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Bacteroides (Porphyromonas) gingivalis Fimbriae Activate Mouse Peritoneal Macrophages and Induce Gene Expression and Production of Interleukin-1

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The purpose of this study was to examine whether *Bacteroides (Porphyromonas) gingivalis* fimbriae, an important structure involved in attachment of the bacteria to periodontal tissues, activate macrophages and subsequently induce gene expression and production of interleukin-1 (IL-1) in the cells. The fimbriae increased glucose consumption and lysozyme activity in BALB/c macrophages, both criteria of macrophage activation of peritoneal macrophages, in a dose-dependent fashion. A marked increase in the mRNA level of the c-*myc* gene, an oncogene, in the cells was observed after a 1-h treatment with the fimbriae, and the level decreased rapidly after 3 h. The fimbriae (4 μg of protein per ml) markedly induced IL-1α and IL-1β gene expression in the cells and IL-1 production. The expression of IL-1α and IL-1β genes measured in terms of specific mRNA increased 1 h after the start of treatment and peaked at 6 h. Such increased expression of IL-1β was also observed in C3H/HeJ mice, a lipopolysaccharide low-responder strain. The fimbriae stimulated transcriptional activity of IL-1β in the cells, but not that of IL-1α. We also observed that fimbriae-induced IL-1 gene expression was not regulated by endogenous prostaglandin triggered by the fimbriae. Therefore, these observations suggest that *B. gingivalis* fimbriae may be involved in the pathogenesis of adult periodontal disease via triggering of IL-1 production by monocytes/macrophages in periodontal diseases.

Bacteroides (Porphyromonas) gingivalis appears to be a key organism associated with periodontal destruction in patients with adult periodontitis (16, 26, 27, 32, 38). Therefore, it is very important to demonstrate the pathogenic mechanism(s) of the organism in adult periodontal disease. *B. gingivalis* fimbriae (34, 35) may be an important cell structure involved in triggering the pathogenesis brought about by the organism, because the fimbriae promote the attachment of the organism to periodontal tissues. However, the effects of the fimbriae on various functions of fibroblasts, macrophages, and lymphocytes in periodontal tissues have not been examined in detail.

Many investigators have demonstrated that monocytes/macrophages exhibit diverse functions. These functions include presentation of antigen to T and B cells, destruction of intracellular microbial pathogens, and generation and secretion of monokines. Sinden and Walker (31) showed that high levels of macrophages and T lymphocytes were found in the gingiva of patients with periodontitis. Therefore, it is very important to understand the precise functional role of macrophages in periodontal tissues. Some recent studies (14, 19, 21, 36) have shown that periodontopathic bacteria strongly induce production of interleukin-1 (IL-1) and tumor necrosis factor by monocytes/macrophages. Although we (13) showed that *B. gingivalis* fimbriae bind to human gingival fibroblasts and stimulate production of thymocyte-activating factor by these cells, as described previously by Ohmori et al. (29), whether the fimbriae induce activation of monocytes/macrophages and stimulate IL-1 production by these cells has not been examined in detail.

IL-1, an inflammatory factor, exhibits diverse functions against many target cells (8). Many recent studies indicated that IL-1 plays an important role as a local regulatory factor in bone remodeling systems (2, 5, 12, 20, 23). For example, IL-1 both potently stimulates bone resorption in vitro (9, 10) and induces differentiation of mouse osteoblastic cell line MC3T3-E1 (15, 28). Therefore, IL-1 may be an important factor in the initiation and development of inflammation and alveolar bone resorption in periodontal tissues. In fact, some investigators (1, 3, 25) have suggested that IL-1 may play a functional role in the pathogenesis of periodontal diseases.

In the present study we examined the effect of *B. gingivalis* fimbriae on IL-1 production by and expression of the IL-1 gene in mouse peritoneal macrophages. We show herein that *B. gingivalis* fimbriae strongly induce gene expression and production of IL-1 in macrophages.

