INFECTION AND IMMUNITY, July 1996, p. 2571–2576 Vol. 64, No. 7
0019-9567/96/$04.00+0

Porphyromonas gingivalis Fimbrillin Is One of the Fibronectin-Binding Proteins

YUKIO MURAKAMI,[1] HIROYOSHI IWAHASHI,[1] HIROHITO YASUDA,[1] TOSHIHIKO UMEMOTO,[2] ISAMU NAMIKAWA,[2] SHIGEO KITANO,[1] AND SHIGEMASA HANAZAWA[1]*

Department of Oral Microbiology, Meikai University School of Dentistry, Keyakidai, Sakado City, Saitama 350-02,[1] and Department of Oral Microbiology, Asahi University School of Dentistry, Hozumi, Motosu, Gifu 501-02,[2] Japan

Received 27 October 1995/Returned for modification 8 December 1995/Accepted 1 April 1996

In this study, we demonstrate that *Porphyromonas gingivalis* fimbrillin, a major component of bacterial fimbriae, is one of the fibronectin-binding proteins and that fibronectin is a potent inhibitor of the adherence of the bacteria to host cells and of the pathogenesis of the bacterium that acts by binding to the fimbriae. A Western blotting (immunoblotting) assay showed that fibronectin binds strongly to *P. gingivalis* fimbrillin. The fimbrial binding to fibronectin was also evidenced by a binding assay involving ^{125}I-labeled fimbriae. Furthermore, fibronectin markedly inhibited the fimbria-induced expression of interleukin-1β and neutrophil-specific chemoattractant KC genes in macrophages. The inhibitory action depended on the fimbrial interaction with heparin-binding and cell attachment domains in the fibronectin structure. The binding of *P. gingivalis* to mouse peritoneal macrophages via its fimbriae was inhibited by fibronectin. Fibronectin also inhibited the bacterial cell-induced expression of interleukin-1β and KC genes in the macrophages. These results demonstrate the importance of fibronectin as a modulator of the pathogenic mechanism of *P. gingivalis*, a pathogen that causes adult periodontal disease.

Adherence to host cells is the initial stage in triggering the pathogenesis brought about by pathogenic bacteria (4). Bacterial fimbriae may be an important cell structure for adherence. In many cases, the adherence takes place through the binding of fimbriae to specific receptors on the host cell surface. Therefore, it is very important to investigate substances that modulate this binding and determine their mechanism of action.

Porphyromonas gingivalis has been recognized widely as the predominant pathogenic organism in adult periodontitis, which is a chronic inflammatory disease. Chemical, immunological, and genetic characterizations of *P. gingivalis* fimbriae have been well demonstrated (39, 40). We (7, 21) previously suggested that the fimbriae attach to fibroblasts and macrophages through the binding of the former to specific receptors. Our suggestion has been confirmed genetically by a recent study showing that a mutation of the *fimA* gene encoding the major subunit of the fimbriae, fimbrillin, prevents bacterial adherence to host cells (6).

Many investigators (3, 5, 11, 14, 17–19, 28–31, 33–38) have shown that several bacteria, including streptococci, *Escherichia coli*, and *Treponema pallidum*, bind to extracellular matrix molecules such as fibronectin, collagen, and laminin. Also, several investigators (12, 16, 23) have shown that *P. gingivalis* is able to bind to the extracellular matrix. These observations suggest that the matrix proteins exert an inhibitory action in the adherence of the organism to host cells via the fimbriae. In the interaction of *P. gingivalis* with fibronectin, although Lantz et al. (16) demonstrated a 150-kDa fibronectin-binding component of the organism, the functions and characterization of this binding protein have not yet been reported. As described above, since *P. gingivalis* fimbriae are essential for the adhesion of the bacterium to the host cell, we considered the possibility that fimbrillin is one of the fibronectin-binding proteins and that the binding of the organism to host cells may be modulated by the interaction of fimbrillin with fibronectin.

Therefore, in the present study, we investigated the ability of *P. gingivalis* fimbriae to bind to fibronectin and also the inhibitory effect of fibronectin on the fimbria-induced expression of inflammatory cytokine genes in mouse peritoneal macrophages. We demonstrate here that fimbrillin strongly binds to fibronectin with high specificity and propose that fibronectin may function as an inhibitor in the pathogenesis of *P. gingivalis* via binding to the fimbriae.

MATERIALS AND METHODS

Reagents. Human fibronectin, collagen types I and V, laminin, and their polyclonal antibodies raised in rabbits were purchased from Sigma Chemical. Co., St. Louis, Mo. Heparin- and gelatin-binding and cell attachment domains of human fibronectin were obtained from Gibco BRL, Gaithersburg, Md. Na^{125}I was from Dupont New England Nuclear, Wilmington, Del. IODO-BEADS iodination reagent was obtained from Pierce, Rockford, Ill. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G and HRP color development reagent were from Bio-Rad, Richmond, Calif. *P. gingivalis* ATCC 33277 lipopolysaccharide (LPS) was prepared as described previously (8).

Preparation of *P. gingivalis* fimbriae. *P. gingivalis* ATCC 33277 fimbriae were prepared and purified from cell washings by the method of Yoshimura et al. (39) as described previously (7). Our previous study (13) demonstrated that the purified fimbriae were able to induce several biological activities and that such inducing activity could not be attributed to contaminants in the preparation. The protein content of the fimbriae was measured by the method of Bradford (2).

Preparation of antiserum to *P. gingivalis* fimbriae. The purified fimbriae (500 μg of protein) prepared from *P. gingivalis* ATCC 33277 were injected subcutaneously into a rabbit two times at a 2-week interval with Freund's complete adjuvant and 2 weeks later intraperitoneally with Freund's incomplete adjuvant. Five days thereafter, whole serum from the immunized rabbit was prepared. Western blotting (immunoblotting) analysis showed that the antiserum recognized 43-kDa fimbrillin with high sensitivity and specificity.

