells. Although the host may benefit from a limited release of LPS (when low levels of mediators are produced), LPS most often acts as a virulence factor (when high levels of mediators are produced)\textsuperscript{8}, that is, it has the ability to induce a number of inflammatory as well as immunopathological reactions through stimulating in inflammatory cells to release a variety of cytokines\textsuperscript{7} leading to the destruction of host tissue.

Cytokines are important regulatory proteins characterized by their pleiotropism and pluripotentiality, and act by binding to high affinity cell surface receptors. They are involved in almost all aspects of cell biology and form interacting networks, with cascades of sequential cell activation. In excess, or when dysregulated, certain cytokines become damaging\textsuperscript{9}. Certain cytokines, including interleukin 1β (IL 1β) and tumor necrosis factor α (TNF α), serve predominantly pro in inflammatory functions and have been reported to be key mediators in pulpal and periapical pathosis\textsuperscript{8,9}. IL 1β and TNF α, which are produced by and act on mononuclear phagocytes and other cell types, have pleiotropic effects, including activation of inflammatory leukocytes and modification of vascular permeability. They also function to up regulate adhesion molecules, immunoglobulin Fc receptors, nitric oxide synthesis, prostaglandin and metalloprotease production and cytokine secretion. Moreover, they promote connective tissue and endothelial cell activation and they can also stimulate bone resorption and inhibit bone formation\textsuperscript{8,9}. These cytokines have been reported to be produced mainly by monocyte/macrophage. However, recent evidence has indicated that polymorphonuclear leukocytes (PMNs) have the ability to release IL 1β and TNF α, though their principal role in inflammatory and immune responses has long been thought to be the phagocytosis and killing of bacteria\textsuperscript{10}.  

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주요어: Porphyromonas endodontialis, Lipopolysaccharide, interleukin 1β, tumor necrosis factor α, interleukin 1 receptor
PMNs are the first cells that migrate into tissues in response to invading pathogens. PMNs are often regarded as terminally differentiated cells, devoid of transcriptional activity and capable of performing little, if any, protein synthesis. However, in the past few years, the validity of this concept has been challenged, principally through the use of molecular biology techniques and other sensitive approaches such as immunohistochemistry. In vitro studies have shown that freshly purified human PMNs, either constitutively or following appropriate stimulation, such as LPS stimulation, have the capacity to express mRNA for a variety of proteins that are involved in PMNs effector functions. These proteins include IL-1β, TNF-α, IL-1 receptor antagonist (IL-1ra), IL-8 and transforming growth factor β (TGF-β1). Therefore, we hypothesized that PMNs stimulated with LPS from endodontopathic bacteria might synthesize IL-1β and TNF-α. In addition, the number of PMNs in inflamed pulp and periapical tissue is much greater than that of macrophage. Thus, assuming PMNs can produce a considerable amount of IL-1β and TNF-α, they might be an important source of these cytokines in inflammatory diseases such as pulp and periapical diseases.

We also paid attention to the fact that LPS stimulated PMN can secrete IL-1ra as well as IL-1β since IL-1ra is known to block the biological actions of IL-1 by competing with IL-1α and IL-1β for the binding to IL-1 receptors. It has been suggested that the balance of production between IL-1 and IL-1ra may be an important determinant for the outcome of several diseases where IL-1 is believed to play a role. Secretion of IL-1ra, therefore, is regarded as one of the principal negative feedback mechanisms to limit the potential pro-inflammatory effects of IL-1β. However, in vitro and in vivo studies have indicated that very large excesses of IL-1ra compared to IL-1β (in the order of 100–1000 times) are required for the inhibition of IL-1 pro-inflammatory activity completely.

In last few years, although many studies have investigated the role of aerobes bacterial lipopolysaccharide, the role of endodontopathic bacterial LPS and the interaction between endodontopathic bacterial LPS and inflammatory cells have received less attention. Porphyromonas endodontalis, highly associated with the root canal, is an asaccharolytic black pigmented bacteria. This bacterium has been detected in radicular cyst fluids and dental pulp where chronic root canal inflammation has occurred. Haapasalo demonstrated that P. endodontalis has been found in half of chronic endodontic lesions and is almost exclusively found in infections of endodontic origin, suggesting a specific association between P. endodontalis and pulp and periapical diseases.

