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# Immunoexpression of CXC-chemokine and recruitment of polymorphonuclear leukocytes in the rat molar periodontal tissue after topical application of lipopolysaccharide

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**Running Title:** Chemokines and periodontitis

## ABSTRACT

Accumulating evidence indicates that an important event in the tissue response in periodontal disease is the recruitment of polymorphonuclear leukocytes (PMN). This study investigated immunohistochemical expression of CXC-chemokines, including macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant-2 (CINC-2) in rat periodontal tissue at 1 and 3 hours and 1, 2, 3 and 7 days after topical application of lipopolysaccharide (LPS; 5 mg/ml in saline) from *E. Coli* into the rat molar gingival sulcus. In normal periodontal tissues before LPS application, a small number of MIP-2 and CINC-2 positive cells were seen in junctional epithelium (JE), especially in its coronal half. After topical application of LPS, a prominent increase of MIP-2 and CINC-2 positive JE cells was observed. Almost all JE cells strongly expressed them at day 1 and day 2, and then the number of chemokine-positive cells returned to normal at day 7. Corresponding to these chemokine expressions, LPS application induced a significant increase in number of PMNs in sub-JE area at 1 hour ($P < 0.01$) and 3 hours ($P < 0.05$) and a significant increase in JE area at 1 day ($P < 0.05$) and 2 days ($P < 0.01$), indicating a dynamic flow of PMNs from the sub-JE area into JE.

These findings indicated that JE cells produced MIP-2 and CINC-2 in response to LPS stimulation and suggested that MIP-2 and CINC-2 may be responsible for PMN migration toward the periodontal pathogen and may play an important role in the initiation of inflammation and subsequent periodontal tissue destruction.

Superficial periodontal tissues are constantly exposed to a plaque-associated bacteria and bacterial lipopolysaccharides ( LPS) which can induce an inflammatory reaction and consequent tissue destruction (27). Inflammation is an essential component of the host defense response to bacterial challenge, and the migration of polymorphonuclear leukocytes ( PMNs) from blood vessel into periodontal tissues is a critical part of the initial inflammatory responses ( 8,18) . In fact, many PMNs are present in diseased gingival connective tissue subjacent to the junctional epithelium ( JE) and continuously migrate into the gingival sulcus through the JE ( 14,28) .

PMNs recruitment into the site of bacterial infection results from many processes including activation of capillary loops, adhesion of PMNs to endothelial cells, transendothelial migration and migration towards the infecting bacteria. Adhesion molecules and chemokines are key mediators for these processes. CXC-chemokines are low molecular weight proteins, which have been reported to be the most powerful mediator for selective PMNs-recruitment and activation. It is well known that interleukin-8 ( IL-8) is the most potent human CXC-chemokine. IL-8 can be produced by various cells including leukocytes, fibroblasts, endothelial cells and keratinocytes, in response to both endogenous and exogenous stimuli (1,10). In particular, it is upregulated by bacterial LPS and proinflammatory cytokines such as interleukin-1 ( IL-1) and tumor necrosis factor-α ( TNF-α) ( 29) . Recent reports had indicated that IL-8 protein and their mRNA were present in human inflamed gingival tissues and that IL-8 protein levels in gingival crevicular fluid ( 4,9) and gingival tissue significantly increased in diseased sites and were related to the influx of PMNs ( 24-26) .

In previous studies, we have reported that initial periodontal tissue destruction is provoked by topical application of 5 mg/ml LPS from *Escherichia coli* into the rat gingival sulcus (6,11,12,26). In summary, infiltration of numerous PMNs in the JE and sub-JE area (6), transient accumulation of exudative macrophages (11), vascular dilatation and inflammatory edema in the sub-JE area, enhancement of proliferative activity of JE cells (26) and stimulation of osteoclastic bone resorption showing a biphasic response peaking at 3 hours and 3 days after LPS treatment (6) were observed. Furthermore, we reported transient over-expression of proinflammatory cytokines, namely TNF-α, IL-1α and IL-1β, in the JE cells with a peak at 3 hours and suggested that JE cells may play an important role in the first line defense against LPS-challenge and the following tissue destruction (12). Considering the inductive production of IL-8 by these proinflammatory cytokines and a possible role as specific mediator of PMNs-influx, it is interesting to investigate the relation between the dynamic changes of IL-8 expression in periodontal tissue and PMNs-migration during an LPS-induced acute inflammatory episode using this animal model. However, the murine counterpart of IL-8 has not been identified yet. It is