Preparation of mouse peritoneal macrophages. Thioglycolate-stimulated peritoneal exudate cells from 6- to 8-week-old BALB/c mice were harvested, and the peritoneal macrophages were prepared as described previously (8). The prepared macrophages were treated for selected times with test samples.

Western blotting assay. The purified fimbriae were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% polyacrylamide gel (15). After SDS-PAGE, the separated samples were transferred to nitrocellulose paper by the method described by Towbin et al. (32). After transfer, the nitrocellulose paper was treated for 2 h with extracellular matrix proteins, washed, and then further incubated for 2 h with polyclonal antibody to each matrix protein. The binding of extracellular matrix protein to the fimbriae was visualized by use of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and HRP color development reagent.

* Corresponding author. Fax: 81-492-85-1035.

Preparation of ^{125}I-fimbriae. Purified fimbriae were iodinated with IODO-BEADS iodination reagent (chloramine-T molecules immobilized on polystyrene beads). In brief, fimbriae (100 μg of protein) were added to a phosphate-buffered saline solution containing three IODO-BEADS and Na^{125}I (18.5 MBq). The reaction was stopped after 15 min, and the beads were then applied to a Sephadex G-25 column to remove the free iodine. Also, *P. gingivalis* ATCC 33277 bacterial cells (10^{11} cells per ml) were iodinated with Na^{125}I as described above and then washed five times with physiological saline to remove the free iodine.

Binding of ^{125}I-fimbriae to fibronectin. Human fibronectin (5 μg per well) was inoculated into the wells of a SUMILON 96-well-type assay plate (Sumitomo Bakeliet Co. Ltd., Tokyo, Japan), which was maintained overnight at 4°C, and then incubated for 4 h at 4°C with ^{125}I-labeled fimbriae (0.5 μg of protein per ml) in the absence or presence of unlabeled fimbriae or test sample. After incubation, the reactive substances were washed 10 times with 15 mM phosphate buffer (pH 7.2). The amount of radioactivity bound to fibronectin was measured in a gamma counter. The experiment was carried out in triplicate. The results were expressed as the mean counts per minute ± standard deviation (SD).

Binding of ^{125}I-fimbriae and ^{125}I-bacterial cells to macrophages. The macrophage monolayer prepared from mouse peritoneal exudate cells (2×10^5 cells) on each well of a 96-well-type multiple microculture plate was fixed with 8% formalin. Then ^{125}I-fimbriae (0.5 μg of protein) or ^{125}I-bacterial cells (2×10^8 cells) were inoculated onto each cell monolayer, and the cells were incubated for 4 h at 4°C in the absence or presence of various doses of fibronectin. Thereafter, the monolayer was washed 10 times with 15 mM phosphate buffer (pH 7.2). The amount of radioactivity bound to the macrophages was measured in a gamma counter. The experiment was carried out in triplicate, and the results were expressed as the mean counts per minute ± SD.

cDNA hybridization probe. A plasmid containing mouse neutrophil chemoattractant KC cDNA sequences was provided by C. D. Stiles. Also, a plasmid bearing mouse interleukin-1β (IL-1β) cDNA was provided by T. Hamilton. In addition, a plasmid with β-actin cDNA was obtained from JCRB, Tokyo, Japan. The methods used for plasmid preparation were described previously (20).

Preparation of RNA and Northern blot analysis. The macrophage monolayers prepared from mouse peritoneal exudate cells (10^7 cells) were treated or not treated with the fimbriae or *P. gingivalis* LPS (provided by T. Umemoto). Thereafter, preparation of total cellular RNA and Northern (RNA) blot analysis were performed as described previously (9). β-Actin was used as an internal standard for the quantification of total mRNA on each lane of the gel.

***P. gingivalis*- and fimbria-induced expression of inflammatory cytokine genes in mouse peritoneal macrophages.** Macrophage monolayers prepared from mouse peritoneal exudate cells (10^7 cells) on 5-cm-diameter plastic plates were incubated with *P. gingivalis* ATCC 33277 bacterial cells (10^9 cells) or their fimbriae (5 μg of protein per ml) that had been pretreated or not treated for 2 h at room temperature with anti-fimbria antiserum or fibronectin. After a 1-h incubation, the monolayers were washed five times with phosphate-buffered saline to remove the test samples. Thereafter, IL-1β and KC gene expressions in the macrophages were analyzed by the Northern blot assay.

RESULTS

***P. gingivalis* fimbriae bind to fibronectin with high specificity.** First, we examined by Western blotting assay the ability of extracellular matrix molecules such as fibronectin, laminin, and collagen types I and V to bind to *P. gingivalis* fimbriae. As shown in Fig. 1, fibronectin strongly bound to the 43-kDa fimbrial protein (fimbrillin), the major subunit. However, such strong binding reactivity was not observed for laminin or collagen types I and V. To verify the binding reactivity between fibronectin and the fimbriae, we tested the binding specificity by an assay of the binding of ^{125}I-labeled fimbriae to fibronectin. The binding activity of ^{125}I-labeled fimbriae toward fibronectin was measured in the presence of unlabeled fimbriae as a competitor. Figure 2a shows that the binding activity of ^{125}I-labeled fimbriae was inhibited in a dose-dependent manner by the competitor. Furthermore, we examined whether the binding of ^{125}I-labeled fimbriae to fibronectin pretreated with anti-fibronectin antibody would be inhibited. The anti-fibronectin antibody pretreatment inhibited the binding in a dilution-dependent fashion (Fig. 2b). These results strongly suggest that the fimbriae are able to bind to fibronectin with high specificity via the fimbrillin.