The purpose of this study was to investigate the capacity of peripheral PMN to secrete IL-1β, TNF-α, and IL-1ra protein as well as to express corresponding cytokine mRNA following stimulation with P. endodontalis LPS. LPS from Escherichia coli, the well-characterized non oral bacterium, was used as a positive control. To examine the inhibitory effects of IL-1ra, a biological assay for IL-1 was also performed.

II. MATERIALS AND METHODS

1. Preparation of Lipopolysaccharides

1) Bacteria

P. endodontalis ATCC 35406 was cultured in BHI broth supplemented with yeast, hemin and menadione. Bacteria were grown at 37°C in an anaerobic chamber containing 85% N2, 10% Hz and 5% CO2. After obtaining sufficient amount of bacteria, the broth which contained bacterial cells was centrifuged at 10,000×g for 15min to collect bacterial pellet. The pellet was washed three times by centrifugation with pyrogen free water and lyophilized.

2) Extraction of crude LPS

LPS was extracted and purified from bacteria by the method described by the hot phenol water extraction method. Briefly, lyophilized cells (1g) were suspended in 30ml of pyrogen free water and
30ml of 90% phenol was then added. The mixture was stirred vigorously at 70°C for 15min and then centrifuged at 10,000×g for 30min. The aqueous phase was removed, and the phenol phase and insoluble precipitate were reextracted with 30ml water.

These two aqueous solutions were combined and dialyzed extensively against distilled water and lyophilized, and was termed crude phenol water extracted LPS. Crude Escherichia coli O111: B4 LPS extracted with hot phenol water procedure was purchased from Sigma Chemical Co. (St Louis, MO, USA).

3) Purification of LPS

Each crude LPS was purified by ultracentrifugation and treatment with nuclease and proteinase according to the method of Koga et al. Briefly, crude LPS (1g) was suspended in 100ml pyrogen free water and centrifuged at 100,000×g for 3h. The pellet was suspended in 20ml of 10mM Tris buffer (pH 7.4) containing 0.1mM ZnCl₂ and 400μg of nuclease P₁ from Penicillin G (Amersham Life Science, Cleveland, OH, USA). The reaction mixture was incubated in 37°C for 16h and then dialyzed extensively against distilled water. The dialyzed solution was centrifuged. The pellet was washed with pyrogen free water by centrifugation and lyophilized. The lyophilized LPS (1mg/ml) was suspended in 0.1mM borate buffer (pH 7.4) containing 2mM CaCl₂ and 1mg of pronase (Boehringer Mannheim GmbH, Mannheim, Germany). The mixture was incubated at 37°C for 24h and then heated at 100°C for 5min, followed by dialysis against distilled water, and then lyophilized. Purified LPS contained only a trace amount of protein (<1%) according to the bichloronic acid (BCA) protein assay (Pierce Chemical Corp., Rockford, IL, USA). The LPS demonstrated a typical ladder like LPS pattern on gel after poly acrylamide electrophoresis and silver staining.

2. Preparation of Human PMN

Venous blood was collected under sterile conditions from 10 healthy medication free volunteers between the ages of 29 and 43 by venipuncture. The blood was anticoagulated with EDTA.

Leukocytes were separated from erythrocytes by dextran (6% dextran, Mw. 400,000~500,000) sedimentation, and PMNs were obtained by centrifugation layered over Lymphoprep (Nycomed Pharma AS, Oslo, Norway). Residual erythrocytes were removed by hypotonic lysis. This procedure yielded a PMN population of >98% viability and >97% purity as judged by Trypan blue dye exclusion test and Giemsa staining. The cells were resuspended in RPMI 1640 supplemented with 100U of penicillin/ml, 100μg of streptomycin/ml and 10% fetal bovine serum (FBS).