likely that IL-8 dose not exist in rodents and that other murine CXC-chemokines replace IL-8. Therefore in the present study, we investigated the immunologic expression of two important murine CXC-chemokines including macrophage inflammatory protein-2 ( MIP-2 ) and cytokine-induced neutrophil chemoattractant-2 ( CINC-2) in rat periodontal tissues after topical application of LPS into the gingival sulcus and compared the immunologic expression of MIP-2 and CINC-2 with local infiltration of PMNs.

## MATERIALS AND METHODS

**Animal experiment.** A total of twenty-one, 7 week-old (about 190 g), male Wistar Strain rats were used in this study. They were divided into 7 groups of 3 rats each. Under intraperitoneal anesthesia of 20% ethyl carbamate (100 mg/100g body weight), a rat was fixed on his back on an experimental stand. A cotton roll (2 mm in diameter and 1 cm in length) saturated with 5 mg/ml LPS from *Escherichia Coli* (Sigma Chemical Co., St. Louis, MO U.S.A.) in sterile physiological saline (Otsuka Med. Co., Tokyo Japan) was placed on the occlusal surface of the right and left upper molar regions for 1 hour. The cotton roll was changed every 20 minutes. Three rats each were killed at 1, 3 hours or 1, 2, 3 or 7 days after the LPS treatment by over dose of ethyl ether. The remaining 3 rats were used as untreated control group.

The experimental protocol was approved by the animal care committee of Hiroshima University.

Tissue samples were resected *en bloc* from the right and left upper molar regions and fixed for 8 hours in a periodic-lysin paraformaldehyde solution at 4℃ . The samples were cut into two parts, which included the first or second molar (about 2 mm thick), respectively at the buccopalatal plane parallel to each disto-palatal root. They were then decalcified in a 10% ethylenediaminetetraacetate (EDTA) solution in a phosphate buffered saline (PBS) (pH 7.4) for 5 days at 4 ℃. The decalcified tissue blocks were embedded in OCT compound (Tissue Tec, Miles Scientific, Naperville, IL). Serial frozen sections (8μ m thick) parallel to the long axis of the tooth, including the root apex were cut and collected on glass slides.

**Polyclonal antibodies.** The CL95575AP polyclonal antibody (CEDARLANE Laboratories Ltd., Ontario, Canada) was employed to detect rat MIP-2. Rat CINC-2 was identified with the anti-rat GRO/CINC-2α, 2β rabbit IgG (IBL, Fujioka, Japan), which was produced using highly purified recombinant peptide for common N-terminal region of rat GRO/CINC-2α, 2β and did not show cross-reactivity with rat GRO/CINC-1 and MIP-2.

**Immunohistochemistry.** The following immunostaining was carried out using a DAKO-LSB2 kit (DAKO Co., Carpinteria, CA). After washing in PBS each section was incubated with normal goat serum for 30 minutes at 4℃ and then incubated with polyclonal

antibodies to CXC-chemokines for 24 hours at 4 ℃ in a humid atmosphere. Polyclonal antibodies to MIP-2 and CINC-2 were diluted in 0.001M PBS containing 5% normal rat serum to 1/100 and 1/500, respectively. After being rinsed with PBS the sections were incubated with biotinylated rabbit anti-mouse IgG serum containing 5% normal rat serum for 30 minutes. The sections were rinsed in PBS and immersed in 0.3% hydrogen peroxide in PBS to block the endogenous peroxidase activity for 1 hour. The sections were rinsed with PBS, incubated with the peroxidase-conjugated streptavidin for 30 minutes and then rinsed with PBS again. The color was developed with 0.025% 3-3'-diaminobenzidine tetrahydrochloride in Tris-HCl buffer plus hydrogen peroxide (Kyowa medics, Tokyo, Japan). The specimens were counter stained with Mayer's hematoxylin, dehydrated and then mounted.