Fibronectin inhibits binding of *P. gingivalis* fimbriae to mouse peritoneal macrophages. Since our previous studies (7, 21) suggested that the fimbriae are able to bind to macrophages and fibroblasts, we assumed that fibronectin would inhibit the fimbrial binding to mouse peritoneal macrophages. Therefore, we examined this assumption. The binding of ^{125}I-labeled fimbriae to the macrophages was inhibited in a dose-dependent fashion by unlabeled fimbriae (Fig. 3a). Also, the binding to the cells was markedly inhibited when the fimbriae were pretreated with fibronectin at 50 μg/ml or greater and then inoculated onto the macrophage monolayer (Fig. 3b).

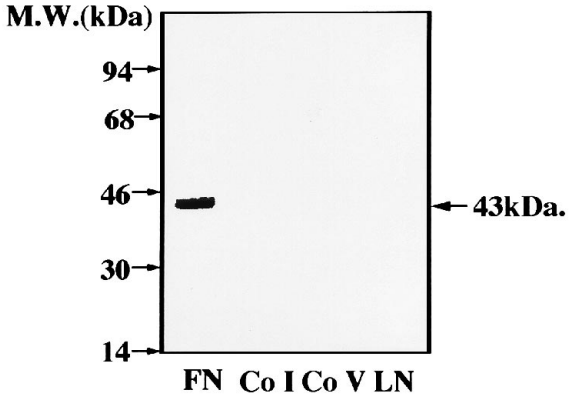

FIG. 1. Binding of fibronectin to *P. gingivalis* fimbriae in a Western blotting assay. Purified fimbriae (10 μg of protein per ml) were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Thereafter, the reactivity to extracellular matrix molecules was analyzed by a Western blotting assay with polyclonal antibodies against fibronectin (FN), laminin (LN), and collagen (Co) types I and V. M.W., molecular mass.

Fibronectin inhibits the fimbria-induced expression of IL-1β and KC genes in mouse peritoneal macrophages. We also previously demonstrated that purified fimbriae were able to induce powerfully the expression of IL-1β and neutrophil chemoattractant KC genes in mouse peritoneal macrophages. Therefore, we next examined by Northern blotting whether fibronectin would be able to inhibit the fimbria-induced expression of both cytokine genes in the macrophages. The macrophages were incubated with fimbriae that had been pretreated or not treated for 2 h with fibronectin, and then the expression of IL-1β and KC genes was measured 1 h later. Although the fimbria-induced expression of both cytokine

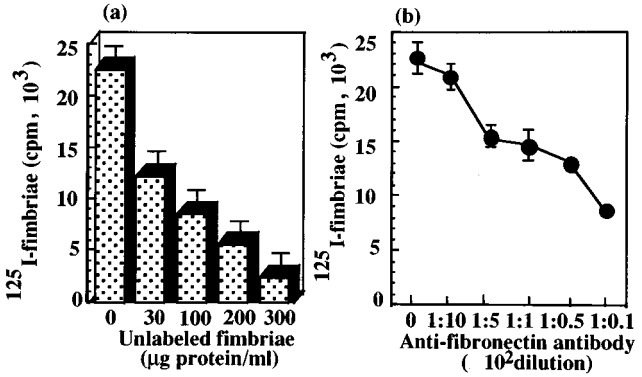

FIG. 2. Binding of ^{125}I-labeled fimbriae to fibronectin. (a) ^{125}I-labeled fimbriae (1 μg of protein) were added to fibronectin that had been used at 5 μg of protein per ml to coat the wells of a microtiter plate overnight. The binding activity of ^{125}I-labeled fimbriae toward the fibronectin was measured at 4 h after their addition. Unlabeled fimbriae were used as a competitor. (b) ^{125}I-labeled fimbriae (1 μg of protein) were added to fibronectin-coated wells of a microassay plate that had been pretreated or not treated for 2 h with anti-fibronectin antibody. The experiment was carried out in triplicate. The results are expressed as the mean counts per minute ± SD.

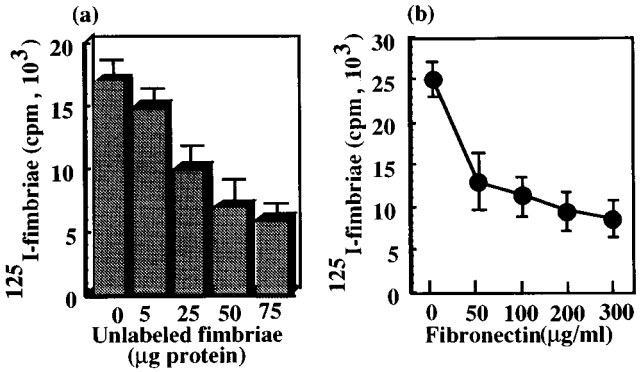

FIG. 3. Fibronectin inhibits the binding of ^{125}I-labeled fimbriae to mouse peritoneal macrophages. (a) ^{125}I-labeled fimbriae (1 μg of protein) were inoculated with or without unlabeled fimbriae into wells of a microculture plate containing formalin-fixed peritoneal macrophages. The binding activity of the ^{125}I-labeled fimbriae toward the cells was measured at 4 h after their addition. (b) ^{125}I-labeled fimbriae (1 μg of protein) inoculated with or without fibronectin into wells of a microculture plate containing formalin-fixed peritoneal macrophages. The binding activity of the ^{125}I-labeled fimbriae toward the cells was measured at 4 h after their addition. The experiment was carried out in triplicate, and the results are expressed as the mean of counts per minute ± SD.

genes was inhibited by fibronectin at 10 μg/ml, the inhibitory effect on IL-1β gene expression was the more obvious (Fig. 4a).