For Northern blot analysis, 4ml of the resuspended cells (5×10⁸ cells/ml) were placed in 50ml tubes and cultured either with LPS (1μg/ml of LPS for 1, 2 or 4h) or without LPS (for 1h) at 37°C in 5% CO₂. For cytokine assays, the resuspended cells were plated at 5×10⁶ cells per well in 24 well plates (Corning, N.Y.). To examine the time related secretion of cytokines, the cells were cultured either with 1μg/ml of LPS or without LPS (as a control) for 2, 6, 12 or 18h at 37°C in 5% CO₂. To examine the dose related secretion of cytokines, the cells were cultured with various concentrations (0, 0.01, 0.1, and 10μg/ml) of LPS for 18h at 37°C in 5% CO₂. All these concentrations were determined by preliminary experiments. Supernatant fluids were collected, centrifuged and stored at 70°C for later cytokine assays.

3. Isolation of RNA and Northern Blot Analysis

Following activation with LPS, the total RNA from PMN was isolated by a single step isolation method originally developed by Chomczynski & Sacchi. RNAse free plastic and water was used throughout.

After each treatment period, the cells were harvested and lysed by resuspending the cell pellet with 1ml of TRIzol reagent (GIBCO Laboratories, Grand Island, NY, USA) and repetitive pipetting. A volume of 200μl of chloroform was added to a volume of 1ml of TRIzol reagent. The
samples were centrifuged at 12,000×g for 15 min at 4°C. Following centrifugation, the colorless upper aqueous phase containing RNA was transferred into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol. Samples were incubated at room temperature for 10 min and centrifuged at 12,000×g for 10 min at 4°C. The RNA pellet was washed twice with 75% RNAse free alcohol. The concentrations of isolated RNA were calculated by absorbance at 260 nm and 280 nm with spectrophotometer.

Aliquots (12 µg) of RNA were separated by electrophoresis through 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Nytran Supercharge Nylon Membrane, Schleicher & Schuell, GmbH, Dassel, Germany) by downward capillary transfer system using TurboBlotter (Schleicher & Schuell). cDNA probes were synthesized using the RT PCR amplification method with oligonucleotide primers specific for IL 1β (5’ ATGGGAGAGTTACCTGAGCTC 3’ and 5’ TTAGGAAGACACAAAAATGATGTGAAACCTC 3’), TNF α (5’ GACAGGGACTGAGGAAGC 3’ and 5’ TCTCAGGGAATGTACGC CAAAGTACACCTGCGCCA 3’), and IL 1α (5’ GACCTGAGCGAAACAGAAAGC 3’ and 5’ GTCTGAGAGCAGGCAGATGC 3’). cDNA probe of β actin was purchased from Bioneer, Taejon, Korea.

Briefly, the first strand cDNA was synthesized using MultiScribe™ Reverse Transcriptase (Perkin Elmer, Foster city, CA, USA) from 2 µl of isolated RNA. PCR amplification of cDNA was performed using oligonucleotide primers specific for each cytokine with Gold Taq DNA Polymerase (Perkin Elmer). After PCR amplification, 10 µl of each PCR product was analyzed by agarose gel electrophoresis in order to confirm that the amplified DNA represented the expected size (81.0bp for IL 1β, 704bp for TNF α, and 371bp for IL 1α).

The PCR products were cloned with TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The PCR products were ligated into plasmid vector (pCR II TOPO) and the recombinant vectors were transformed into TOP 10 E. coli competent cells. After transformation, the transformed plasmid DNAs were isolated with S.N.A.P MiniPrep Kit (Invitrogen) and the isolated plasmid DNAs were analyzed by restriction analysis, that is, the plasmid was digested with EcoRI. The digested plasmids were electrophoresed on 1% agarose gel to confirm that the digestion procedure was completed properly. The cDNA bands were excised from the agarose gel and purified with QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA, USA).

The purified cDNA probes were labeled with DIG High Prime DNA Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany).

The blotted membranes were prehybridized for 2 h at 50°C, then the DIG labeled cDNA probes were added at a concentration of 25 ng/ml and the membranes were hybridized overnight at 50°C. Blots were washed for 5 min twice in 2×SSC plus 0.1% SDS at room temperature and then washed for 15 min twice in 0.5×SSC plus 0.1% SDS at 68°C. The membranes were detected with DIG Chemiluminescent Detection Kit (Boehringer Mannheim). The mRNA band intensities were measured by densitometric analysis using Quantity One (BioRad, Hercules, CA, USA). β actin was used as an internal standard for quantification of total mRNA on each lane of the gel.

