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

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국문초록

Porphyromonas endodontalis 의 lipopolysaccharide가 다형핵백혈구의 IL-1β, TNF-α, IL-1ra 생성에 미치는 영향에 대한 연구

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목 적

Inflammatory cytokine으로 알려진 interleukin 1β, tumor necrosis factor α는 치수 및 치근단질환에서 주요한 역할을 하며, 골흡수를 자극하고 골형성을 방해하는 것으로 알려져 왔다. 이들 cytokine은 주로 단핵세포/대식세포가 형성하는 것으로 알려져 왔으나 최근 연구에 의하면, PMN도 또한 이런 cytokine들을 형성할 수 있다는 것이 보고되었다. 오랫동안 염증반응이나 면역반응에서 PMN의 역할이 주로 포식작용 을 통해 병원균을 제거하는 것이라고만 생각되어져 왔던 것을 생각하면, 새로운 발견이라 할 수 있다. 또, PMN은 IL-1ra도 생성하는 것으로 보고되었는데, IL-1ra란 IL-1의 생물학적 작용을 방해하는 인자이므로, IL-1과 밀접한 관련을 가지는 질환의 발전에 있어서 IL-1과 IL-1ra의 balance가 매우 중요한 역할을 할 것으로 생각된다. 즉, IL-1ra는 IL-1β의 proinflammatory effect를 제한할 수 있는 negative feedback mechanism이라고 할 수 있다.

이 연구의 목적은 치수 및 치근단 조직의 감염에 있어서 주요 원인균인 Porphyromonas endodontalis의 LPS가 PMN의 IL-1β, TNF-α, IL-1ra 생성에 미치는 영향을 단백질과 mRNA 수준에서 관찰하는 것이다. 잘 알려진 non-oral bacterium인 E. coli의 LPS를 positive control로 사용하였으며, IL-1ra가 IL-1β의 생물학적 작용을 방해하는 작용을 관찰하기 위해, IL-1의 biological assay도 시행하였다.

방 법

P. endodontalis ATCC 35406을 혐기성 조건에서 배양하고, hot phenol-water extraction의 방법으로 LPS를 추출(crude LPS)한 후, 제조회사로부터 구입한 E. coli의 crude LPS와 함께 정제하였다. 건강한 자원자들을 대상으로 말초혈액을 채취한 후 dextran sedimentaion을 거쳐, Lymphoprep을 이용하여 PMN층을 분리하였다. 얻어진 세포들은 RPMI 1640 (supplemented with fetal bovine serum, antibiotics)에 5×10^6cells/ml이 되도록 resuspend시킨 후 각기 다른 농도 (0, 0.01, 0.1, 1 and 10μg/ml)의 LPS를 처리하여, 각기 다른 시간 (Northern blot : 1, 2, 4시간, ELISA : 2, 6, 12, 18시간)동안 37℃ in 5% CO_2 의 조건으로 배양하였다. 상층액은 -70℃에 보관하였다가 추후에 ELISA 를 이용한 단백질 농도 측정과 IL-1 biological assay에 사용되어졌으며, 배양된 세포로부터 RNA를 추출하여 Northern hybridization을 통해 mRNA expression을 관찰하였다.

결 과

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

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2. E. coli LPS는 P. endodontalis LPS에 비해 통계적 유의성 있게 많은 양의 protein synthesis와 mRNA 발현을 나타냈다.
3. 세 가지 cytokine 모두에서 mRNA는 두 가지 LPS 모두 1시간 만에 peak를 나타냈고, IL-1β만이 4시간 만에 두 번째 peak를 나타냈다.
4. PMN으로부터 cytokine을 분비하게 하는 데는 0.01㎍/ml의 LPS 정도면 충분하였으며, 세 가지 cytokine 모두에서 분비된 cytokine 양은 dose-dependent manner를 나타냈다.
5. 모든 sample의 IL-1ra:IL-1β ratio가 anti-inflammatory range에 속하지 못했다. 결과적으로, LPS로 자극된 PMN supernatant내에 상당한 수준의 biological IL-1 activity가 발견되었다.
6. IL-1ra:IL-1β ratio는 배양시간이 증가함에 따라 감소하였다. 이는 시간이 지남에 따라 IL-1β의 level은 현저히 증가하지만, IL-1ra의 level은 큰 변화가 없는 것에 따른 결과이며, 결과적으로 시간이 증가함에 따라 IL-1ra의 IL-1β에 대한 inhibitory effect가 점점 감소한다는 것을 의미한다.