Furthermore, by using anti-fibronectin antibody, we examined the specificity of the inhibitory effect of fibronectin on the fimbria-induced expression of both cytokine genes. The fimbriae were treated or not treated with fibronectin that had been pretreated or not pretreated with anti-fibronectin antibody, and then the macrophages were incubated in the absence or presence of the fimbriae. Subsequently (1 h later), the expression of IL-1β and KC genes in the macrophages was analyzed. Figure 4b shows that the inhibitory effect of fibronectin on the fimbria-induced expression of both cytokine genes was almost completely eliminated by pretreatment with anti-fibronectin antibody at a dilution of 1:500. These results strongly suggest that fibronectin inhibited the fimbria-induced expression of both cytokine genes with high specificity.

***P. gingivalis* LPS is not involved in the binding of the fimbriae to fibronectin.** Although we were unable to detect LPS in the purified fimbrial preparation by silver staining after SDS-PAGE, it is very important to clarify whether the LPS is involved in the interaction between the fimbriae and fibronectin. Therefore, we used Northern and Western blotting assays to examine the possible involvement of LPS in their interaction. Western blotting analysis showed that fibronectin was unable to bind to the LPS (data not shown). Also, the LPS-induced expression of IL-1β and KC genes in mouse peritoneal macrophages was not inhibited by pretreatment of the LPS with fibronectin (Fig. 5). These results strongly indicate that LPS is not involved in the interaction between the fimbriae and fibronectin.

Heparin-binding and cell attachment domains are involved in fibronectin inhibition of the fimbria-induced expression of IL-1β and KC genes in mouse peritoneal macrophages. Since the existence of cell attachment and heparin- and gelatin-binding domains in the fibronectin structure is well known, we explored by the Northern blotting assay which domain in the fibronectin structure is involved in binding to the fimbriae and contributes to fibronectin inhibition of the fimbria-induced expression of the cytokine genes. In this experiment, the fimbriae were treated or not treated with fibronectin or each domain. Thereafter, the fimbriae were inoculated onto the macrophage monolayer, and IL-1β and KC gene expression in the cells was analyzed 1 h later. As shown in Fig. 6, the fimbria-induced expression of both cytokine genes was inhibited by pretreating the fimbriae with either the heparin-binding or cell attachment domain of the fibronectin structure. We also observed, by Western blotting analysis, that fibronectin binding to the fimbriae was inhibited by pretreating the fimbriae with heparin-binding or cell attachment domains (data not shown).

These observations suggest that heparin-binding and cell attachment domains in the fibronectin structure are involved in the fimbrial binding and contribute to the inhibitory action of the protein toward fimbria-induced gene expression in the macrophages.

Fibronectin inhibits *P. gingivalis* binding to mouse peritoneal macrophages via its fimbriae. As shown in Fig. 3, fibronectin inhibition of fimbrial binding to peritoneal macrophages suggested that the binding of the bacterial cells themselves would also be inhibited by the protein. Therefore, we examined the effect of fibronectin on *P. gingivalis* binding to mouse peritoneal macrophages via its fimbriae. As shown in Fig. 7a, we first demonstrated that the bacterial binding to the cells was mediated by its fimbriae by showing that anti-fimbrial antiserum inhibited the binding in a dilution-dependent fashion. Then we tested fibronectin and found that it also inhibited the bacterial cell binding to the cells (Fig. 7b). Together, these parallel results strongly suggest that fibronectin actually is able to inhibit the bacterial cell binding to the macrophages via interaction with the fimbriae in situ.

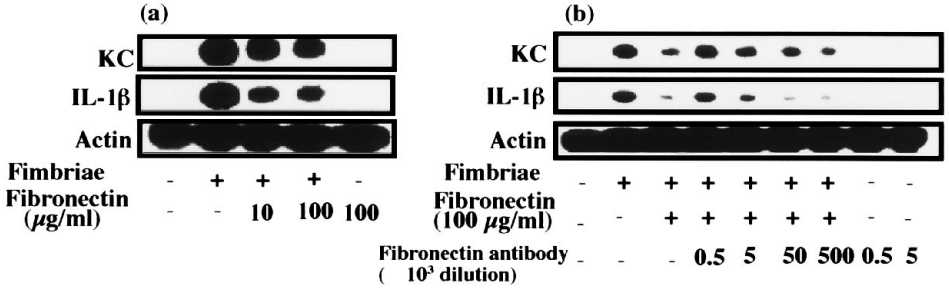

FIG. 4. Fibronectin inhibits the fimbria-induced expression of IL-1β and KC genes in the peritoneal macrophages. (a) Cells from BALB/c mice were incubated for 1 h with or without fimbriae (5 μg of protein per ml) that had been pretreated or not treated with fibronectin. Then their total RNAs were prepared, and Northern blot analysis was performed with IL-1β, KC, and β-actin cDNAs as probes. (b) Cells from BALB/c mice were incubated for 1 h with or without fimbriae (5 μg of protein per ml) and fibronectin that had been pretreated or not treated with anti-fibronectin antibody. Then their total RNAs were prepared, and Northern blot analysis was performed with IL-1β, KC, and β-actin cDNAs as probes.