MATERIALS AND METHODS

Preparation of *B. gingivalis* fimbriae. *B. gingivalis* fimbriae were prepared and purified from the cell washing by the method of Yoshimura et al. (34). Briefly, *B. gingivalis* ATCC 33277 cells were cultured anaerobically for 3 days at 37°C in a general anaerobic medium (GAM; Nissui Pharmaceutical Co., Tokyo, Japan). The bacterial cell suspension was agitated by a stirrer and magnetic bar for 30 min in 20 mM Tris-HCl buffer (pH 7.2) and then centrifuged at 8,000 × *g* for 20 min. The supernatant was precipitated with 40% saturated $(NH_4)_2SO_4$, and the precipitated materials were collected by centrifugation and purified by DEAE-Sepharose CL-6B (Pharmarcia Japan, Tokyo, Japan) column chromatography. The purified preparation was observed on sodium dodecyl sulfate (SDS)-polyacrylamide gels as a single band having a molecular mass of 43,000 Da. No significant contamination of lipopolysaccharides (LPS) was observed in the

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purified sample for the following previously stated (13) reasons: (i) no LPS in the fimbriae sample was detected by SDS-polyacrylamide gel electrophoresis; (ii) the fimbriae did not stimulate a mitogenic response in the spleen cells of C3H/HeN mice, and (iii) endotoxicity of the fimbria preparation was not detected by a colorimetric *Limulus* amoebocyte lysate assay.

Protein content of the fimbriae was measured by the method of Bradford (4).

Preparation of mouse peritoneal macrophages. BALB/c and C3H/HeJ mice, 6 weeks of age, were injected intraperitoneally with 3 ml of thioglycolate medium (Difco Laboratories, Detroit, Mich.). Peritoneal macrophages were prepared from the mice as described earlier (14).

Induction of IL-1 production by mouse peritoneal macrophages. The macrophages prepared from peritoneal exudate cells (10^5 per well) were incubated at 37°C in 5% CO_2 in each well of a Falcon 96-well (flat-well-type) microculture plate with serum-free RPMI 1640 supplemented with test samples. At various times of incubation, the culture medium was harvested and centrifuged, and the supernatant was passed through a 0.22-μm-pore-size Millipore filter membrane. IL-1 activity in the filtered supernatant was then measured.

Assay of IL-1 activity. IL-1 activity was quantified by measurement of the incorporation of [^{3}H]thymidine into C3H/HeJ mouse thymocytes treated with a suboptimal dose of phytohemagglutinin P (Difco) as described previously (14). The results were expressed as the arithmetic means of counts per minute of [^{3}H]thymidine uptake ± standard deviations for triplicate cultures.

Measurement of glucose consumption and lysozyme activity of peritoneal macrophages. The macrophages prepared from peritoneal exudate cells (10^5 per well) were incubated in each well of a Falcon 96-well (flat-well-type) microculture plate containing phenol red-free Eagle's minimum essential medium. After 24 h of incubation with test samples, the culture medium was taken from each well for measurement of glucose consumption and lysozyme activity. Glucose consumption was measured with a glucose B test Wako kit (Wako Junyaku Co., Osaka, Japan). Lysozyme activity was measured as standard egg lysozyme by the method described by Osserman and Lawlor (30). Each assay was carried out in triplicate.

cDNA hybridization probe. Plasmids containing IL-1α and IL-1β sequences described previously (22, 33) were provided by P. T. Lomedico and T. Hamilton, respectively. Also, plasmids with β-actin and c-*myc* sequences were obtained from JCRRB (Tokyo, Japan). The methods used for plasmid preparation were described earlier (24).

Preparation of RNA and Northern (RNA) blot analysis. At the end of induction, total cellular RNA was extracted by the guanidine isothiocyanate procedure (7). It was subjected to electrophoresis on a 1% agarose–0.2 M sodium phosphate gel and blotted onto a nylon membrane (MSI Magnagraph) by capillary transfer. The membranes were baked and then prehybridized for 12 h at 42°C in a prehybridization solution consisting of 50% formamide, 1% SDS, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 1× Denhardt solution (0.02% Ficoll, 0.02% bovine serum albumin [BSA], 0.02% polyvinylpyrolidone), 0.25 mg of denatured salmon sperm DNA per ml, and 50 mM sodium phosphate buffer (pH 6.5). Hybridization to primer-extended cDNA probes labeled with 5′-[α-^{32}P]dCTP (Amersham Japan, Tokyo, Japan) for IL-1β, IL-1α, c-*myc*, and β-actin was carried out overnight at 42°C in fresh prehybridization solution containing 10% dextran sulfate. After hybridization, the membranes were washed sequentially with 2× SSC–0.1% SDS, 1× SSC, and 0.1× SSC at 55°C for 30 min each. The membranes were dried, and exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. β-Actin was used as an internal standard for quantification of total mRNA on each lane of the gel.