주요어 : Porphyromonas endodontalis, Lipopolysaccharide, interleukin 1β, tumor necrosis factor α, interleukin 1 recep-

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[1,2].

Gram negative bacteria have in the outer leaflet of their outer membrane a class of macroamphiphiles 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[3-7].

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)[4], that is, it has the ability to induce a number of inflammatory as well as immunopathological reactions through stimulating inflammatory cells to release a variety of cytokines[7-15] 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[16]. Certain cytokines, including interleukin 1β(IL 1β) and tumor necrosis factor α(TNF α), serve predominantly pro inflammatory functions and have been reported to be key mediators in pulpal and periapical pathosis[8,17].

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 upregulate 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[18-30]. 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[3,31].

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[31,32]. These proteins include IL 1β, TNF α, IL 1 receptor antagonist (IL 1ra), IL 8 and transforming growth factor β 1(TGF β1)[31 33]. 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 pulpal and periapical tissue is much greater than that of macrophage[31]. 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 pulpal 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[3,33]. 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[34]. 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 proinflammatory activity completely[35,36].

In last few years, although many studies have investigated the role of aerobic 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[8]. Haapasalo[37] 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 pulpal 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℃ in an anaerobic chamber containing 85% N_2, 10% H_2 and 5% CO_2. 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[38]. 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℃ 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.[39]. 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 $ZnCl_2$ and 400㎍ of nuclease P1 from Penicillin citrinum(Amersham Life Science, Cleveland, OH, USA). The reaction mixture was incubated in 37℃ 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 0.1M borate buffer(pH 7.4) containing 2mM $CaCl_2$ and 1mg of pronase(Boerheinger Mannheim GmbH, Mannheim, Germany). The mixture was incubated at 37℃ for 24h and then heated at 100℃ for 5min, followed by dialysis against distilled water, and then lyophilized. Purified LPS contained only a trace amount of protein (<1%) according to the bicinchoninic acid (BCA) protein assay (Pierce Chemical Corp., Rockford, IL, USA). The LPS demonstrated a typical ladder like LPS pattern on gel after polyacrylamide 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 23 and 43 by venipuncture. The blood was anticoagulated with EDTA.

Leukocytes were separated from erythrocytes by dextran (6% dextran, Mwt. 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, 100㎍ of streptomycin/ml and 10% fetal bovine serum(FBS).

For Northern blot analysis, 4ml of the resuspended cells(5×10^6 cells/ml) were placed in 50ml tubes and cultured either with LPS (1㎍/ml of LPS for 1, 2 or 4h) or without LPS(for 1h) at 37℃ in 5% CO_2. For cytokine assays, the resuspended cells were plated at 5×10^6 cells per well in 24 well plates(Corning, N.Y). To examine the time related secretion of cytokines, the cells were cultured either with 1㎍/ml of LPS or without LPS(as a control) for 2, 6, 12 or 18h at 37℃ in 5% CO_2. To examine the dose related secretion of cytokines, the cells were cultured with various concentrations(0, 0.01, 0.1, 1 and 10㎍/ml) of LPS for 18h at 37℃ in 5% CO_2. All these concentrations were determined by preliminary experiments. Supernatant fluids were collected, centrifuged and stored at 70℃ 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 [40]. 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 200㎕ 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℃. Following centrifugation, the colorless upper aqueous phase containing RNA was trans ferred into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5ml of isopropyl alcohol. Samples were incubated at room temperature for 10min and centrifuged at 12,000×g for 10min at 4℃. The RNA pellet was washed twice with 75% RNAse free alcohol. The concentrations of isolated RNA were calculated by absorbance at 260nm and 280nm with spec trophotometer.

Aliquots(12μg) of RNA were separated by elec trophoresis 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 syn thesized using the RT PCR amplification method with oligonucleotide primers specific for IL 1β (5′ ATGGCAGAAGTACCTGAGCTC 3′ and 5′ TTAGGAAGACACAAATTGCATGGTGAAGTCAGT 3′)[41], TNF α(5′ CCATGAGCACTGAAAG CATGA 3′ and 5′ TCACAGGGCAATGATCC CAAAGTA GACCTGCCCA 3′)[42] and IL 1ra(5′ GACCTGA GCGAGAACAGAAAGC 3′ and 5′ GTTGAAGAG GAGGCAGAGTCC 3′)[43] and 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 2μl of isolat ed RNA. PCR amplification of cDNA was per formed using oligonucleotide primers specific for each cytokine with Gold Taq DNA Polymerase (Perkin Elmer). After PCR amplification, 10μl of each PCR product was analyzed by agarose gel electrophoresis in order to confirm that the am plified DNA represented the expected size (810bp for IL 1β, 704bp for TNF α and 371bp for IL 1ra).