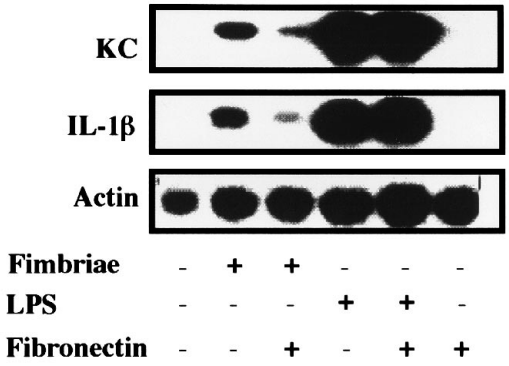

FIG. 5. *P. gingivalis* LPS is not involved in the binding of the fimbriae to fibronectin. Cells from BALB/c mice were incubated for 1 h with or without LPS (1 μg/ml) or fimbriae (5 μg of protein per ml) that had been pretreated or not treated for 2 h with fibronectin (100 μg/ml). Then their total RNAs were prepared, and Northern blot analysis was performed with IL-1β, KC, and β-actin cDNAs as probes.

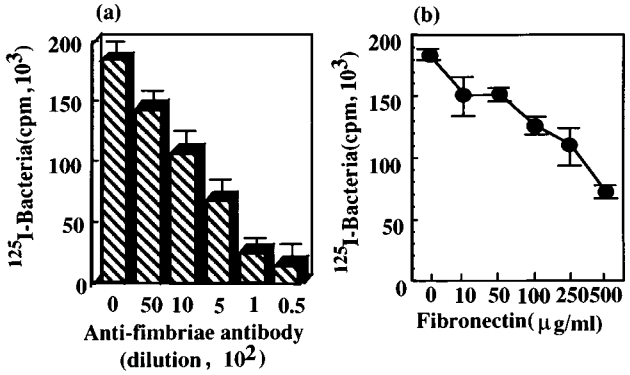

FIG. 7. *P. gingivalis* binding to mouse peritoneal macrophages via its fimbriae is inhibited by fibronectin. ^{125}I-labeled bacterial cells (2×10^8 cells) were pretreated or not treated for 2 h with anti-fimbria antiserum (a) or fibronectin (b) and then inoculated onto formalin-fixed peritoneal macrophages in the wells of a microculture plate. The binding activity of ^{125}I-labeled fimbriae to the cells was measured 4 h after their addition. The experiment was carried out in triplicate, and the results are expressed as the mean counts per minute ± SD.

Fibronectin inhibits *P. gingivalis*-induced expression of IL-1β and KC genes in mouse peritoneal macrophages. As described above, since *P. gingivalis* binding to the peritoneal macrophages via its fimbriae was inhibited by fibronectin, it is also important to demonstrate whether fibronectin inhibits the expression of IL-1β and KC genes induced by the bacterial cells via the fimbriae. Again, we first examined whether the bacterial cell-induced expression of both cytokine genes in the cells would be inhibited by the anti-fimbriae antiserum. Figure 8 shows that this was indeed the case. These results suggest that the bacterial cell-induced expression of the cytokine genes is mediated in part by the fimbriae. Then we investigated whether fibronectin also is able to inhibit the bacterial cell-induced expression of the cytokines. As shown in Fig. 9, the bacterial cell-induced IL-1β and KC gene expression was inhibited by fibronectin. These observations strongly imply that fibronectin functions as an inhibitor in the pathogenesis of *P. gingivalis* via its binding to the fimbriae.

DISCUSSION

Fibronectin is present in soluble form in plasma and saliva and in insoluble form in the extracellular matrix of many tissues. Several studies (19, 28) have shown that fibronectin is involved in the adherence of *Staphylococcus* and *Treponema* organisms to epithelial cells. On the other hand, it has been shown that the adherence of *E. coli* to epithelial cells is inhibited by fibronectin (27). These observations suggest that fibronectin serves as a potent modulator of the bacterial cell infection.

We (7, 21) previously suggested that a *P. gingivalis* fimbrial receptor is located on the human gingival fibroblasts and mouse peritoneal macrophages and that *N*-acetyl-D-galactosamine in the receptor may play an functional role in the interaction of the cells with the fimbriae. As described above, since fibronectin functions as an inhibitor in bacterial adhesion to host tissues, it was of interest to understand whether fibronectin is able to bind to *P. gingivalis* fimbriae and, if so, whether the matrix protein functions as a modulator in the pathogenesis of the organism via its interaction with the fimbriae. The present study demonstrated that the fimbriae bind to fibronectin with high affinity and consequently inhibit the expression of inflammatory cytokine genes induced in mouse peritoneal macrophages by the fimbriae.

It is well known that *P. gingivalis* is able to bind to extracel-

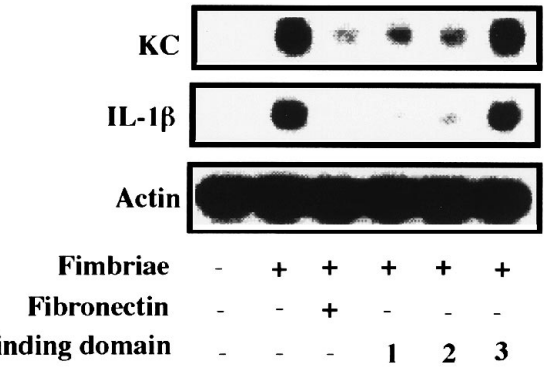

FIG. 6. Heparin-binding and cell attachment domains are involved in fibronectin inhibition of the fimbria-induced expression of IL-1β and KC genes in the peritoneal macrophage. Cells from BALB/c mice were incubated for 1 h with or without fimbriae (5 μg of protein per ml) that had been pretreated or not treated for 2 h with fibronectin (100 μg/ml) or each domain (1, heparin binding; 2, cell attachment; 3, gelatin binding [each at 20 μg/ml]). Then their total RNAs were prepared, and Northern blot analysis was performed with KC and β-actin cDNAs as probes.