TABLE 1. Effect of *B. gingivalis* fimbriae on activation of mouse peritoneal macrophages[a]

Fimbria concn (μg/ml)	Glucose consumption[b] (%)	Lysozyme activity[b] (μg/ml)
0	16.0 ± 1.2	0
1	20.2 ± 2.1	0
2	22.7 ± 2.4*	20 ± 1.0*
4	31.1 ± 2.5*	40 ± 3.0*
8	39.5 ± 2.9*	50 ± 4.0*

[a] The cells from BALB/c mice were incubated with or without fimbriae, and the supernatants were harvested 24 h after the incubation.

[b] The results are expressed as means ± standard deviations for triplicate cultures. The asterisk indicates statistical significance ($P < 0.01$) compared with control (no fimbriae added).

Nuclear transcriptional assay. Macrophages from peritoneal exudate cells (2×10^7 cells) from mice injected with thioglycolate medium were treated with fimbriae (4 μg of protein per ml), and their nuclei were isolated as described previously (11). Transcription initiated in intact cells was allowed to proceed for 30 min at 30°C in the presence of 5′-[α-^{32}P]UTP, and the RNA was isolated and hybridized to slot-blotted cDNA probes (5 μg per slot). Blots were hybridized for 72 h and autoradiographed for 3 days. The β-actin gene was utilized as an internal standard.

Reagents. *B. gingivalis* LPS (B-LPS) was prepared as described previously (14). Indomethacin, prostaglandin E_2 (PGE_2), and *Escherichia coli* 0111:B4 LPS (E-LPS) extracted by the method of Westphal and Jahn (37) were purchased from LBL, Inc., Campbell, Calif.

Statistical analysis. All statistical analyses were done by using Student's *t* test.

RESULTS

***B. gingivalis* fimbriae activate some functions of mouse peritoneal macrophages.** First, we examined the effect of fimbriae on some criteria commonly used to indicate macrophage activation. Table 1 shows the results. When the peritoneal macrophages were cultured with fimbriae for 24 h, the glucose consumption and lysozyme activity of the cells increased in a dose-dependent manner. A significant increase in both was observed at a dose of 2 μg of fimbrial protein per ml.

***B. gingivalis* fimbriae induce c-*myc* gene expression in mouse peritoneal macrophages.** We assessed the inductive effect of fimbriae on activation of the peritoneal macrophages in terms of the expression of c-*myc*. As shown in Fig. 1, incubation with fimbriae altered expression of c-*myc* in the cells. After a 1-h treatment with fimbriae, a marked and dose-dependent increase in the c-*myc* mRNA level was observed. However, this striking increase was short-lived, for the level decreased rapidly after 3 h. On the other hand, the mRNA level of β-actin, an internal control for the quantification of total mRNA on the gel, was not affected in the fimbriae-treated cells. These results provide support that the fimbriae induce activation of peritoneal macrophages.

Inducing effect of *B. gingivalis* fimbriae on IL-1 production by peritoneal macrophages. Peritoneal macrophages were