Specificity was ascertained by substituting PBS and normal rabbit serum for each antibody.

**Histometric analysis of PMN infiltration.** The number of PMNs infiltrated into JE (JE area) and gingival connective tissue subjacent to JE (sub-JE area) was statistically analyzed. For the morphometric analysis, 10 representative specimens stained with hematoxylin and eosin from each experimental group were selected and the number of PMNs in the JE area and sub-JE area was counted. These two counting areas are illustrated in Fig. 1. 1)**PMNs in JE area:** the palatal gingival tissue of each selected specimen was photographed under a magnification of X 33. On the color prints (enlarged at final magnification of X 180), the number of PMNs seen in JE area was counted. After tracing the JE area on translucent paper using the same prints, the JE area traced was measured by the LA 500 image analysis system (PIAS Inc., Osaka, Japan). The number of PMNs in a unit area (1 $mm^2$) was calculated. 2)**PMNs in sub-JE area** : Using the same specimen, PMNs infiltrated in a 0.13 X 0.13 $mm^2$ connective tissue area subjacent to JE were counted under a light microscope equipped with an ocular micrometer and the number of PMNs seen in a unit area (1 $mm^2$) was also calculated. The results were shown as mean ± SE. According to the Fisher's system, the mean values obtained were analyzed for statistical differences using Wilcoxon's test for non-paired examination.

## RESULTS

**Histological findings.** In the normal gingival tissue of untreated control rats, the JE showed minimal migration of PMNs through intercellular spaces. In the connective tissue area subjacent to the JE, a small number of PMNs was seen but no obvious inflammatory changes were observed (Fig.2A). LPS application caused edematous changes, dilatation of blood capillaries and infiltration of PMNs into the gingival connective tissue subjacent to the JE. Numerous PMNs migrated into the enlarged intercellular spaces of JE (Fig.2B). These findings

have appeared in 1-hour specimens and persisted until 3 days after LPS application. In the case with severe intra JE infiltration of PMNs, initial pocket formation was seen associated with JE cell damage by PMNs. The inflammatory changes gradually decreased with time and disappeared by day 7.

Neither lateral JE nor apical migration of the JE was seen throughout the experimental periods.

**Localization of MIP-2 expression cells.** Although weakly positive reactions for MIP-2 were partially seen in the JE of the normal gingival tissue( Fig. 3A), oral gingival epithelium and oral sulcular epithelium were negative. Various types of cells seen in gingival connective tissue and periodontal ligament did not express MIP-2.

At 3 hours after topical application of LPS, the expression of MIP-2 in JE was enhanced and the number of MIP-2 positive cells was increased. The expression of MIP-2 gradually enhanced over time. At 1 and 2 days after LPS application, almost all cells in JE were intensively positive for MIP-2 ( Fig.3B) . Numerous PMNs with weak positivity for MIP-2 were seen in widely enlarged intercellular spaces between MIP-2 positive JE cells. Both the intensity of MIP-2 expression and the number of MIP-2 positive cells decreased at day 3 (Fig.3C) and returned to normal range by day 7. There was no MIP-2 expression in the gingival connective tissue and periodontal ligament throughout the experimental periods.

**Localization of CINC-2α expression cells.** Fig. 4 illustrates the localization pattern of CINC-2 protein in dentogingival junction of the control and experimental rats. Positive reactions for CINC-2α were seen in a small number of JE cells in the control rats. Especially strong positivity was seen in the coronal half of JE (Fig. 4A). Some basal cells in oral sulcular epithelium were positive for CINC-2. LPS application enhanced CINC-2 expression in gingival tissue. CINC-2 expression in JE was enhanced and many epithelial cells were positive for CINC-2 at 1 days after LPS application. In addition to intensely positive stained cells of JE in its coronal half, several cells in its apical part and PMNs were weakly positive for CINC-2 (Fig. 4B). At 3 days after LPS-application, CINC-2 expression was remarkably reduced( Fig. 4C) .

