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Bacteroides gingivalis Fimbriae Stimulate Production of Thymocyte-Activating Factor by Human Gingival Fibroblasts

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In a previous report (Y. Ohmori, S. Hanazawa, S. Amano, T. Miyoshi, K. Hirose, and S. Kitano, Infect. Immun. 55:947–954, 1987), we showed that human gingival fibroblasts spontaneously produce thymocyte-activating factor (FTAF), which stimulates mitogen-induced thymocyte proliferation. In the present study, we examined the effect of *Bacteroides gingivalis* fimbriae on FTAF production by the cells, because the fimbriae may be involved in attachment of the organism to periodontal tissues. We show here that the fimbriae bind to the cells, which may subsequently lead to the stimulation of FTAF production by the cells.

Gingival fibroblasts are the predominant cell type in periodontal tissues and may be involved in the regulation of immunological and inflammatory responses in their microenvironment. Therefore, it is very important to understand the precise functional role of these cells in periodontal tissues. Recently, we showed that human gingival fibroblasts spontaneously produce thymocyte-activating factor (FTAF), which stimulates mitogen-induced thymocyte proliferation, and also that FTAF induces the production from the cells themselves of prostaglandin metabolites, which in turn inhibit proliferation of the fibroblasts (8). These findings suggest that human gingival fibroblasts regulate their own proliferation in an autocrine manner.

Although *Bacteroides gingivalis* is believed to be closely associated with adult periodontal disease (6, 7, 9), the pathogenetic mechanism(s) involved in the initiation and development of the disease is not yet understood in detail. Yoshimura et al. (10) recently found novel fimbriae present on the cell surface of *B. gingivalis*. They purified the fimbriae from the organism and further characterized their physical, chemical, and immunological properties (11). The fimbriae may be an important cell structure in triggering the pathogenesis brought about by *B. gingivalis*, because the fimbriae may be involved in attachment of the organism to periodontal tissues. However, the precise interactions of the fimbriae with gingival fibroblasts, monocytes-macrophages, and lymphocytes in periodontal tissues are not yet understood. Therefore, as an approach for determining the effect of the fimbriae on the function of human gingival fibroblasts, we examined the effect of the fimbriae on FTAF production by human gingival fibroblasts. We show here that *B. gingivalis* fimbriae bind specifically to gingival fibroblasts, resulting in the subsequent stimulation of FTAF production by the cells.

A human gingival fibroblast cell line (Gin-1, CRL 1292) was obtained from the American Type Culture Collection, Rockville, Md. The cells were maintained, passaged, and cultured as described previously (8). *B. gingivalis* 381 fimbriae were used in this study. *B. gingivalis* was cultured anaerobically for 3 days at 37°C in EX-1 diffusate medium as described by Mashimo and Ellison (5). The fimbriae were prepared and purified from the cell washings by the method of Yoshimura et al. (10). The purified preparation was observed on sodium dodecyl sulfate-polyacrylamide gels as a single band having a molecular weight of 43,000. *B. gingivalis* lipopolysaccharide (LPS) was extracted and purified by the method described in our previous report (3). Purified fimbria-treated Gin-1 cell cultures were prepared as follows. A suspension of Gin-1 fibroblasts was adjusted to a density of 5×10^4 cells per ml in alpha minimum essential medium (alpha-MEM) containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), and the cells (1.2 ml) were seeded into 30-mm Falcon culture dishes and cultured for 6 days at 37°C in a 5% CO_2 incubator. After cultivation, the cells were washed three times with alpha-MEM and cultured in serum-free alpha-MEM supplemented or not supplemented with various concentrations of the purified fimbriae. At selected times, the culture supernatants were harvested, sterilized by passage through a Millex filter (0.22-μm pores; Milipore Corp., Bedford, Mass.), and frozen at −20°C until used. These culture supernatants were examined for their ability to stimulate thymocyte proliferation. FTAF activity was measured by a [^{3}H]thymidine ([^{3}H]TdR) incorporation assay with C3H/HeJ mouse thymocytes as described previously (3). The results were expressed as the arithmetic mean of the change in counts per minute of [^{3}H]TdR uptake ± the standard deviation (SD) in triplicate cultures, unless otherwise noted (change in counts per minute = [counts per minute in thymocytes plus culture medium plus phytohemagglutin {PHA}] − [counts per minute in thymocytes plus PHA]). To assay the binding of ^{125}I-labeled *B. gingivalis* fimbriae to human gingival fibroblasts, we mixed 200 μg of fimbriae, 25 μl of reconstituted Enzymobead reagent (Bio-Rad Japan, Tokyo, Japan), 1 mCi of carrier-free ^{125}I (Amersham Japan, Tokyo, Japan), and 200 mM Tris (pH 7.2) in a total volume of 100 μl. The reaction was initiated by the addition of 20 μl of 1% β-D-glucose. After 15 min of incubation on ice, 20 μl of 25 mM sodium azide was added. The ^{125}I-labeled fimbriae were purified by gel filtration on Sephadex G-75. A Gin-1 cell suspension (0.2 ml) adjusted to a density of 10^4 cells per ml was seeded into each well of a Falcon 96-well flat microculture plate, and the cells were cultured overnight in alpha-MEM containing 10% fetal bovine serum. The cultured cells were washed three times with alpha-MEM and incubated for 5 h at 4°C with or without 0.1 μg of ^{125}I-labeled fimbriae or with ^{125}I-labeled fimbriae and various doses of unlabeled fimbriae. After incubation, the cells were washed three times