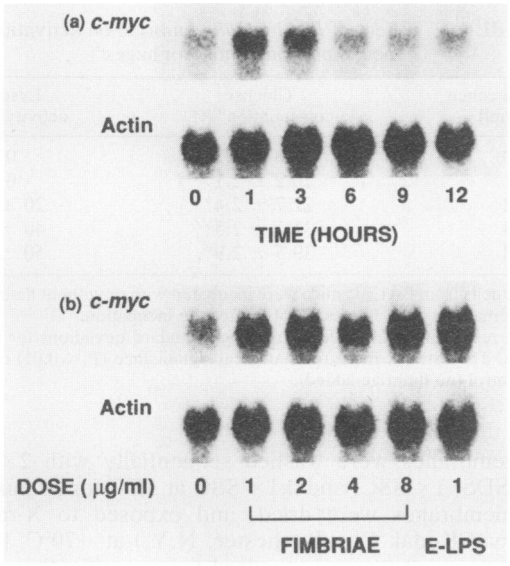

FIG. 1. Inducing effect of *B. gingivalis* fimbriae on c-*myc* mRNA level of mouse peritoneal macrophages. (a) The cells from BALB/c mice were incubated with or without the fimbriae (4 μg of protein per ml), and total RNA was prepared at various times after the start of incubation. (b) The cells from BALB/c mice were incubated for 1 h with or without various concentrations of fimbriae, and total RNA was prepared. E-LPS was used as a positive control. Northern blot analysis was performed with c-*myc* and β-actin cDNAs used as probes.

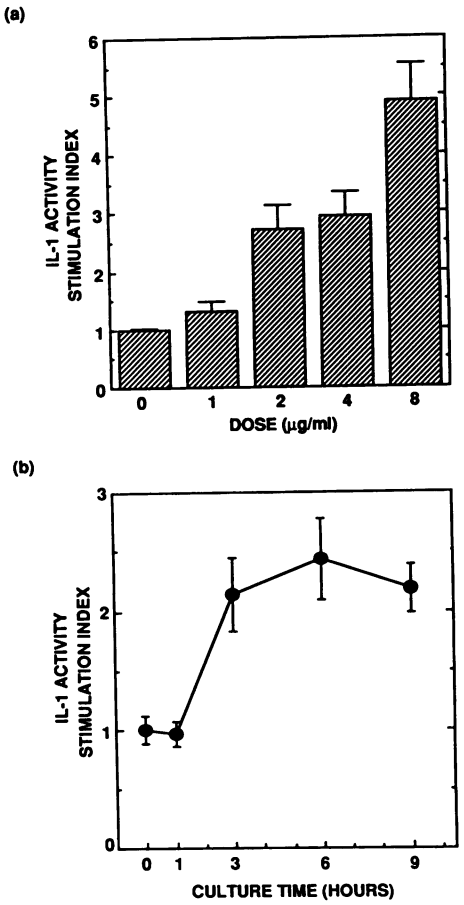

FIG. 2. Stimulatory effect of *B. gingivalis* fimbriae on IL-1 production by mouse peritoneal macrophages. (a) The cells from BALB/c mice were incubated for 6 h with or without various concentrations of fimbriae, and culture supernatants were harvested 6 h after incubation. (b) The cells from BALB/c mice were incubated for various periods of time with or without fimbriae (4 μg of protein per ml), and culture supernatants were harvested and assayed for IL-1, as described in Materials and Methods. The results are expressed as the means ± standard deviations for of triplicate cultures.

treated with fimbriae for selected periods of time, and their culture supernatants were assayed for IL-1 activity. Figure 2 shows that the fimbriae induced IL-1 production in a dose- and culture time-dependent fashion. Significant induction of IL-1 was observed after 3 h of treatment with 4 μg of fimbrial protein per ml.

***B. gingivalis* fimbriae induce IL-1 gene expression of peritoneal macrophages.** BALB/c mouse peritoneal macrophages were treated for various periods of time with the fimbriae, and their total RNA was prepared and analyzed by Northern blotting for IL-1α and IL-1β gene expression. Figure 3 shows the results. Although fimbriae at a concentration of 2 μg of protein per ml markedly increased the IL-1β mRNA level, induction of IL-1α mRNA in the cells required 4 μg of protein per ml to achieve roughly the same effect. Both mRNA levels increased in a dose-dependent fashion. IL-1α and IL-1β mRNAs first appeared 1 h after the start of fimbriae (4 μg of protein per ml) treatment, and the peak expression of each was observed at 6 h. Since the β-actin mRNA level in the fimbriae-treated cells was the same as that of untreated cells (control), the increased expression of their IL-1 genes brought about by the fimbriae is a specific response.