4. Enzyme-Linked Immunosorbent Assay (ELISA)

The cell free supernatants were examined for IL 1α, TNF α and IL 1α using a quantitative ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocols.

5. Biological Assay of IL-1

The biological activity of IL-1 was assayed using the C3H/HeJ mouse thymocyte mitogen proliferation assay. Six to twelve week old lipopolysaccharide unresponsive C3H/HeJ mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were used in this assay, ensuring that the cells did not respond to any lipopolysaccharide that may have been present in the culture supernatants.

Briefly, the thymuses were removed after the mice were anesthetized intraperitoneally with
Ketalar (Ketamine hydrochloride, Yuhan Co., Seoul, Korea) and 2 × 10⁶ thymocytes were cultured in a final volume of 200μl in RPMI 1640 medium (supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 50μM 2 ME, 100U/ml penicillin and 100μg/ml streptomycin sulfate) with PMN supernatants in the presence of a submitogenic concentration of concanavalin A (1μg/ml). Cultures were incubated for 48h in an atmosphere of 5% CO₂ in air at 37°C, pulsed with 3.75kBq of [³H] thymidine (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England), and further incubated for 24h at 37°C. Cells were harvested and incorporated counts were measured with a scintillation counter after the cells were digested in an Aquasol 2 (Packard, Meriden, CT, USA). Test samples were assayed in triplicate. To compare the immunoreactive IL 1β and its biological activity, recombinant human IL 1β (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England) was added instead of PMN supernatants in each assay. The amounts of IL 1β (pg/ml) in the PMN supernatants were transformed to the equivalent cpm of [³H] thymidine incorporation using a standard recombinant human IL 1β dilution curve.

6. Statistical Analysis

All statistical analyses were performed according to Student's t test and Mann Whitney rank sum test. The statistical significance was set at P < 0.05.

II. RESULTS

1. Detection of Cytokine mRNA by Northern Blot Analysis

![Diagram and graphs showing the effect of LPS on the expression of IL-1β, TNF-α, and IL-1ra in PMN.](image)

**Fig. 1.** Time course (1h, 2h, 4h) effects of LPS from P. endodontalis and E. coli on the expression of IL-1β, TNF-α, and IL-1ra mRNA in PMN. The equivalent loading of each sample was verified by measurement of β-actin mRNA. **Upper:** The figure shows the representative results of three separate Northern blot analyses. (−) indicates unstimulated PMN. **Lower:** Relative signal intensities of IL-1β, TNF-α, and IL-1ra that of β-actin were quantified with Quantity 2.1 image analyzer. The data indicate the mean values of three separate experiments.
Fig. 2 Effect of incubation time with LPS from \textit{P. endodontalis} and \textit{E. coli} on cytokine production in the supernatants of PMN. PMN (5 $\times 10^6$ cells/ml) from human peripheral blood were incubated at 37°C with 1 µg/ml of each LPS. The data represent mean values with standard deviation. All of the experimental groups represented significantly higher cytokine concentrations ($p<0.05$) than control groups. + indicates significant differences ($p<0.05$) between cells stimulated with \textit{P. endodontalis} LPS and cells stimulated with \textit{E. coli} LPS ($n=10$ for each group).

Fig. 3. Dose-response to LPS from \textit{P. endodontalis} and \textit{E. coli} of cytokine production in the supernatants of PMN. The cells were treated with various concentrations of each LPS for 18h. The data represent mean values with standard deviation. All of the experimental groups represented significantly higher concentrations ($p<0.05$) than control groups. + indicates significant differences ($p<0.05$) between cells stimulated with \textit{P. endodontalis} LPS and cells stimulated with \textit{E. coli} LPS ($n=10$ for each group).
Fig. 4. A. The ratio of mean amounts of IL-1α and IL-1β in human peripheral PMN after stimulation with 1 μg/ml of LPS from P. endodontalis and E. coli. B. The biological IL-1 activity in the supernatants of PMNs was compared with the same doses of recombinant human IL-1β (rhIL-1β) detected by ELISA. (a) and (b) indicate that PMN were incubated with LPS for 6h and 18h, respectively. The data represent mean values (n=10 for each group). The [3H]-thymidine incorporation levels of thymocytes incubated with concanavalin A alone were 122 to 256 cpm (P-endo represents P. endodontalis).