The PCR products were cloned with TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The PCR products were ligated into plasmid vec tor (pCR Ⅱ TOPO) and the recombinant vectors were transformed into TOP 10 E. coli competent cells. After transformation, the transformed plas mid 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 GENECLEAN Kit (Bio101, Carlsbad, CA, USA). The purified cDNA probes were labeled with DIG High Prime DNA Labeling Kit (Boerheinger Mannheim GmbH, Mannheim, Germany).

The blotted membranes were prehybridized for 2h at 50℃, then the DIG labeled cDNA probes were added at a concentration of 25ng/ml and the membranes were hybridized overnight at 50℃. Blots were washed for 5 min twice in 2×SSC plus 0.1% SDS at room temperature and then washed for 15min twice in 0.5×SSC plus 0.1% SDS at 68 ℃. The membranes were detected with DIG Chemilumines cent Detection Kit (Boerheinger Mannheim). The mRNA band intensities were measured by densitometric analysis using Quantity 2.1(Bio Rad, 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 1β, TNF α and IL 1ra using a quantitative ELISA kit(R&D system, Minneapolis, MN, USA) ac cording 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 comitogen prolif eration assay[44]. Six to twelve week old lipopolysac charide 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 2×10^5 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% CO_2 in air at 37℃, pulsed with 3.75kBq of (^{3}H) thymidine(Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England), and further incubated for 24h at 37℃. 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 1 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 (^{3}H) 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 risk rate was set at $P<0.05$.

Ⅲ. RESULTS

1. Detection of Cytokine mRNA by Northern Blot Analysis

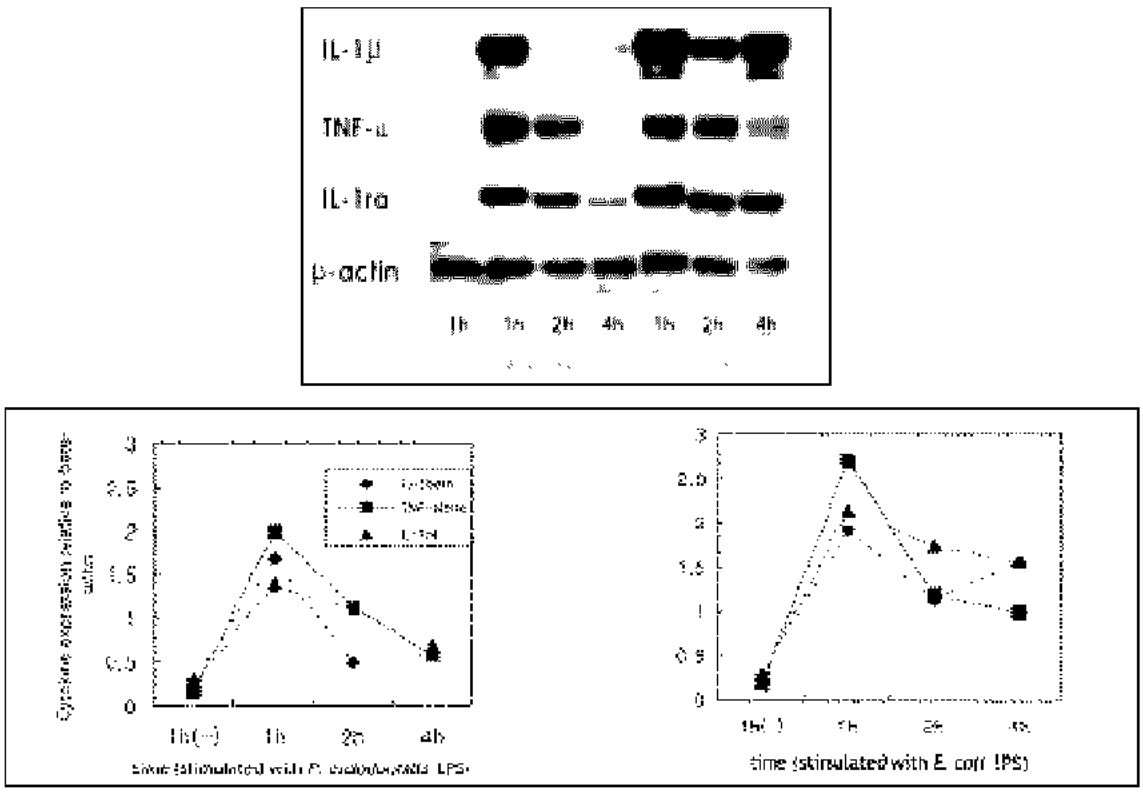

Fig. 1. Time course(1h,2h,4h) effects of LPS from P. endodontalis and E. coli on the expression of IL-1β, TNF-α and IL-1ra mRNA in PMN. The equivalent loading of each sample was verified by measurement of β-actin mRNA. **Upper:** The figure shows the representative results of three separate Northern 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.