In addition, the epithelial remnants of Malassez show the staining of CINC-2( Fig. 4A, inset). Positive staining in the epithelial remnants of Malassez was constantly observed through the experimental period.

**Histometric findings.** The temporal changes in the number of PMNs in sub-JE and JE areas are demonstrated in Fig. 5. In the untreated control group, the mean number of PMNs in sub-JE and JE areas was 355 ± 96.6, 1066.5 ± 68.7 cells/mm$^2$, respectively. In sub-JE area, PMNs increase at 1 and 3 h and 1 d after LPS treatment and then gradually decreased( $P < 0.01$

at 1 h and P <0.05 at 3 h). However in the JE area, the mean number of PMNs increased gradually and reached a maximum ( 2608.9± 543.8 cells/mm$^2$) at day 1 and then decreased. The significant differences were detected at 1 day ( P< 0.05) and at 2 days( P< 0.01) .

## DISCUSSION

The CXC-chemokines are powerful mediators of PMN recruitment. A representative member of CXC-chemokines is IL-8, which is a major chemoattractant for PMNs in humans (10,28). In rats, no homologue to IL-8 has been identified. So far, four CXC-chemokines, including CINC ( or CINC-1), CINC-2α, CINC-2β and MIP-2 ( or CINC-3), have been identified in rats ( 3,13,17). They are structurally related one another and share many functions. It has been demonstrated that they have an ability to attract PMNs and have effects on other PMN functions, including adhesion molecule expression, intracellular calcium influx, and phagocytosis (13,18). It was also reported that CINC-2α and MIP-2 as the major chemoattractants in conditioned medium of the granulation tissue. The chemotactic potency of CINC-2α and MIP-2 is higher than that of CINC-1 at the concentration of 1-10 nM ( 17). Takano et al. (22) also reported that CINC-2 and MIP-2 (CINC-3) play an important role in PMN recruitment in the rat air pouch/LPS induced inflammation. In the present study, therefore we immunohistochemically examined the expression of MIP-2 and CINC-2( CINC-2α and CINC-2β) in rat periodontal tissues after LPS-challenge.

In untreated control animals, we observed that JE cells, especially in coronal half of JE, constitutively expressed MIP-2 and CINC-2. In addition, a minimum number of PMNs were also constantly seen in JE and sub-JE areas. These CXC-chemokines produced from JE cells may be responsible for PMN recruitment in JE and sub-JE areas under physiological condition. Gamonal et al. (4) examined the levels of IL-1, IL-8, IL-10 and RANTES in gingival crevicular fluid from clinically healthy gingival site. They demonstrated that IL-8 was the only cytokine detected in gingival crevicular fluid from healthy sites and that their concentrations were relatively lower than those in gingival crevicular fluid from inflamed sites. Tonetti et al. revealed that focally distributed IL-8 mRNA positive cells were constitutively detected in JE and suggested that IL-8 expression in JE may be important for the maintenance of a host-parasite equilibrium in the gingival sulcus. (24,26).

LPS application caused transient over expression of MIP-2 and CINC-2 in JE cells with a peak at 1 and 2 days. Corresponding with this peak, a significant increase of PMNs in JE and sub-JE area was seen. Therefore, an excessive production of MIP-2 and CINC-2α from JE cells may be responsible for selective PMN-recruitment from blood vessels and migration into the gingival sulcus through JE. A similar pattern of IL-8 production accompanied with PMNs

infiltration has been reported in a variety of mucosal infections( 20,27) . For example, colonic epithelial cells continuously exposed to pathogenic bacteria produced TNFα in response to bacterial invasion, and TNF-α was able to activate the inflammatory response in the intestinal mucosa by secondarily upregulated IL-8 and MCP-1 production from the epithelial cells( 20) .