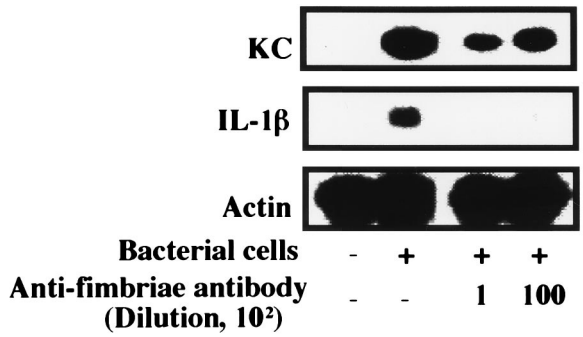

FIG. 8. *P. gingivalis*-induced expression of IL-1β and KC genes in mouse peritoneal macrophages is inhibited by anti-fimbria antiserum. Cells from BALB/c mice were incubated for 1 h with or without bacterial cells (10^9 cells) that had been pretreated or not treated for 2 h with anti-fimbria antiserum. Then their total RNAs were prepared, and Northern blot analysis was performed with KC, IL-1β, and β-actin cDNAs as probes.

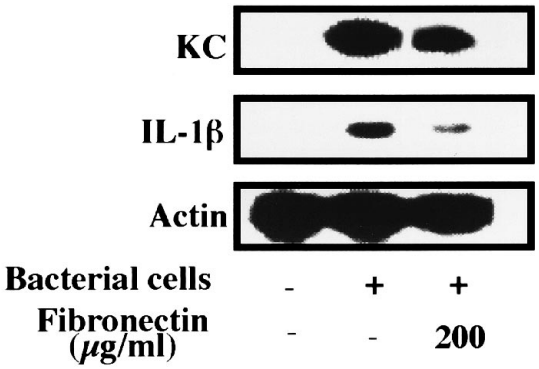

FIG. 9. Fibronectin inhibits *P. gingivalis*-induced expression of IL-1β and KC genes in peritoneal macrophages via binding to the fimbriae. Cells from BALB/c mice were incubated for 1 h with or without bacterial cells (10^{10} cells) that had been pretreated or not treated for 2 h with fibronectin (200 μg/ml). Then their total RNAs were prepared, and Northern blot analysis was performed with KC, IL-1β, and β-actin cDNAs as probes.

lular matrix proteins, like collagen, fibronectin, and laminin. Although Lantz et al. (16) showed that a 150-kDa component from the organism binds to fibronectin, the chemical character and nature of this fibronectin-binding component were not determined in detail. Since *P. gingivalis* fimbriae are important structures for bacterial adherence to host tissues, it was of much interest to demonstrate whether the fimbriae could bind to several extracellular matrix molecules. Our Western blotting analysis showed that fibronectin was able to bind to 43-kDa fimbrillin with high affinity, although laminin and collagen types I and V did not do so. This high affinity between fimbriae and fibronectin was demonstrated by a binding assay in which ^{125}I-labeled fimbriae were incubated with fibronectin in the presence of unlabeled fimbriae as a competitor. Since the binding of ^{125}I-labeled fimbriae to fibronectin pretreated with anti-fibronectin antibody was markedly inhibited (although ^{125}I-fibronectin binding to the fimbriae in the presence of unlabeled fibronectin has not been examined), these observations strongly suggest that 43-kDa fimbrillin is a fibronectin-binding protein.

Recently, Svanborg et al. (29) showed that *E. coli* fimbriae induce IL-6 production by urinary epithelial cells. We (7, 13, 21, 22) also previously demonstrated that *P. gingivalis* fimbriae were able to induce the expression of inflammatory cytokines in human gingival fibroblasts and mouse peritoneal macrophages. These findings suggested an important role for their fimbriae in the pathogenic mechanism of these organisms following their adherence to host cells. Therefore, it was of interest to us to explore whether fibronectin functions as an inhibitor of the cytokine expression through inhibition of the binding of *P. gingivalis* to macrophages, because the fimbria-induced inflammatory cytokines may contribute to the pathogenic mechanism(s) triggered by the organism. Although several studies (12, 23) have demonstrated the binding of *P. gingivalis* to fibronectin, the inhibitory action of fibronectin in the pathogenic mechanism of the organism has not yet been investigated. Thus, we investigated the inhibitory effect of fibronectin on *P. gingivalis* fimbria-induced expression of IL-1β and KC genes in mouse peritoneal macrophages. We observed that the fimbria-induced expression of both cytokine genes was inhibited by the fibronectin pretreatment. Since the inhibitory effect of fibronectin on the fimbria-induced expression of both cytokine genes was eliminated by pretreatment with anti-fibronectin antibody, fibronectin may be an inhibitor involved in the pathogenesis of *P. gingivalis* via its binding to the fimbriae.

It has been demonstrated that there are three distinct binding sites, i.e., cell attachment and gelatin- and heparin-binding domains, in the fibronectin structure. Several studies (24, 25, 35) have examined the binding domain and ability of fibronectin to bind to bacterial cells. Visai et al. (35) examined the binding of enterotoxigenic *E. coli* to fibronectin and its proteolytic fragments and showed that the bacterial cell components are able to interact with an N-terminal fragment and heparin-binding domain in the fibronectin structure. However, they did not demonstrate the character or nature of the components of the bacterial cells that bound to the fibronectin structure. Since it was of much interest to us to examine which domain in the fibronectin molecule is able to bind to *P. gingivalis* fimbriae, we used the Northern blotting assay to investigate the inhibition of the fimbria-induced expression of IL-1β and KC genes by three distinct domains of the fibronectin structure. Consequently, we observed that the fimbria-induced expression of the cytokine genes was inhibited by the heparin-binding and cell attachment domains. These results thus suggest the presence of some fimbrial structure that is able to recognize these two domains. Although we do not yet have any information on the chemical structure of the fimbrial receptor that may be localized on the cell surface of human fibroblasts and mouse macrophages, we assume that there is a similarity in chemical structure between the fimbrial receptor on the cell surface and the fimbrial binding domains in the fibronectin structure.