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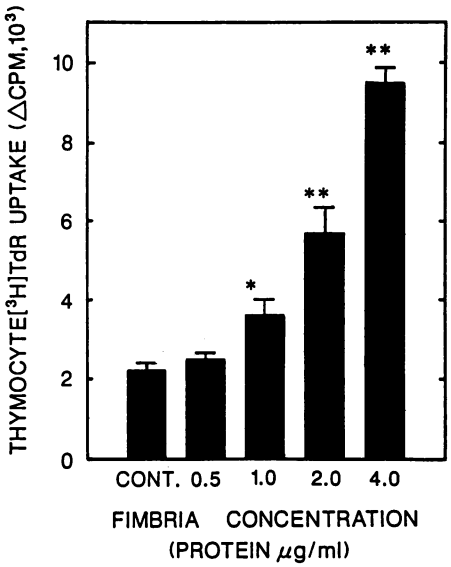

FIG. 1. Effect of *B. gingivalis* fimbriae on FTAF production by Gin-1 cells. Confluent monolayers were cultured for 24 h in serum-free alpha-MEM supplemented with various doses of fimbriae. After 24 h, the culture medium was harvested and measured for FTAF activity at a dilution of 1:4. Each column and bar represent the arithmetic mean ± SD for triplicate cultures. The levels of [^{3}H]TdR uptake in control (CONT.) thymocyte cultures were as follows: RPMI medium only, 505 ± 42 cpm; PHA only, 2,162 ± 262 cpm; and PHA plus fimbriae (4 μg/ml), 2,217 ± 196 cpm. *, $P < 0.01$, significantly different from control; **, $P < 0.005$, significantly different from control.

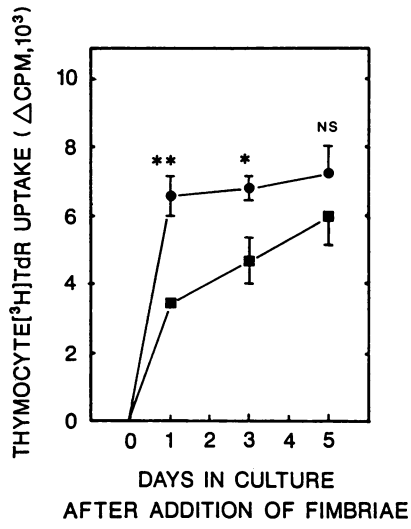

FIG. 2. Time course of the stimulatory effect of *B. gingivalis* fimbriae on FTAF production by Gin-1 cells. Confluent monolayers were cultured in serum-free alpha-MEM supplemented with (●) or lacking (■) *B. gingivalis* fimbriae (2 μg/ml). At selected times, the culture medium was harvested and measured for FTAF activity. Each point and bar represent the arithmetic mean ± SD for triplicate cultures. The levels of [^{3}H]TdR uptake in control thymocyte cultures were as follows: RPMI medium only, 508 ± 32 cpm; PHA only, 2,192 ± 231 cpm; and PHA plus fimbriae, 2,334 ± 224 cpm. *, $P < 0.01$, significantly different from control; **, $P < 0.005$, significantly different from control; NS, not significantly different from control.

with alpha-MEM and harvested with a rubber policeman. The radioactivity was measured in a gamma counter. The experiment was carried out in triplicate. The assay for mitogenicity was done as described previously (3).

We first examined the effect of purified fimbriae on FTAF production by Gin-1 cells. Confluent cells were cultured for 24 h with various doses of purified fimbriae in serum-free alpha-MEM, and the culture media were examined for FTAF activity. The fimbriae stimulated FTAF production by Gin-1 cells in a dose-dependent manner (Fig. 1). Significant stimulation was observed at a dose of 1 μg/ml. We next examined the time course of the stimulatory effect of the fimbriae on FTAF production. Fimbriae (2 μg/ml) were added to confluent cultures of Gin-1 cells, and the culture media from treated cultures were harvested at selected times and examined for FTAF activity. Although a stimulatory effect was observed as early as day 1 after treatment with the fimbriae, no significant stimulatory effect was observed on day 5 (Fig. 2). For understanding the mechanism underlying the stimulation of FTAF production by purified fimbriae, it is important to determine whether the fimbriae bind specifically to the Gin-1 cell surface. Therefore, we examined fimbriae for their ability to bind to Gin-1 cells by using ^{125}I-labeled fimbriae. The binding ability was determined by a competition binding assay with unlabeled fimbriae. The binding was effectively blocked by increasing doses of unlabeled fimbriae (Fig. 3). This result suggests that purified fimbriae from *B. gingivalis* 381 bind specifically to Gin-1 cells. It is well known that bacterial LPS is a potent stimulator of interleukin-1 and interleukin-1-like cytokine production by human monocytes, keratinocytes (4), and glioma cells (2). Therefore, it is very important to rule out LPS contamination of the fimbria preparation used for the stimulation of FTAF production. LPS was not detected on a silver-stained gel following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fimbriae (data not shown). Furthermore, the fimbriae did not induce a mito