Several in vitro and in vivo studies showed that gingival epithelial cells are a major source of various cytokines and chemokines in response to periodontal pathogens. Sfakiuanakis et al. (15,16) demonstrated that *Actinobacillus actinomycetemcomitans* extracts induced dose- and time- dependent expression of IL-1α, IL-1β and IL-8 in cultured gingival epithelial cells and that IL-1 inhibitors inhibited IL-8 induction by IL-1α and IL-β . TNFα and IL-1α also induced the dose-dependent expression of IL-8 in cultured gingival epithelial cells( 2) . Moreover, using in situ hybridization method, Tonneti et al. demonstrated that IL-8 mRNA positive cells were selectively located in the JE or pocket epithelium in close spatial relationship with subgingival plaque microorganisms ( 26) . In our previous studies with the present animal model, we reported that JE cells revealed transient expression of IL-1α, IL-1β and TNF-α peaking at 3 hours after LPS application ( 12) . The results support that JE cells recruit PMNs by their CXC-chemokine production induced via their own proinflammatory cytokine secretion and indicate that JE cells may be responsible for initiation of periodontal inflammation and the acute transformation of periodontal disease.

Another possible role of CXC-chemokines produced from JE cells may be the upregulation of the proliferative activity of the JE cells themselves. It is reported that the recombinant rat MIP-2 stimulated proliferation of alveolar epithelial cells( 3) and that cultured gastric epithelial cells treated with GRO/CINC-1 showed a significant increase in cell number and BrdU incorporation in a dose dependent manner ( 20) . In addition, Jarnbring et al ( 7) immunohistochemically demonstrated that IL-8 was expressed in PCNA positive proliferating keratinocytes in periodontal patient group and suggested that IL-8 may have role in keratinocyte proliferation. In this animal model, we previously reported that number of PCNA-positive JE cells was significantly increased by LPS application on day 2( 23) . Although in this animal model, the lateral proliferation or down growth of JE cells along the root surface was not observed, MIP-2 and CINC-2 produced in JE cells may also play an important role on upregulation of the proliferative activity of the JE cells.

Interestingly, in both the untreated animals and the LPS treated animals, epithelial remnants of Malassez showed constitutive expression of CINC-2. We also demonstrated constitutive expression of IL-1β in the epithelial remnants( 12) . Therefore IL-β constitutively produced by the epithelial remnant may cause the following CINC-2 production and the inductive CINC-2 may exert some critical effects in the functions of this epithelium such as their

survival in periodontal ligament.

In summary, in gingival tissue after topical application of LPS, JE cells are a major source of CXC-chemokines including MIP-2 and CINC-2. In the period associated with the marked enhancement of CXC-chemokine production, a prominent increase of PMNs infiltration in JE and sub JE area was also detected. These findings suggest that MIP-2 and CINC-2 may be responsible for PMNs migration toward the periodontal pathogen and may play an important role in the initiation of inflammation and subsequent periodontal tissue destruction. Further studies will be required to clarify the critical role of JE cells in the pathogenesis of periodontitis and the possibility of new cytokine-theraphy of periodontitis targeting CXC-chemokines.

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## FIGURE LEGENDS

Fig.1 A diagram illustrating the two areas used for counting the number of polymorphonuclear leukocytes.

(1) **JE area;** all PMNs in the JE were counted.

(2) **sub-JE area;** PMNs seen in a 0.13 × 0.13 $mm^2$ connective tissue area subjacent to JE near the contact point (arrow) of JE and OSE at the basal cell layer.