Fibronectin is detected in soluble form in saliva, and several investigators have suggested that fibronectin is involved in the modulation of bacterial adherence (10, 26). Thus, it was very attractive to us to demonstrate whether fibronectin actually inhibits the binding of *P. gingivalis* to host cells via binding to its fimbriae and also whether the bacterial cell-induced expression of inflammatory cytokine genes is inhibited by fibronectin. Interestingly, the binding of the bacterial cells to macrophages was inhibited markedly by anti-fimbria antiserum, and the bacterial cell-induced expression of inflammatory cytokine genes was also inhibited by pretreatment with anti-fimbria antiserum or fibronectin. Although the inhibitory action of the anti-fimbria antiserum toward bacterial binding is partly due to bacterial aggregation, because we did not use the Fab fragment of the fimbrial antibody, these observations strongly suggest that fibronectin may play a regulatory role in the pathogenic mechanism for *P. gingivalis* fimbrillin via the fimbriae. Since a recent study (1) demonstrated the presence of a receptor for *P. gingivalis* in saliva, it is possible that several chemical substances in saliva contribute to the modulation of infection of the host by the organism.

In summary, our present study demonstrates that *P. gingivalis* fimbrillin is one of the fibronectin-binding proteins and suggests that fibronectin may play a functional role as a potent modulator in the pathogenic mechanism of the organism via binding to the fimbriae.

ACKNOWLEDGMENTS

We thank C. D. Stiles for generous gifts of KC cDNAs and T. Hamilton for kindly providing the IL-1β cDNA.

REFERENCES

1. **Amano, A., H. T. Sojar, J. Y. Lee, A. Sharma, M. J. Levine, and R. J. Genco.** 1994. Salivary receptors for recombinant fimbrillin of *Porphyromonas gingivalis*. Infect. Immun. **62:**3372–3380.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

