INFECTION AND IMMUNITY, July 1994, p. 3033–3037
0019-9567/94/$04.00+0

Vol. 62, No. 7

Degradation of Endogenous Plasma Membrane Fibronectin Concomitant with *Treponema denticola* 35405 Adhesion to Gingival Fibroblasts

RICHARD P. ELLEN,* MEJA SONG, AND CHRISTOPHER A. G. McCULLOCH

Department of Periodontics, Faculty of Dentistry, University of Toronto, Toronto, Canada M5G 1G6

Received 15 November 1993/Returned for modification 12 January 1994/Accepted 11 April 1994

***Treponema denticola* adhesion and degradation of fibronectin (Fn) on human gingival fibroblasts (HGF) were studied by immunofluorescence and enzyme-linked immunosorbent assays. The number of adherent bacteria increased and the amount of immunoreactive Fn decreased as a function of increasing *T. denticola* concentration. The distribution of cell-bound Fn was punctate in micrographs. Anti-human Fn impaired bacterial adhesion to HGF. Phenylmethylsulfonyl fluoride inhibited Fn degradation but not adhesion. Sonicated extracts and diluted spent growth medium degraded HGF Fn but, unlike intact *T. denticola* cells, they hardly stimulated F-actin rearrangements.**

Treponema denticola and other oral spirochetes are considered significant in periodontal disease etiology because their population densities correlate with clinical signs of disease. Treponemes are numerous in inflamed pockets associated with both marginal gingivitis and periodontitis (9), and they also invade gingival soft tissues in acute necrotic ulcerative gingivitis and advanced periodontitis (8, 9, 14). Chymotrypsin-, trypsin-, and elastase-like enzymes and iminopeptidase enzymes of *T. denticola* are considered important virulence factors that may enhance its tissue penetration (10, 12, 15). *T. denticola* can degrade in vitro models of basement membrane and intercellular matrix (6). Therefore it has the potential to gain access in vivo and to interact directly with stromal cells of the superficial connective tissues.

Using in vitro cell culture infection models, we and others have reported several cytopathic responses of human gingival fibroblasts (HGF) following contact with *T. denticola* (1, 3, 18). These alterations include (i) plasma membrane folds, blebs, and gross rounding of normally flattened fibroblasts; (ii) F-actin rearrangement into a perinuclear array; (iii) cell detachment from the substratum; and (iv) reduction in proliferative capacity and cell death. Conceivably, these responses are induced following contact of *T. denticola* with specific molecules on the HGF plasma membrane.

Most *T. denticola* strains adhere avidly to extracellular proteins like fibronectin (Fn) which are synthesized by HGF and bind to the outer surface of the HGF plasma membrane. A few laboratories have demonstrated that *T. denticola* adheres to plasma Fn immobilized on plastic surfaces, and we have shown that most strains adhere well in a polar orientation (4, 7). While adhesion to such surfaces is inhibited by pretreating the Fn coating with anti-Fn antibodies and is enhanced by pretreating *T. denticola* with plasma Fn (4), similar treatments have evidently led to results that are dependent on the strain of *T. denticola* when HGF have been used as the adhesion substrate (18). As endogenous HGF Fn interacts with integral membrane proteins that interface with the cytoskeleton, adhesion of bacterial agonists to Fn or degradation of Fn by bacterial proteases might be significant in initiating signal transmission to the cytoplasm. We have studied one of the first steps in this process by determining whether endogenous HGF Fn is degraded concomitantly with the adhesion of *T. denticola* to the plasma membrane or exposure of HGF to protease activities of *T. denticola* extracts and spent growth medium.

The *T. denticola* type strain, ATCC 35405, was originally obtained from E. Chan, McGill University, Montreal, and was maintained in the culture collection at the University of Toronto Faculty of Dentistry. Previously, we showed that it adheres to plasma Fn-coated coverslips in a typical polar orientation and stimulates cytopathic reactions in HGF (1, 4). Stocks were maintained by growth in spirochete medium supplemented with 0.3% Noble agar and were subcultured once every 3 weeks (4). For experiments, the cultures were grown in spirochete broth for 72 h (mid- to late log phase) at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) in an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. Cells were harvested by centrifugation for 5 min at 12,000 × *g*, washed twice with phosphate-buffered saline (PBS) (pH 7.2), resuspended in alpha minimal essential medium (α-MEM) without serum or antibiotics, and adjusted to various cell densities determined by microscopic count.