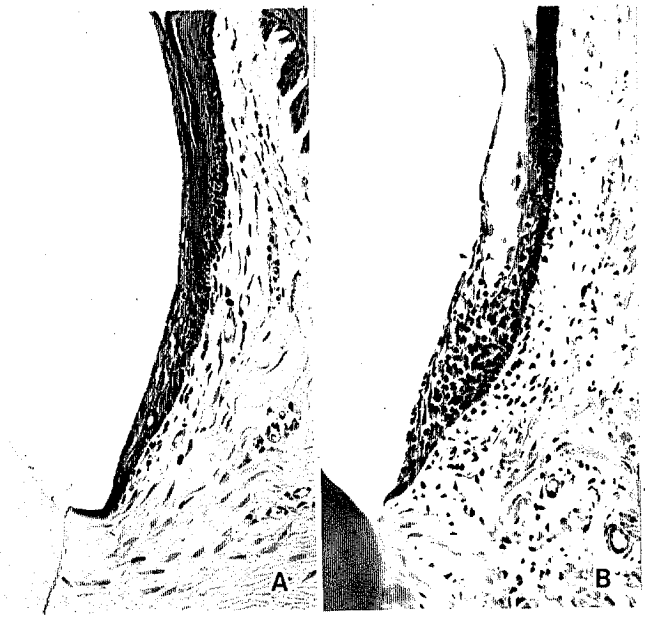
AB; alveolar bone, C; the contact point of JE and OSE at basal layer ES; enamel space, OGE; oral gingival epithelium, OSE oral sulcular epithelium, JE; junctional epithelium, T; tooth.

Fig.2 Histological findings in gingival tissues. A: **untreated control animal.** A small number of PMNs was observed in junctional epithelium. B**3 hour after LPS application**. Numerous PMNs migrated into the elongated intercellular spaces of junctional epithelial cells.( × 150, HE stain)

Fig.3 Immunohistochemical staining of MIP-2 in the gingival tissues. A**untreated control animal.** Positive staining is partially seen in the junctional epithelial cells. B**1 day after LPS application**. Almost all junctional epithelial cells are strongly positive for MIP-2. PMNs infiltrated into the gingival pocket, junctional epithelium and gingival connective tissue adjacent to junctional epithelium show weakly positive staining. GP; gingival pocket. C: **3 day after LPS application.** Number of MIP-2 positive cells are remarkably decreased. ( × 150, SAB method).

Fig.4 Immunohistochemical staining of CINC-2 in the gingival tissues. **Auntreated control animal.** Junctional epithelial cells in its coronal part were strongly positive for CONC-2. Several CINC-2 positive basal cells are seen in the oral sulcular epithelium. Epithelial remnants of Malassez show intense staining of CINC-2( inset) . B:**1 day after LPS application.** Junctional epithelial cells in its coronal part are still strongly positive for CONC-2. Several junctional epithelial cells in the apical part and PMNs were also weakly positive for CINC-2. GP; gingival pocket. C: **3 day after LPS application.** Positive reaction in gingival tissue is not detected. ( A, B, C× 150, inset, × 300, SAB method) .