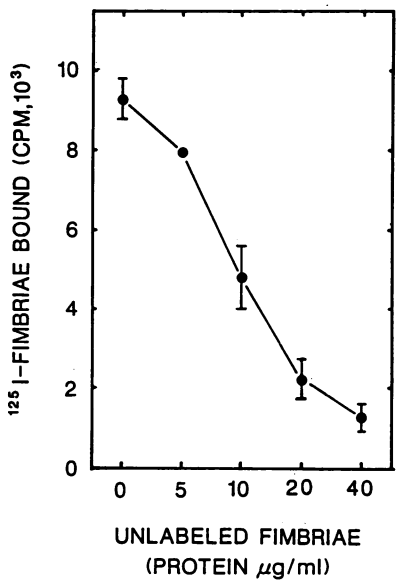

FIG. 3. Binding of ^{125}I-labeled fimbriae of *B. gingivalis* to Gin-1 cells. Gin-1 cells (10^4 cells per well) were incubated for 5 h at 4°C with ^{125}I-labeled fimbriae (0.1 μg per well) in the presence or absence of various doses of unlabeled fimbriae. After incubation, the cells were washed and harvested, and the radioactivity was measured. Each bar and point represent the mean ± SD for triplicate cultures.

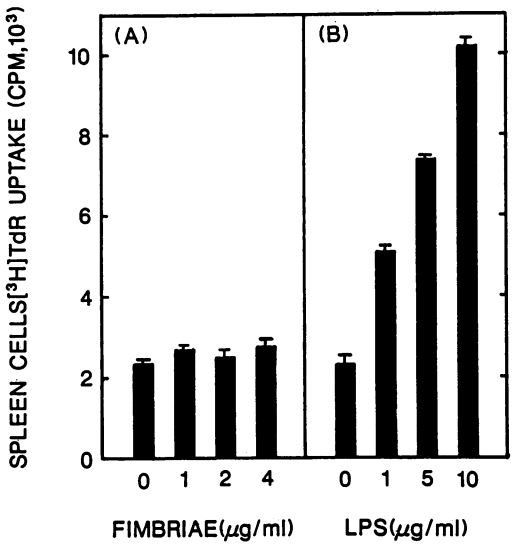

FIG. 4. Effect of *B. gingivalis* fimbriae and LPS on mitogenic responses in spleen cells from C3H/HeN mice. Spleen cells (10^5 cells per well) in U-bottomed microculture plates (Nunc, Roskilde, Denmark) were incubated for 72 h with various doses of fimbriae (A) or LPS (B) as described previously (3). [^{3}H]TdR (0.5 μCi per well) was added to each culture 24 h before termination of the culture. Each column and bar represent the mean ± SD for triplicate cultures.

genic response in C3H/HeN mouse spleen cells at the dose that stimulated FTAF production (Fig. 4). This result indicates that *B. gingivalis* fimbriae and not LPS is the stimulator of FTAF production in this case.

Charon et al. (1) have shown that thymocyte-activating factor can be detected in gingival fluid and that this activity is higher in fluid obtained during gingival inflammation. These findings suggest that FTAF may function in the regulation of the immune response in human periodontal tissue during the initiation and development of periodontal disease. It was shown previously that FTAF production was stimulated by the treatment of Gin-1 cells with extracts of sonicated *B. gingivalis* (8). Our present results suggest that the stimulatory effect of *B. gingivalis* on FTAF production may be dependent in part on the fimbriae of the organism. Although it is well known that LPS from gram-negative enterobacteria is a potent stimulator of interleukin-1 and interleukin-1-like cytokine production, *B. gingivalis* LPS is probably not involved in the fimbria-induced stimulation of FTAF production, because (i) the LPS was not detected by electrophoresis of the fimbriae on sodium dodecyl sulfate-polyacrylamide gels (data not shown); (ii) the fimbriae did not stimulate a mitogenic response in C3H/HeN spleen cells (Fig. 4); and (iii) pyrogenic activity in the purified fimbriae was not detected by a colorimetric assay for the determination of pyrogenicity (unpublished data). Since the fimbriae bound specifically to the surfaces of Gin-1 cells (Fig. 3), the stimulatory effect of the fimbriae may be mediated by some structure on the fibroblast membrane. However, at present we do not know the precise stimulatory mechanism. Although fimbriae have been considered an important cell structure for mediating the attachment of *B. gingivalis* to periodontal tissue, little is known about the function of the fimbriae with respect to gingival fibroblasts, monocytes-macrophages, and lymphocytes in periodontal tissues. Therefore, our present finding that *B. gingivalis* fimbriae stimulated FTAF production by Gin-1 cells strongly suggests that the fimbriae play a functional role in the induction of immunological and inflammatory responses in periodontal tissue by stimulating the release of a soluble factor(s) from gingival fibroblasts during the initiation and development of periodontal disease.

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