An HGF cell line derived from explants was cultured as described previously (1). Briefly, cells were cultured in α-MEM with 15% fetal bovine serum and antibiotics and were maintained in a 5% CO_2 atmosphere at 37°C. For experiments, HGF were plated at a concentration of 10^4 cells per well in 96-well plates (Corning Glass Works, Corning, N.Y.) and were incubated for 1 or 2 weeks before use to prevent the HGF from detaching during the repeated wash cycles used for the enzyme-linked immunosorbent assay (ELISA). The α-MEM was changed every 4 to 5 days. Confluent monolayers were washed twice with PBS. One hundred microliters of washed *T. denticola* suspensions containing different densities of bacteria was added to each well and incubated for periods up to 2 h. Nonadherent treponemes were removed by being rinsed once, then thoroughly washed twice with PBS.

T. denticola adhesion to HGF was quantified by a modified urease ELISA. One hundred microliters of a 1/50 dilution of anti-*T. denticola* 35405 antiserum was added to each well and

* Corresponding author. Mailing address: Department of Periodontics, University of Toronto, Faculty of Dentistry, 124 Edward Street, Toronto, Canada M5G 1G6. Phone: (416) 979-4928, ext. 4408. Fax: (416) 979-4936.

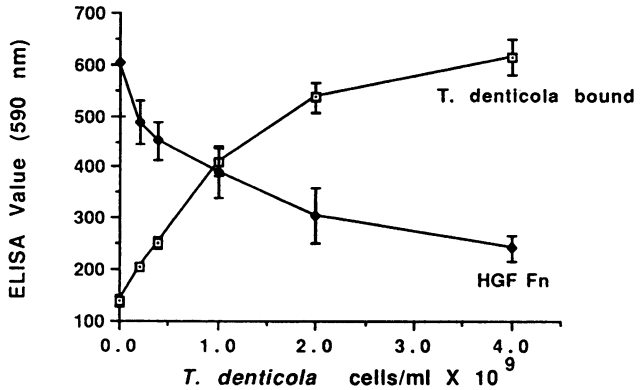

FIG. 1. Binding curve for *T. denticola* and amount of immunoreactive Fn remaining on HGF surface at various *T. denticola* concentrations during 2-h assay. Points represent ELISA values (means ± standard deviations) of quadruplicates. For the binding curve, an ELISA value of 550 approximated 4.7×10^6 *T. denticola* cells per ml.

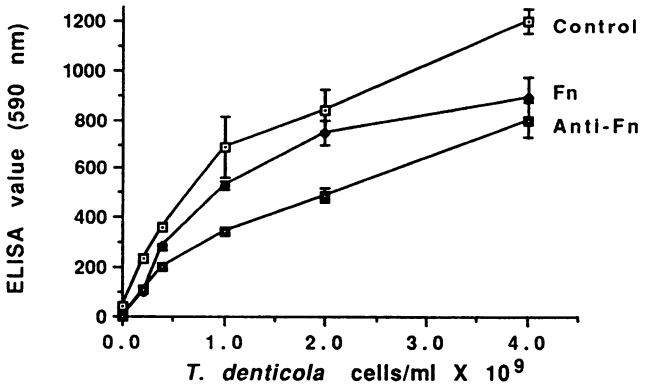

FIG. 2. Binding curves for *T. denticola* represented by ELISA values (means ± standard deviations) when plasma Fn or anti-human Fn was added to the assay buffer.