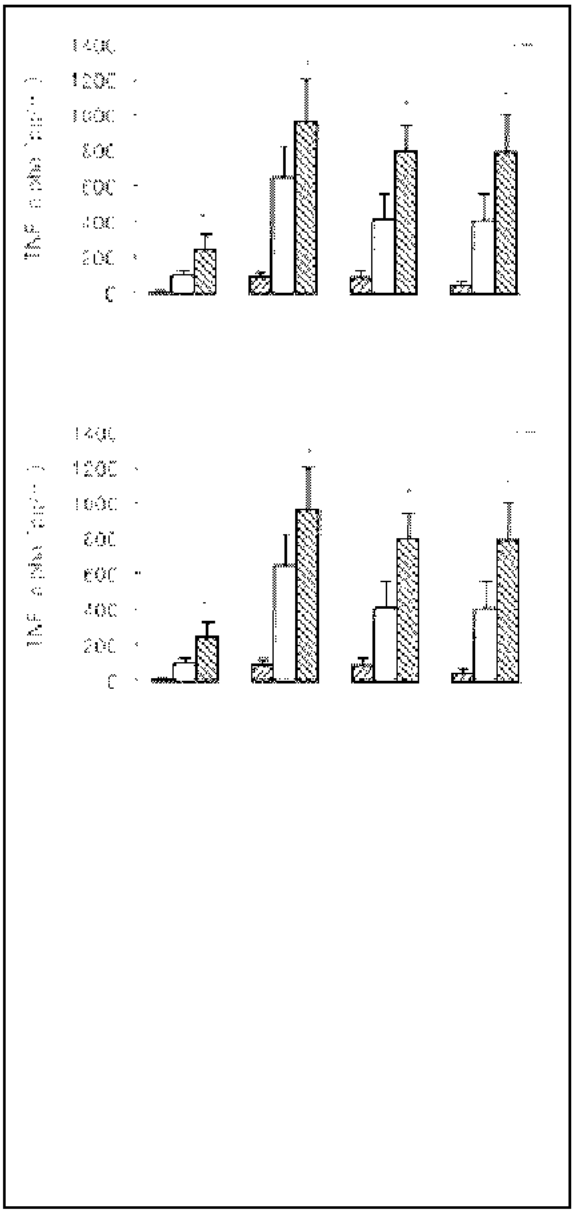

Fig. 2. Effect of incubation time with LPS from P. endodontalis and E. coli on cytokine production in the supernatants of PMN. PMN(5 $\times 10^6$ cells/ml) from human peripheral blood were incubated at 37°C with 1μg/ml of each LPS. The data represent mean values with standard deviation. All of the experimental groups represented significantly higher cytokine concentrations ($p<0.05$) than control groups. + indicates significant differences($p<0.05$) between cells stimulated with P. endodontalis LPS and cells stimulated with E. coli LPS (n−10 for each group).