It is well-known that gene expression and production of IL-1 in monocytes/macrophages are markedly induced by LPS. Therefore, we examined whether LPS was involved in the induction of mRNA level and production of IL-1 in the fimbriae-treated peritoneal macrophages by using C3H/HeJ mice, a LPS low-responder strain. The peritoneal macrophages from C3H/HeJ and BALB/c mice were treated for 6 h with fimbriae or B-LPS (10 μg/ml), and the IL-1β mRNA level in the cells was then measured. B-LPS induced expression of the IL-1β gene in the BALB/c cells, but not in the C3H/HeJ ones. In contrast, the fimbriae markedly increased IL-1β mRNA level in the macrophages from both C3H/HeJ and BALB/c mice (Fig. 4). These results show that LPS does not contribute to the fimbriae-induced IL-1β gene expression.

Effect of *B. gingivalis* fimbriae on transcriptional activity of IL-1α and IL-1β genes in the peritoneal macrophages. In order to demonstrate whether the fimbriae-induced increase in IL-1α and IL-1β mRNA levels result from increased transcriptional activity, we performed transcriptional activity assay using nuclei isolated from peritoneal macrophages treated with fimbriae (4 μg of protein per ml). [^{32}P]UTP-radiolabeled RNA was hybridized to slot-blotted cDNA fragments for IL-1α, IL-1β, and β-actin. As shown in Fig. 5, the fimbriae stimulated transcriptional activity of the IL-1β gene. However, such a stimulatory effect was not observed for the IL-1α gene. These results show that the increase in mRNA for the IL-1β gene in the fimbriae-treated macrophages depends on stimulation of transcriptional activity of the gene but that this is not the case for the IL-1α gene.

***B. gingivalis* fimbriae-induced IL-1 gene expression in the**

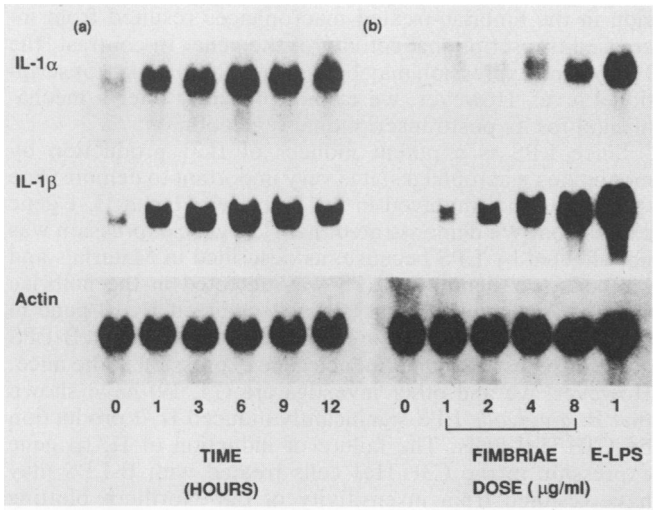

FIG. 3. *B. gingivalis* fimbriae increase IL-1α and IL-1β mRNA levels of mouse peritoneal macrophages. (a) The cells from BALB/c mice were incubated with or without fimbriae (4 μg of protein per ml), and total RNA was prepared at various times after the start of incubation. (b) The cells from BALB/c mice were incubated for 6 h with or without various concentrations of the fimbriae or E-LPS (1 μg/ml). Total RNA was then prepared, and Northern blot analysis was performed by use of IL-1α, IL-1β, and β-actin cDNA probes. E-LPS was used as a positive control.

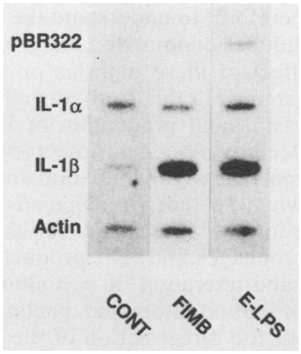

FIG. 5. Stimulatory effect of *B. gingivalis* fimbriae on transcriptional activity of IL-1β gene, but not on that of IL-1α, of mouse peritoneal macrophages. The cells from BALB/c mice were incubated with or without the fimbriae (4 μg of protein per ml) or E-LPS (10 ng/ml), and their nuclei were prepared at 6 h after the start of incubation. Transcriptional activity assay was performed with IL-1α, IL-1β, and β-actin cDNAs as described in Materials and Methods. E-LPS was used as a positive control, and pBR322 was used as a negative control. FIMB, fimbriae; CONT, control.