3. **Butler, K. M., C. J. Baker, and M. S. Edwards.** 1987. Interaction of soluble fibronectin with group B streptococci. Infect. Immun. **55:**2404–2408.
4. **Ceri, H., and Y. Westra.** 1988. Host binding proteins and bacterial adhesion: ecology and binding model. Biochem. Cell Biol. **66:**541–548.
5. **Faris, A., K. Krovacek, G. Froman, and T. Wadstrom.** 1987. Binding of fibronectin to *Escherichia coli* isolated from bovine mastitis from different geographical regions. Vet. Microbiol. **15:**129–136.
6. **Hamada, N., K. Watanabe, C. Sasakawa, M. Yoshikawa, F. Yoshimura, and T. Umemoto.** 1994. Construction and characterization of a *fimA* mutant of *Porphyromonas gingivalis*. Infect. Immun. **62:**1696–1704.
7. **Hanazawa, S., K. Hirose, Y. Ohmori, S. Amano, and S. Kitano.** 1988. *Bacteroides gingivalis* fimbriae stimulate production of thymocyte-activating factor by human gingival fibroblasts. Infect. Immun. **56:**272–274.
8. **Hanazawa, S., K. Nakada, Y. Ohmori, T. Miyoshi, S. Amano, and S. Kitano.** 1985. Functional role of interleukin-1 in periodontal disease: induction of interleukin-1 production by *Bacteroides gingivalis* lipopolysaccharide in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. Infect. Immun. **50:**262–270.
9. **Hanazawa, S., A. Takeshita, S. Amano, T. Semba, T. Nirazuka, H. Katoh, and S. Kitano.** 1993. Tumor necrosis factor-alpha induces expression of monocyte chemoattractant JE via fos and jun genes in clonal osteoblastic MC3T3-E1 cells. J. Biol. Chem. **268:**9526–9532.
10. **Hasty, D. L., and W. A. Simpson.** 1987. Effects of fibronectin and other salivary macromolecules on the adherence of *Escherichia coli* to buccal epithelial cells. Infect. Immun. **55:**2103–2109.
11. **Hook, M., M. J. McGavin, L. M. Switalski, R. Raja, G. Raucci, P. E. Lindgren, M. Lindberg, and C. Signas.** 1990. Interactions of bacteria with extracellular matrix proteins. Cell Differ. Dev. **32:**433–438.
12. **Isogai, E., K. Hirose, N. Fujii, and H. Isogai.** 1992. Three types of binding by *Porphyromonas gingivalis* and oral bacteria to fibronectin, buccal epithelial cells and erythrocytes. Arch. Oral Biol. **37:**667–670.
13. **Kawata, Y., S. Hanazawa, S. Amano, Y. Murakami, T. Matsumoto, K. Nishida, and S. Kitano.** 1994. *Porphyromonas gingivalis* fimbriae stimulate bone resorption in vitro. Infect. Immun. **62:**3012–3016.
14. **Kukkonen, M., T. Raunio, R. Virkola, K. Lahteenmaki, P. H. Makela, P. Klemm, S. Clegg, and T. K. Korhonen.** 1993. Basement membrane carbohydrate as a target for bacterial adhesion: binding of type I fimbriae of *Salmonella enterica* and *Escherichia coli* to laminin. Mol. Microbiol. **7:**229–237.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Lantz, M. S., R. D. Allen, L. W. Duck, J. L. Blume, L. M. Switalski, and M. Hook.** 1991. Identification of *Porphyromonas gingivalis* components that mediate its interactions with fibronectin. J. Bacteriol. **173:**4263–4270.
17. **Lindgren, P. E., C. Signas, L. Rantamaki, and M. Lindberg.** 1994. A fibronectin-binding protein from *Streptococcus equisimilis*: characterization of the gene and identification of the binding domain. Vet. Microbiol. **41:**235–247.
18. **Ljungh, A., L. Emody, H. Steinruck, P. Sullivan, B. West, E. Zetterberg, and T. Wadstrom.** 1990. Fibronectin, vitronectin, and collagen binding to *Escherichia coli* of intestinal and extraintestinal origin. Int. J. Med. Microbiol. **274:**126–134.
19. **Mamo, W., G. Froman, and T. Wadstrom.** 1988. Interaction of sub-epithelial connective tissue components with *Staphylococcus aureus* and coagulase-negative staphylococci from bovine mastitis. Vet. Microbiol. **18:**163–176.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 365–389. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Murakami, Y., S. Hanazawa, K. Nishida, H. Iwasaka, and S. Kitano.** 1993. *N*-Acetyl-D-galactosamine inhibits TNF-α gene expression induced in mouse peritoneal macrophages by fimbriae of *Poryphromonas* (*Bacteroides*) *gingivalis*, an oral anaerobe. Biochem. Biophys. Res. Commun. **192:**826–832.
22. **Murakami, Y., S. Hanazawa, A. Watanabe, K. Naganuma, H. Iwasaka, K. Kawakami, and S. Kitano.** 1994. *Porphyromonas gingivalis* fimbriae induce a 68-kilodalton phosphorylated protein in macrophages. Infect. Immun. **62:**5242–5246.
23. **Naito, Y., and R. J. Gibbons.** 1988. Attachment of *Bacteroides gingivalis* to collagenous substrata. J. Dent. Res. **67:**1075–1080.
24. **Obara, M., M. S. Kang, D. S. Rocher, A. Kornblihtt, J. P. Thiery, and K. M. Yamada.** 1987. Expression of the cell-binding domain of human fibronectin in *E. coli*. Identification of sequences promoting full to minimal adhesive function. FEBS Lett. **213:**261–264.
25. **Olsen, A., A. Jonsson, and S. Normark.** 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature (London) **338:**652–655.
26. **Pellat, B., T. Planchenault, D. V. Keil, and C. Pellerin.** 1988. Fibronectin-degrading activity in human cerevicular fluid, gingival explants culture medium and bacterial plaques. J. Biol. Buccale **16**(2)**:**51–57.
27. **Simpson, W. A., D. L. Hasty, and E. H. Beachey.** 1985. Inhibition of the adhesion of *Escherichia coli* to oral epithelial cells by fibronectin, p. 40–44. *In* S. E. Mergenhagen and B. Rosan (ed.), Molecular basis of oral microbial adhesion. American Society for Microbiology, Washington, D.C.
28. **Steiner, B. M., S. Sell, and R. F. Schell.** 1987. *Treponema pallidum* attachment to surface and matrix proteins of cultured rabbit epithelial cells. J. Infect. Dis. **155:**742–748.
29. **Svanborg, C., W. Agace, S. Hedges, R. Lindstedt, and M. L. Svensson.** 1994. Bacterial adherence and mucosal cytokine production. Ann. N. Y. Acad. Sci. **730:**162–181.
30. **Switalski, L. M., H. Murchison, R. Timpl, R. Curtiss III, and M. Hook.** 1987. Binding of laminin to oral and endocarditis strains of viridans streptococci. J. Bacteriol. **169:**1095–1101.
31. **Talay, S. R., W. P. Valentin, P. G. Jerlstrom, K. N. Timmis, and G. S. Chhatwal.** 1992. Fibronectin-binding protein of *Streptococcus pyogenes*: sequence of the binding domain involved in adherence of streptococci to epithelial cells. Infect. Immun. **60:**3837–3844.
32. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
33. **Umemoto, T., Y. Nakatani, Y. Nakamura, and I. Namikawa.** 1993. Fibronectin-binding proteins of a human oral spirochete, *Treponema denticola*. Microbiol. Immunol. **37:**75–78.
34. **Umemoto, T., and I. Namikawa.** 1994. Binding of host-associated *Treponema* proteins to collagen and laminin: a possible mechanism of spirochetal adherence to host tissues. Microbiol. Immunol. **38:**655–663.
35. **Visai, L., S. Bozzini, T. E. Petersen, L. Speciale, and P. Speziale.** 1991. Binding sites in fibronectin for an enterotoxigenic strain of *E. coli* B342289c. FEBS Lett. **290:**111–114.
36. **Visai, L., P. Speziale, and S. Bozzini.** 1990. Binding of collagens to an enterotoxigenic strain of *Escherichia coli*. Infect. Immun. **58:**449–455.
37. **Westerlund, B., P. Kuusela, T. Vartio, D. I. Van, and T. K. Korhonen.** 1989. A novel lectin-independent interaction of P fimbriae of *Escherichia coli* with immobilized fibronectin. FEBS Lett. **243:**199–204.
38. **Winkler, J. R., S. R. John, R. H. Kramer, C. I. Hoover, and P. A. Murray.** 1987. Attachment of oral bacteria to a basement-membrane-like matrix and to purified matrix proteins. Infect. Immun. **55:**2721–2726.
39. **Yoshimura, F., K. Takahashi, Y. Nodasaka, and T. Suzuki.** 1984. Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. J. Bacteriol. **160:**949–954.
40. **Yoshimura, F., T. Takasawa, Y. Yoneyama, T. Yamaguchi, H. Shiokawa, and T. Suzuki.** 1985. Fimbriae from the oral anaerobe *Bacteroides gingivalis*: physical, chemical, and immunological properties. J. Bacteriol. **163:**730–734.

Editor: J. R. McGhee