As shown in the representative experiments of Fig. 1, untreated PMN expressed appreciable levels of cytokine transcripts, whereas each case of treatment with LPS greatly augmented all three cytokine mRNA.

Fig. 1 shows that the peak expression of IL-1β mRNA in PMN treated with each LPS was apparent at 1h, then decreased by 2h, but repeatedly, increased again by 4h. As seen in Fig. 1, TNFα mRNA expression in PMN treated with P. endodontalis and E. coli peaked at 1h and then decreased by 4h. Fig.1 also shows that IL-1α mRNA expression in PMN treated with P. endodontalis LPS peaked at 1h and then decreased gradually, whereas the expression in PMN treated with E. coli LPS was apparent at 1h and continued to maintain a similar level until 4h.

The expression of all three cytokine mRNA in PMN treated with E. coli LPS was greater than those treated with P. endodontalis LPS.

2. Cytokine Assay

The levels of IL-1β, TNFα and IL-1α released by freshly isolated human PMN in response to P. endodontalis and E. coli LPS were examined. Fig. 2 shows that the levels of all three cytokines released from cells stimulated with either P. endodontalis or E. coli LPS were significantly higher than that of the unstimulated control cells (p<0.05). The levels of the three cytokines released from cells stimulated with E. coli LPS were higher than those released from cells stimulated with P. endodontalis LPS. Production of
IL 1β and IL 1ra by PMN stimulated with P. endodontalis LPS continued to increase until 12h, however, production of these cytokines stimulated with E. coli LPS reached a peak at 12h and maintained similar levels until 18h. Interestingly, the peaks of TNF α production by PMN stimulated with P. endodontalis and E. coli LPS were at 6h and 12h, respectively, and their production decreased slightly afterwards.

Fig. 3 shows that the levels of all three cytokines incurred a significant dose dependent increase when PMN were treated with 0.01, 0.1, 1, 10 μg/ml of P. endodontalis and E. coli LPS compared with the control(p<0.05), but that the levels of all three cytokines secreted by PMN stimulated with E. coli LPS were higher than those secreted by cells stimulated with P. endodontalis.

3. Effect on IL-1β Biological Activity

Fig. 4A shows the ratios between the amounts of IL 1ra and IL 1β at different incubation times. As seen in Fig. 4A, the ratios decreased as incubation time increased. The supernatants of PMN stimulated with each LPS exhibited less biological IL 1 activity than the equivalent doses of recombinant human IL 1β detected by ELISA, however, considerable levels of biological IL 1 activity in the supernatants of PMN stimulated with each LPS were still found (Fig. 4B).

IV. DISCUSSION

The inflammatory periapical lesions are a common sequela of infected pulp necrosis. Numerous cell types, including polymorphonuclear leukocytes, T and B lymphocytes, macrophages, and plasma cells, have been identified in periapical lesions. These inflammatory cells have the potential to mediate the entire spectrum of immunologic phenomena. Bone resorption is often a feature of inflammatory disease, and IL 1 and TNF α production by inflammatory cells may be the mechanism by which inflammatory osteolysis is effected.

IL 1 and TNF α are key elements in the proinflammatory cytokine cascade that is activated in response to infection or immunologic insult. Whereas IL 1 by itself is much more potent than TNF, these mediators also synergize to stimulate bone resorption. IL 1 and TNF α in turn induce the expression of IL 6, which, besides its immunoregulatory effects, increases osteoclast formation and has been reported to stimulate bone resorption.

IL 1ra is an anti-inflammatory cytokine, which binds to the IL 1 receptor but does not initiate IL 1 signal transduction. It was reported that administration of IL 1ra to animals reduced the severity of diseases such as hemodynamic shock, lethal sepsis, inflammatory bowel disease and experimentally induced arthritis.