peritoneal macrophages is PG independent. Since PGE_2 has been shown to regulate gene expression and production of IL-1 in monocytes/macrophages, we used indomethacin, a cyclooxygenase inhibitor, to examine whether endogenous PG is involved in the fimbriae-induced increase in IL-1 mRNA level and production of IL-1 in peritoneal macrophages. As shown in Fig. 6, indomethacin did not affect the mRNA level of IL-1 in the fimbriae-treated cells. We also examined the effect of exogenous PGE_2 on the mRNA level in the cells treated with fimbriae. These results are also shown in Fig. 6. No marked increase in IL-1 mRNA level in fimbriae-treated cells was effected by 100 ng of PGE_2 per ml. These results suggest that PGs do not regulate IL-1 gene expression in the fimbriae-treated cell.

DISCUSSION

B. gingivalis fimbriae have been considered an important cell structure for mediating the attachment of the organism to periodontal tissues. This consideration has been supported by our previous report (13) indicating that the fimbriae bound specifically to the surfaces of human gingival fibroblasts. However, little is known about the function of fimbriae with respect to monocytes/macrophages. The latter cells play a functional role in inflammatory reactions and bone resorption through the release of cytokine(s) from

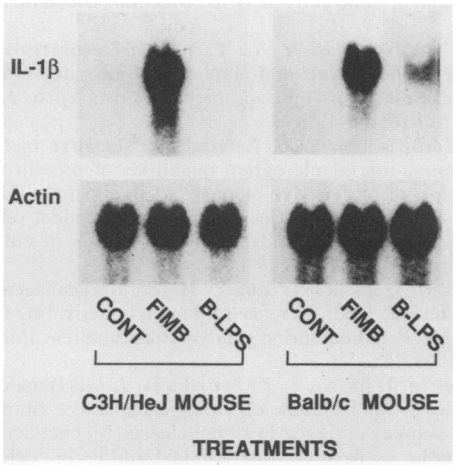

FIG. 4. *B. gingivalis* fimbriae induce gene expression of IL-1β of C3H/HeJ mouse peritoneal macrophages. The cells from C3H/HeJ and BALB/c mice were incubated for 6 h with or without fimbriae (4 μg of protein per ml) or B-LPS (10 μg/ml). Total RNA was prepared, and Northern blot analysis was carried out with IL-1β and β-actin cDNA used as probes. FIMB, fimbriae; CONT, control.

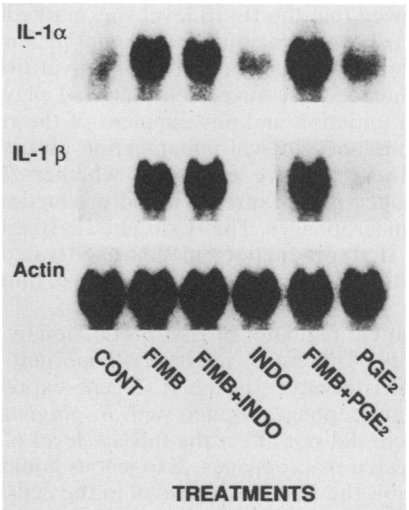

FIG. 6. *B. gingivalis* fimbria-induced IL-1α and IL-1β gene expression is PG independent. The cells from BALB/c mice were incubated with or without fimbriae (4 μg of protein per ml), indomethacin (10^{-6} M), or PGE_2 (100 ng/ml), and their total RNAs were prepared at 6 h after the start of incubation. Northern blot analysis was performed with IL-1α, IL-1β, and β-actin cDNA used as probes. FIMB, fimbriae; CONT, control; INDO, indomethacin.

themselves. Therefore, to understand the pathogenesis of *B. gingivalis* in adult periodontal disease, it is very important to examine the effect of these fimbriae on monocytes/macrophages. We examined in this study the effect of the fimbriae on gene expression and production of IL-1, an important cytokine produced by monocytes/macrophages and one that plays a functional role in inflammation and bone resorption. We have shown here that *B. gingivalis* fimbriae strongly induce expression of IL-1α and IL-1β genes of mouse peritoneal macrophages and IL-1 production.