Fig.5 Number of PMNs in the JE area and sub JE area ( cells/ $mm^2$) after topical application of LPS. N=10 for each group. Asterisks indicate a significant difference compared with the value in control group (*; $P < 0.05$, **; $P < 0.01$).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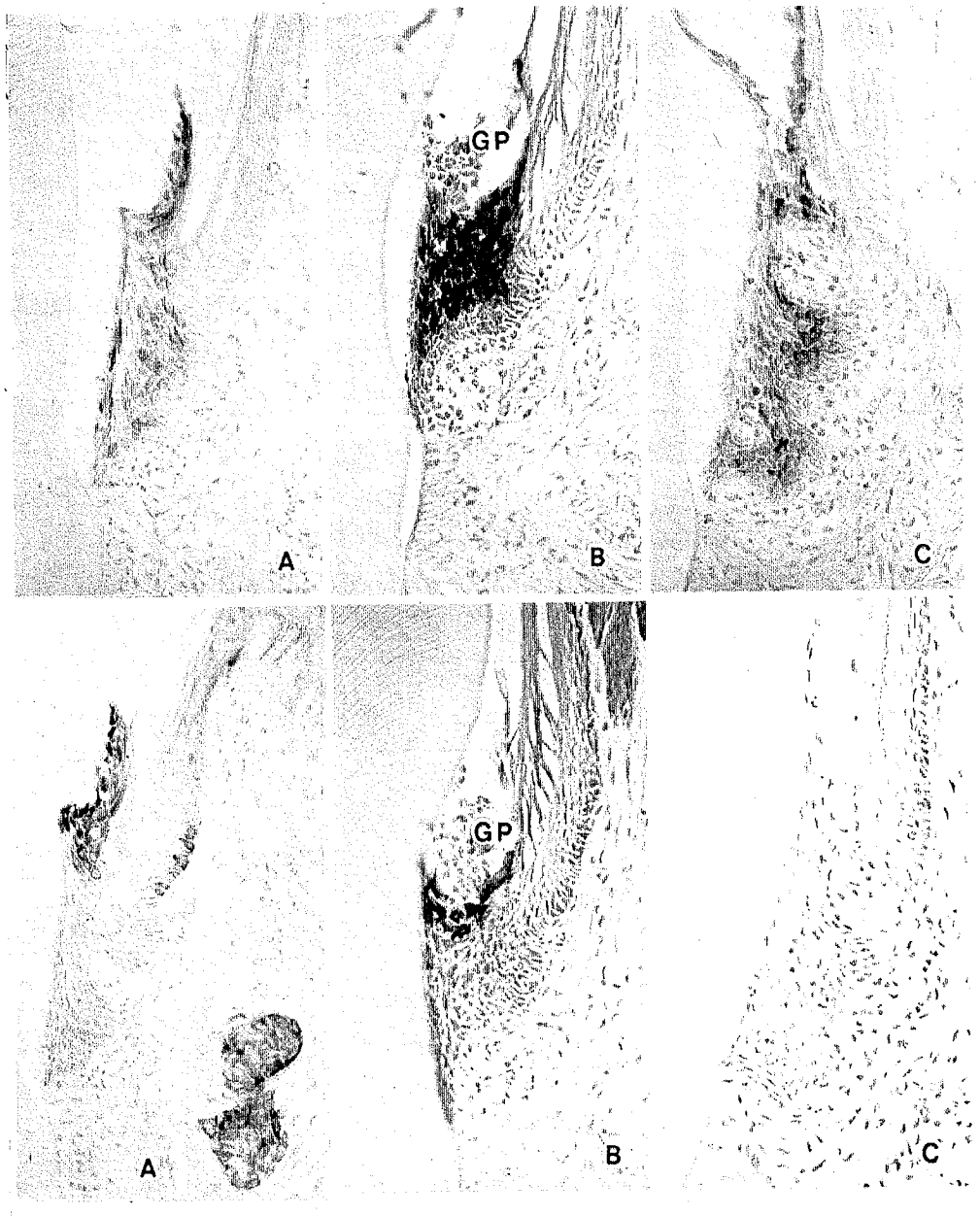
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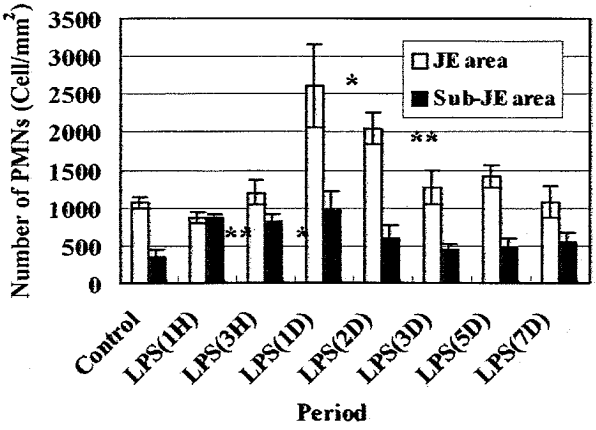
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LPS(3H)
LPS(1D)
LPS(2D)
LPS(3D)
LPS(5D)
LPS(7D)
Period