The major cell that produces IL 1β, TNF α and IL 1ra in human peripheral blood has been thought to be the monocyte. PMNs originate from the same stem cell as monocytes and share other functions such as phagocytosis and the killing of bacteria. The results presented here show unequivocally that the mature circulating human PMN is also capable of synthesizing and secreting these cytokines. PMNs are terminally differentiated, short lived cells, incapable of proliferation or self renewal. Thus, their ability to synthetize immunomodulatory cytokines might be viewed as a phenomenon of little physiological significance. However, a mounting body of evidence indicates that PMN survival can be greatly extended following exposure to microenvironmental signals in volved in infection and immunity, such as LPS, in activated streptococci, IL 1β, TNF α, IL 6, IFN γ, G CSF, and GM CSF. Therefore, these observations raise the possibility that PMN viability in vivo may be considerably greater than is currently believed.

This study also determined that the levels of protein and mRNAs of all three cytokines in PMN stimulated with each LPS were significantly higher than the control, suggesting that LPS plays an important role in inflammatory diseases, such as, pulpal and periapical diseases. It
was also found that E. coli LPS augmented greater amounts of protein synthesis of those cytokines than P. endodontalis LPS and that the mRNA expression of those cytokines in PMN stimulated with E. coli LPS was greater and lasted longer than that in PMN stimulated with P. endodontalis LPS. These findings suggest that P. endodontalis LPS acts differently from E. coli LPS in cytokine production. In other studies, similar results have been found. Hosoya et al.\(^4\) reported that the levels of IL 1β protein and mRNA production in human dental pulp cells stimulated with P. endodontalis LPS were higher than that of the unstimulated control cells. And Yoshimura et al.\(^3\) demonstrated that E. coli LPS stimulated PMN to produce greater amounts of IL 1β, TNF α and IL 8 than did P. gingivalis and/or C. oralis LPS.

It has been known that LPS from different or organisms vary in their effects on host cells. This may be due to differences in the chemical structure of the LPS in these organisms. It is well documented that the lipid A of LPS from black pigmented bacteria (BPB) contains different fatty acids from the lipid of enterobacterial LPS, and is monophosphorylated, and these are regarded as reasons why it is less endotoxic than enterobacterial LPS. Matsushita et al.\(^5\) reported that the IL 1β and IL 6 inducing activities of BPB LPS were weaker than those of E. coli LPS. These findings support the results of this study that E. coli LPS induced greater amounts of cytokine and its mRNA than P. endodontalis. Moreover, Firoozkooi et al.\(^6\) suggested that the carbohydrate moiety of LPS and the length of the O chain, which constitutes a polymer of oligosaccharides of LPS, may also have significant functions in terms of virulence. Therefore, further study will be necessary to investigate the relationship between each LPS component and its virulence.

The results of this study show that mRNA expression of all three cytokines in PMN treated with either P. endodontalis or E. coli LPS peaked at 1h. This finding is consistent with the previous study of Palma et al.\(^4\). They demonstrated that E. coli LPS induced the transcription of mRNA for IL 1β, TNF α and IL 6 in PMN, which peaked at 1h when they compared the mRNA expression at 1h with that at 3h. But interestingly, only IL 1β mRNA expression showed a second peak at 4h in this study. Since TNF α can induce the synthesis of IL 1β, it is possible that secreted TNF α by PMN treated with each LPS might have stimulated IL 1β mRNA expression and consequently resulted in the second peak at 4h in the present study. However, PMN can also produce IL 8, transforming growth factor β, IL 6, macrophage in inflammatory protein 1α and interferon α as well as IL 1β, TNF α and IL 1α after treatment with LPS, and the PMN centered cytokine network is very complex. Therefore, there may be other possibilities in this process.

In this study, the concentrations of cytokines increased in a time dependent manner. However, the concentration of TNF α increased in a time dependent manner until 6h and then decreased slightly. This result is in agreement with that of other studies.\(^5\) Yoshimura et al.\(^3\) reported that the concentration of TNF α produced by PMN stimulated with periodontopathic bacteria was higher at 6h than at 18h. And Rossesto et al.\(^7\) reported that the concentration of TNF α in gingival crevicular fluid was decreased as the severity of the disease increased.