Destruction and alteration of periodontal tissues caused by infection of periodontopathic bacteria are thought to result from both the direct action of the bacterial products and indirect actions of inflammatory macrophages activated by the bacterial cell components. Therefore, it is important to examine whether *B. gingivalis* fimbriae activate macrophage functions. We showed that the fimbriae significantly activated selected functions of mouse peritoneal macrophages, i.e., glucose consumption and lysozyme activity. Their activating effects were also confirmed by markedly increased expression of c-*myc* gene in cells treated with the fimbriae. Introna et al. (18) showed that early expression of the c-*myc* gene in macrophages is related closely to their activation. Therefore, c-*myc* gene expression may be one marker of macrophage activation. The fimbriae induced a marked increase in the c-*myc* mRNA level in mouse peritoneal macrophages 1 h after the start of treatment, and this increase was dose dependent. Since it is well-known that activated macrophages produce various inflammatory cytokines, these results indicating macrophage activation suggested to us the possibility that the fimbriae also induce production of IL-1, a potent inflammatory and bone resorbing factor, by the macrophages.

Recent studies (2, 5, 12, 20) have revealed that IL-1 has several significant avenues of participation in connective and bone metabolism, including stimulation of bone resorption, inhibition of collagen synthesis of osteoblasts, and induction of collagenase by fibroblasts. Also and most interestingly, Charon et al. (6) have detected IL-1 in gingival fluid from inflamed regions of periodontal patients. Recently, Honig et al. (17) showed that the IL-1β level was markedly increased in gingival tissues from adult periodontal patients, although no IL-1β could be found in normal gingival tissues. These lines of evidence have suggested that IL-1 plays an important role in initiation and development of the resorption of alveolar bone and gingival inflammation observed in these patients. Therefore, we examined whether *B. gingivalis* fimbriae induce gene expression and production of IL-1 in peritoneal macrophages. The IL-1α and IL-1β mRNA levels, as well as IL-1 production, in fimbriae-treated cells were markedly stimulated in a dose- and culture time-dependent fashion.

PG is a potent regulator of IL-1 production by monocytes/macrophages. Therefore, it is very important to examine whether PG is involved in the IL-1 gene expression of the peritoneal macrophages treated with *B. gingivalis* fimbriae. Indomethacin did not affect the mRNA level of IL-1 in the fimbriae-treated macrophages. Exogenous addition of PGE_2 did not inhibit the IL-1 mRNA level in the cells. Therefore, our results indicate no involvement of PGs.

We also examined whether the fimbriae-induced IL-1 gene expression depended on stimulation of transcriptional activity of IL-1 genes. The fimbriae markedly stimulated transcriptional activity of the IL-1β gene. However, such a stimulatory effect was not observed for the IL-1α gene. These results show that the increase in IL-1β gene expression in the fimbriae-treated macrophages resulted from increased transcriptional activity of the gene. In contrast, the IL-1α gene expression may be regulated at the posttranscriptional level. However, we cannot presently offer a mechanism(s) for its posttranscriptional regulation.

Since LPS is a potent inducer of IL-1 production by monocytes/macrophages, it is very important to demonstrate that LPS is not involved in the fimbriae-induced IL-1 gene expression. We demonstrated that IL-1 gene expression was not affected by LPS because, as described in Materials and Methods, no significant LPS was detected in the fimbriae sample. Also, the fimbriae strongly induced IL-1β gene in C3H/HeJ mice, a LPS low-responder strain, although B-LPS at the dose tested did not induce gene expression in the mice. However, we and other investigators (14, 19) have shown that *B. gingivalis* LPS significantly induced IL-1 production by C3H/HeJ cells. The failure of induction of IL-1β gene expression in the C3H/HeJ cells treated with B-LPS may have resulted from insensitivity of our Northern blotting analysis, judging from the low insensitivity of IL-1β gene expression from LPS responder BALB/c mouse peritoneal macrophages with B-LPS.

In conclusion, we have demonstrated that *B. gingivalis* fimbriae strongly induced expression of IL-1 genes of mouse peritoneal macrophages, resulting in the subsequent secretion of IL-1 into the culture medium of the cells. These results suggest that *B. gingivalis* fimbriae may contribute to the pathogenesis of adult periodontal disease by triggering IL-1 production by the monocytes/macrophages in periodontal tissues.

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