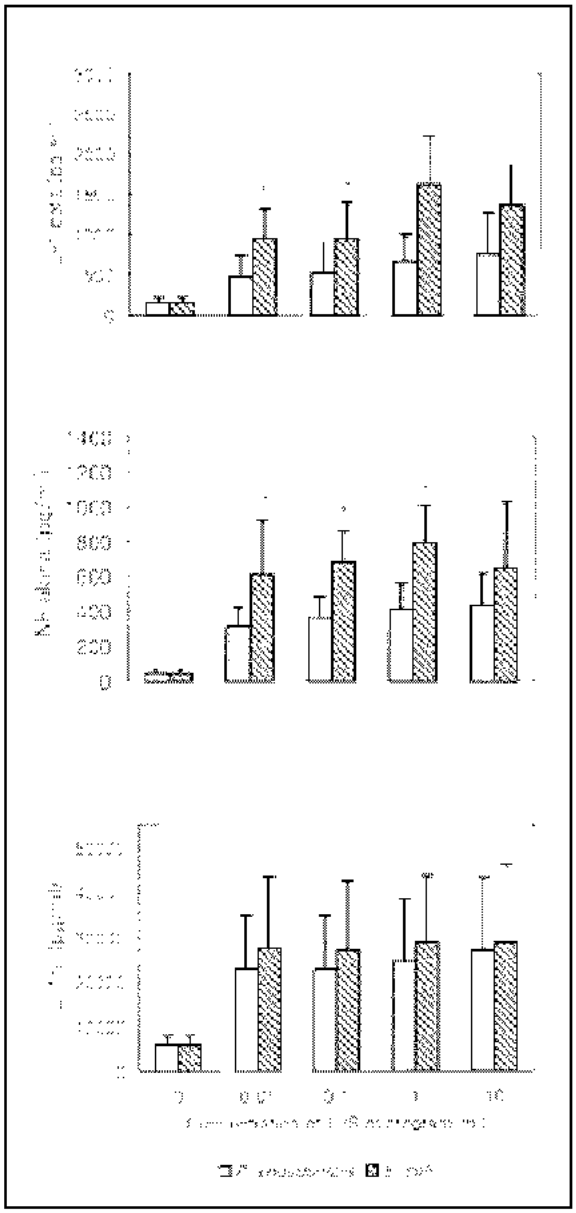

Fig. 3. Dose-response to LPS from P. endodontalis and E. coli of cytokine production in the supernatants of PMN. The cells were treated with various concentrations of each LPS for 18h. The data represent mean values with standard deviation. All of the experimental groups represented significantly higher concentrations ($p<0.05$) than control groups. + indicates significant differences($p<0.05$) between cells stimulated with P. endodontalis LPS and cells stimulated with E. coli LPS (n−10 for each group).