In terms of LPS concentration, 0.01μg/ml of LPS was enough to stimulate cytokine production by PMN. And the levels of secretion for all three cytokines were effected in a dose dependent manner by PMN stimulated with P. endodontalis LPS, but the maximum levels of cytokine secretion by PMN stimulated E. coli LPS occurred at a concentration of 1μg/ml. It is possible that higher concentrations of E. coli LPS may inhibit cytokine production or damage peripheral PMN. These findings are supported by those of a previous study\(^8\).

Since it has been reported that IL 1α exhibits dose responsive inhibition of IL 1α and IL 1β mediated augmentation of mitogen induced murine thymocyte proliferation, a thymocyte mitogen proliferation assay was performed in this study.
It is commonly assumed that the ratio of IL 1ra to IL 1β determines the severity of an inflammatory response. Other in vitro and in vivo studies have indicated that very large excesses of IL 1ra over IL 1β are required to shift the IL 1ra:IL 1β ratio in favor of IL 1ra sufficiently to result in complete inhibition of IL 1 bioactivity. Such excesses of IL 1ra are required because of the extreme sensitivity of the IL 1 type 1 receptor and rapid in vivo clearance. In this study, none of the IL 1ra:IL 1β ratios were in the anti-inflammatory range. Consequently, although the supernatants of PMN stimulated with each LPS had less biological IL 1 activity than the same doses of recombinant human IL 1β detected by ELISA, considerable levels of biological IL 1 activity in the supernatants were still detected. And the results of this study also show that the ratios decreased as incubation time increased, that is, the ratio became increasingly proinflammatory, due to the increasing level of IL 1β, without a corresponding significant increase in IL 1ra. These findings suggest that the antagonistic action of IL 1ra produced by PMN stimulated with LPS from P. endodontalis and E. coli toward IL 1 bioactivity could be largely eliminated by relatively high concentrations of IL 1β produced by PMN stimulated with each LPS. Accordingly, each LPS can produce proinflammatory effects.

In summary, PMN may represent a first line of defense against endodontopathic bacteria and should be considered not only as active and central elements of the inflammatory response, but also as cells that, through cytokine secretion, may significantly influence the direction and evolution of the immune process. Thus, PMN can play a key role in pulpal or periodontal inflammatory reactions. In this study, LPS from P. endodontalis stimulated PMN to produce IL 1β, TNF-α, and IL 1ra through the enhancement of gene expression of these cytokines. But P. endodontalis LPS was less potent than E. coli LPS in the production of these proinflammatory cytokines. Secreted IL 1β and TNF-α from PMN induced by these bacteria activate PMN themselves and other cell types including monocytes/macrophages and lymphocytes, stimulate further cytokine secretion, and subsequently, cause pulp or periodontal tissue destruction. Secreted IL 1ra might inhibit IL 1 bioactivity slightly during the initial phase of the pathologic process, but such inhibition might be abolished by high concentrations of secreted IL 1β during the advanced phase of the pathologic process.

V. CONCLUSIONS

This study evaluated the effects of Porphyromonas endodontalis lipopolysaccharide (LPS) on the production of interleukin 1 β, tumor necrosis factor α and interleukin 1ra protein and mRNA by PMN, which were evaluated by ELISA and Northern hybridization. Escherichia coli LPS was used as a positive control.

The following results were obtained:
1. The levels of protein secretion and mRNA expression of these cytokines in PMN stimulated with each LPS were significantly higher than the unstimulated control cells (p<0.05).
2. E. coli LPS augmented greater amounts of cytokines than P. endodontalis LPS (p<0.05).
3. mRNA expressions of all three cytokines in PMN treated with either P. endodontalis or E. coli LPS were peaked at 1h. And only IL 1β mRNA expression showed the second peak at 4h.
4. 0.01μg/ml of LPS was enough to stimulate cytokine production by PMN. And the levels of secretion for all three cytokines were effected in a dose dependent manner.
5. None of the IL 1ra:IL 1β ratios were in the anti-inflammatory range. Consequently, considerable levels of biological IL 1 activity in the supernatants were still detected.
6. The ratios decreased as incubation time increased, that is, the ratio became increasingly proinflammatory, due to the increasing level of IL 1β, without a corresponding significant increase in IL 1ra.

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