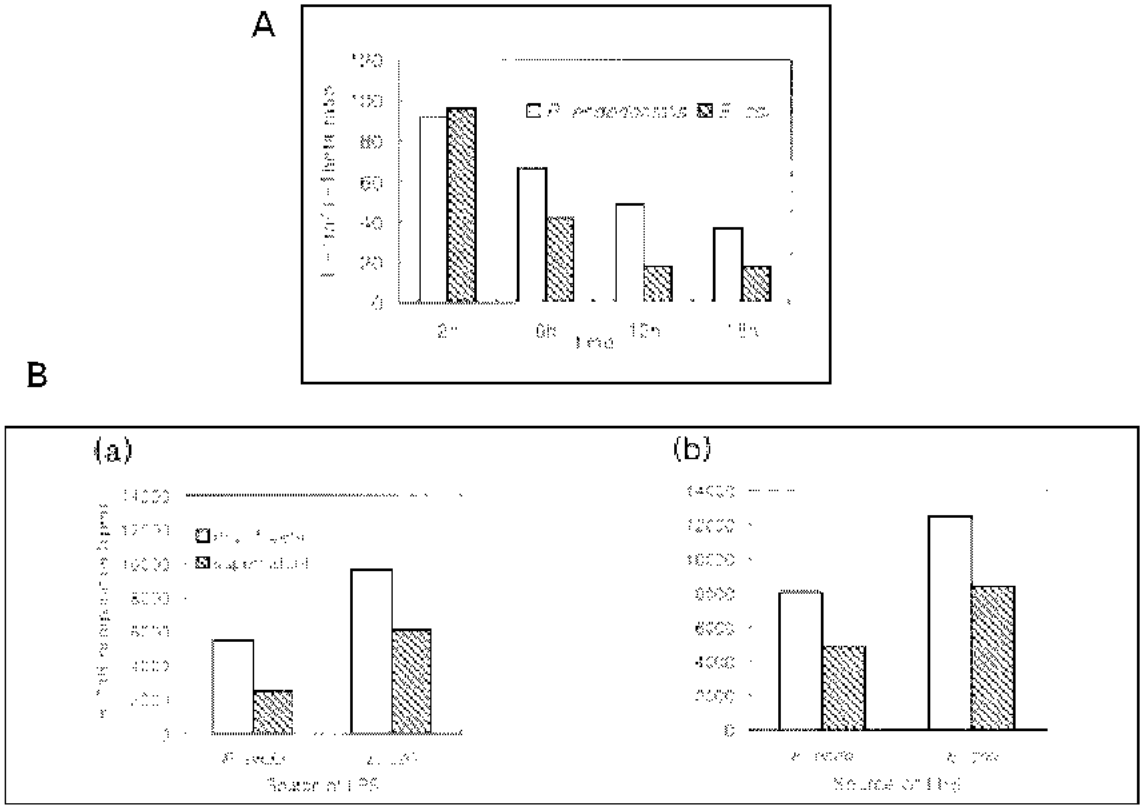

Fig. 4. **A.** The ratio of mean amounts of IL-1ra and IL-1β in human peripheral PMN after stimulation with 1 μg/ml of LPS from P. endodontalis and E. coli. **B.** The biological IL-1 activity in the supernatants of PMNs was compared with the same doses of recombinant human IL-1β (rhIL-1β) detected by 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 [^{3}H]-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 ap parent at 1h, then decreased by 2h, but repeat edly, increased again by 4h. As seen in Fig. 1, TNF α 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 1ra mRNA expression in PMN treated with P. endodontalis LPS peaked at 1h and then de creased 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, TNF α and IL 1ra 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 cy tokines 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 stim ulated with P. endodontalis LPS. Production of

IL 1β and IL 1ra by PMN stimulated with P. endodontalis LPS continued to increase until 18h, however, production of those 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[45,46]. 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[47]. Whereas IL 1 by itself is much more potent than TNF, these mediators also synergize to stimulate bone resorption[23]. IL 1 and TNF α in turn induce the expression of IL 6[47], which, besides its immunoregulatory effects, increases osteoclast formation and has been reported to stimulate bone resorption[48].

IL 1ra is an anti inflammatory cytokine, which binds to the IL 1 receptor but does not initiate IL 1 signal transduction[3,33]. 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[49].

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 synthesize 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 involved in infection and immunity, such as LPS, in activated streptococci, IL 1β, TNF α, IL 6, IFN γ, G CSF, and GM CSF[50,51]. 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 mRNA 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.[8] 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. ochracea LPS.

It has been known that LPS from different 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 A of enterobacterial LPS and is monophosphorylated, and these are regarded as reasons why it is less endotoxic than enterobacterial LPS[52]. Matsushita et al.[53] 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, Firoozkoohi et al.[4] 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.[54]. 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 inflammatory protein 1α and interferon α as well as IL 1β, TNF α and IL 1ra after treatment with LPS, and the PMN centered cytokine network is very complex[31]. 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[3,55]. 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 Rossomando et al.[56] 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[56].

Since it has been reported that IL 1ra exhibits dose responsive inhibition of IL 1α and IL 1β mediated augmentation of mitogen induced murine thymocyte proliferation[35], a thymocyte comitogen 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[36]. Such excesses of IL 1ra are required because of the extreme sensitivity of the IL 1 type I receptor and rapid in vivo clearance[35]. 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 periapical 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 those bacteria activate PMN themselves and other cell types including monocytes/macrophages and lymphocytes, stimulate further cytokine secretion, and subsequently, cause pulpal or periapical 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 1 β 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.

REFERENCES

1. Jiang Y, Magli L, Russo M. Bacterium dependent induction of cytokines in mononuclear cells and their pathologic consequences in vivo. Infet Immun 1999;67:2125 2130.
2. Baumgartner J, Falkler WJ. Bacteria in the apical 5mm of infected root canals. J Endod 1991;17:380 383.
3. Yoshimura A, Hara Y, Kaneko T, Kato I. Secretion of IL 1β, TNF α, IL 8 and IL 1ra by human polymor phonuclear leukocytes in response to lipopolysaccha rides from periodontopathic bacteria. J Periodont Res 1997;32:279 286.
4. Firoozkoohi J, Zandi H, Olsen I. Comparison of lipopolysaccharides from Bacteroides, Porphyromonas, Prevotella, Campylobacter and Wolinella spp. by Tricine SDS PAGE. Endod Traumatol 1997;13:13 18.
5. Van Winkelhoff AJ, Van Steenbergen TJM, De Graaff J. The role of black pigmented Bacteroides in human oral infections. J Clin Periodontol 1988;15:145 155.
6. Slots J and Genco RJ. Microbioal pathogenicity : Black pigmented Bacteroides species, Capnocytophaga species, and Actinobacillus actino-mycetemcomitans in human periodontal disease : Virulence factors in colonization, survival, and tissue destruction. J Dent Res 1984;63(3) :412 421.
7. Shapira et al. Strain dependent activation of mono cytes and inflammatory macrophages by LPS of P. gin givalis. Infect Immun 1998;66(6):2736 2742.
8. Hosoya S, Matsushima K. Stimulation of interleukin 1 β production of human dental pulp cells by Porphyromonas endodontalis lipopolysaccharide. J Endod 1997;23:39 42.
9. Baqui AA et al. Antigen activation of THP 1 human monocytic cells after stimulation with lipopolysaccha ride from oral microorganisms and granulocyte macrophage colony stimulating factor. J Periodon Res 1999;34(4):203 13.
10. Tanamoto K et al. The lipid A moiety of Porphyromonas gingivalis lipopolysaccharides specifi cally mediates the activation of C3H/HeJ mice. J Immunol 1997;158(9): 4430 6.
11. Reddi K, et al. Lipid A associated proteins from peri odontopathogenic bacteria induce interleukin 6 produc tion by human gingival fibroblasts and monocytes. FEMS Immunol Med Microbiol 1995;11(2):137 44.
12. Wilson M. Biological activities of lipopolysaccharides from oral bacteria and their relevance to the pathogen esis of chronic periodontitis. Sci Prog 1995;78(1):19 34.
13. Nagaoka S et al. IL 8 gene expression by human den tal pulp fibroblast in cultrues stimulated with Prevotella intermedia lipopolysaccharide. J Endod 1996;22(1):9 12.
14. Ogawa T, et al. Immunobiological activities of chemi cally defined lipid A from lipopolysaccharide of Porphyromonas gingivalis. Microbiology 1994;140(5):1209 16.
15. Gemmell E and Seymour GJ. Interleukin 1, inter leukin 6 and transforming growth factor β production by human gingival mononuclear cells following stimula tion with Pophyromonas gingivalis and Fusobacterium nucleatum. J Periodont Res 1993;28:122.
16. Schytte Blix IJ, Helgeland K, Hvattum E, Lyberg T. Lipopolysaccharide from Actinobacillus action-mycetemcomitans stimulates production of inter leukin 1β, tumor necrosis factor α, interleukin 6 and interleukin 1 receptor antagonist in human whole blood. J Periodont Res 1999;34:34 40.
17. Safavi KE, Rossomando EF. Tumor necrosis factor identified in periapical tissue exudates of teeth with apical periodontitis. J Endod 1990;17:12 14.
18. Stashenko P, Jandinski JJ, Fujiyoshi P, Rynar J, Scransky SS. Tissue levels of bone resorptive cytokines in periodontal disease. J Periodontol 1991;62:504 509.
19. Roberts FA, Hockett RD, Bucy RP, Michalek SM. Quantitative assessment of inflammatory cytokine gene expression in chronic adult periodontitis. Oral Microbial Immunol 1997;12:336 344.
20. Gowen M, Wood DD, Ehrie EJ, McGuire MKB, Russel RGG. As interleukin 1 like factor stimulates bone reso prtion in vitro. Nature 1983;306:378 380.
21. Dewhirst FE, Stashenko P, Mole JE, Tsurumachi T. Purification and partial sequence of human osteoblast activating factor : Identify with interleukin 1β. J Immunol 1985;135:2562 2568.
22. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibi tion of bone formation in vitro by human tumor necro sis factors. Nature 1986;319:516 518.
23. Stashenko P, Dewhirst FE, Peros WJ, Kent RL, Ago JM. Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorp tion. J Immunol 1987;138: 1464 1468.
24. Stashenko P, Dewhirst FE, Rooney ML, DesJardins LA, Heeley JD. Interleukin 1β is a potent inhibitor of bone resorption in vitro. J Bone Min Res 1987;2:559 565.
25. Konig A, Muhlbauer RC, Fleisch H. Tumor necrosis factor α and interleukin 1 stimulate bone resorption in vivo as measured by urinary(3H) tetracycline excretion from prelabeled mice. J Bone Min Res 1988;6:621 627.
26. Sabatini M, Boyce B, Aufdemorte T, Bonewald L, Mundy GR. Infusions of recombinant interleukins 1α and 1β cause hypercalcemia in normal mice. Proc Natl Acad Sci(USA) 1988;85:5232 5239.
27. Nguyen L, Dewhirst FE, Hauschka PV, Stashenko P. Interleukin 1β stimulates bone resorption and inhibits bone formation in vivo. Lymphokine Cytokine Res 1991;10:15 21.
28. Tatakis DN, Schneeberger G, Dziak R. Recomobinant interleukin 1 stimulates prostaglandin E2 production by osteoblastic cells : Synergy with parathyroid hor mone. Calcif Tissue Int 1988;42:358 362.
29. Mizel SB, Dayer JM, Krane SM, Mergenhagen SE. Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lympho cyte activating factor. Proc Natl Acad Sci(USA) 1981;78:2474 2477.
30. Stashenko P, Obernesser MS, Dewhirst FE. Effect of immune cytokines of bone. Immunol Invest 1989;18:239 249.
31. Cassatella MA. The production of cytokines by poly morphonuclear neutrophils. Immunol Today 1995;16:21 26.
32. Llyod AR, Oppenheim JJ. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the affer

ent limb of the immune response. Immunol Today 1992;13:169 172.
33. Re F, Mengozzi M, Muzio M, Dinarello CA, Mantovani A, Colotta F. Expression of interleukin 1 receptor antagonist(IL 1ra) by human circulating polymor phonuclear cells. Eur J Immunol 1993;23:570 573.
34. Nerad JL, Griffiths JK, van der Meer JWM. Interleukin 1β, IL 1 receptor antagonist, and TNF α production in whole blood. J Leuk Biol 1992;52:687 692.
35. Arend WP, Welgus HG, Thompson RC, Eisenberg SP. Biological properties of recombinant human monocyte derived interleukin 1 receptor antagonist. J Clin Invest 1990 May;85(5):1694 1697.
36. Lennard AC. Interleukin 1 receptor antagonist. Crit Rev Immunol 1995;15:77 105.
37. Haapasalo M. Black pigmented gram negative anaer obes in endodontic infections. FEMS Immunol Med Microbiol 1993;6:213 217.
38. Westphal O, Jann K. Bacterial lipopolysaccharides. Extraction with phenol water and further applications of the procedure. Methods Carbohydr Chem 1965; 5: 83 91.
39. Koga T, Nishihara T, Fujiwara T, Nisizawa T, Okahashi N, Noguchi T, Hamada S. Biochemical and immunobiological properties of lipopolysaccharide from Bacteroides gingivalis and comparison with LPS from Escheria coli. Infect Immun 1985; 47(3): 638 647.
40. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate phenol chlo roform extraction. Anal Biochem 1987; 162: 156 159.
41. Nishida T, Nishino N, Takano M, Kawai K, Bando K, Masui Y, Nakai S, Hirai Y. cDNA cloning of IL 1α and IL 1β from mRNA of U937 cell line. Biochem Biophys Res Commun 1987;143:345 352.
42. Wang AM, Creasey AA, Ladner MB, Lin LS, Strickler J, Van Arsdell JN, Yamamoto R, Mark DF. Molecular cloning of the complementary DNA for human tumor necrosis factor. Sicence 1985;228:149 228.
43. Mulero JJ, Pace AM, Nelken ST, Loeb DB, Correa TR, Drmanac R, Ford JE. IL1HY1 : A novel interleukin 1 receptor antagonist gene. Biochem Biophys Res Commun 1999;263:702 706.
44. Economou J, Shin H. Lymphocyte activating factor. Generation and physiochemical characterization. J Immunol 1978;121:1446 1452.
45. Barkhordar RA, DeSouza YG. Human T lymphocyte subpopulations in human periapical lesions. Oral Surg Oral Med Oral Pathol 1988;65:736 766.
46. Torabinejad M, Kettering JD. Identification and rela tive concentration of B and T lymphocytes in human chronic periapical lesions. J Endod 1985;11:122 125.
47. Dinarello CA. Interleukin 1 and its biologically related cytokines. Adv Immunol 1988;44:153 205.
48. Vaes G. Cellular biology and biochemical mechanism of bone resorption. Clin Orthop 1988;231:239 271.
49. Dinarello CA. Interleukin 1 and interleukin 1 antago nism. Blood 1991;77:1627 1652.
50. Colotta F, Re F, Polentarutti N, Sozzani S and Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 1992;80(8):2012 2020.
51. Brach MA, deVos S, Gruss JH, Herrmann F. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte macrophage colony stimu lating factor is caused by inhibition of programmed cell death. Blood 1992;80(11) :2920 4.
52. Hofstad T, Skaug N, Sveen K. Stimulation of B lym phocytes by LPS from anaerobic bacteria. Clin Inf Dis 1993;16(suppl.4):S200 2.
53. Matsushita K, Tajima T, Tomita K, takada H, Nagaoka S, Torii M. Inflammatory cytokine production and specific antibody responses to lipopolysaccharide from endodontopathic black pigmented bacteria in patients with multilesional periapical periodontitis. J Endod 1999;25:795 799.
54. Palma C, Cassone A, Serbousek D, Pearson CA, Djeu JY. Lactoferrin release and interleukin 1, interleukin 6, and tumor necrosis factor production by human polymorphonuclear cells stimulated by various