was incubated at 37°C for 30 min, followed by a rinse and two washes with PBS and Tween 20. One hundred microliters of 1/100 urease-conjugated goat anti-rabbit gamma globulin (Sigma, St. Louis, Mo.) was added, and the plates were incubated for 30 min. Following a rinse and two washes, 100 μl of the substrate bromcresol purple was added and incubated for 60 min at room temperature. Absorbance was quantified with a Titertek Multiscan Plus plate reader (Flow Laboratories, McLean, Va.) at a wavelength of 590 nm. Controls consisted of substitutions of α-MEM for the *T. denticola* suspension in the bacterial challenge protocol and preimmune serum for the anti-*T. denticola* serum in the ELISA. Preliminary experiments without HGF had established that ELISA absorbance values were proportional to input concentrations of *T. denticola* used to coat wells of microtiter plates. The ELISA curves were linear up to 10^9 bacteria, assuming that all the bacteria bound. The cell concentration in *T. denticola* suspensions was also standardized by chymotrypsinlike degradative activity for the synthetic peptide *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPPNA), and the number of *T. denticola* cells bound to HGF monolayers was quantified by measuring SAAPPNA degradation by HGF washed free of nonadherent bacteria. Immunoreactive Fn was measured in parallel but independent samples. Following 2 h of exposure to *T. denticola* suspensions or the control medium, the amount of surface Fn remaining on HGF monolayers was quantified by ELISA. The first antibody was 1/100 rabbit anti-human Fn (Calbiochem, San Diego, Calif.), with the rest of the conditions the same as described above. Differences in mean ELISA values were analyzed by Student's *t* test using unpaired data.

The degree of *T. denticola* adherence and the amount of immunoreactive HGF Fn varied reciprocally with the *T. denticola* cell concentration (Fig. 1). The greater the number of *T. denticola* cells per ml of suspension, the greater was the ELISA value in the bacterial adhesion assay and the lower was the amount of Fn detected in parallel samples. Quantification of HGF-bound *T. denticola* by SAAPPNA-degrading activity yielded a binding curve virtually identical to that for the ELISA (data not shown). HGF infection experiments were repeated, incorporating Fn or anti-Fn antibodies in the assay buffer in an attempt to inhibit bacterial adhesion to HGF Fn. Human plasma Fn (Sigma) at 0.5 mg/ml and rabbit anti-human Fn at 20 μg/ml decreased but did not fully inhibit *T. denticola* 35405 adhesion in these assays (Fig. 2, $P < 0.05$ for anti-Fn at all bacterial input concentrations and for Fn at the highest bacterial input concentration). At the *T. denticola* input densities used in these experiments, the ELISA values for anti-Fn-containing wells were less than those for the controls, and the percent inhibition for anti-Fn was greater at the lower *T. denticola* input concentrations. Adding 15% fetal calf serum to the adherence assay reduced the ELISA values across all *T. denticola* input concentrations; bovine serum albumin at 10 mg/ml impaired adhesion only for low bacterial input concentrations up to 4×10^8 per ml (data not shown).

As we have previously found the chymotrypsin inhibitor phenylmethylsulfonyl fluoride (PMSF) to inhibit the *T. denticola*-stimulated detachment of HGF from its substratum (1), PMSF (Sigma) and an additional protease inhibitor for comparison, amidinophenylmethane-sulfonyl fluoride (APMSF), were used to pretreat the *T. denticola* cells for 1 h, followed by one wash, prior to their addition to the HGF cells. From 20-mg/ml stock solutions in ethanol, 10 μl was added in 1.0 ml of *T. denticola* suspensions (optical density at 550 nm of 1.0), yielding a final concentration of 200 μg/ml. While APMSF had little effect ($P = 0.64$), PMSF was inhibitory for Fn degradation ($P < 0.001$) but not *T. denticola* adhesion ($P = 0.15$; Fig. 3). These data suggest that activity of the surface-associated chymotrypsinlike protease is not crucial for *T. denticola* adhesion to HGF.

Immunoreactive HGF Fn was also degraded by sonicated

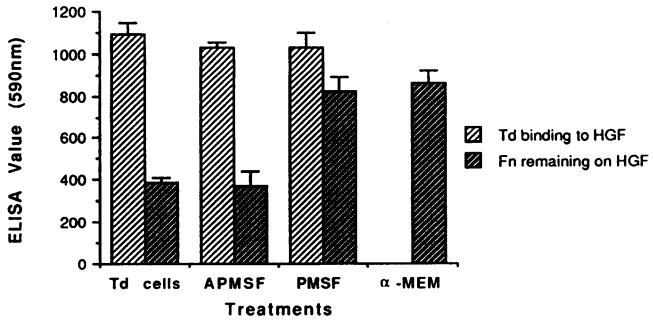

FIG. 3. Adhesion of *T. denticola* and amount of immunoreactive Fn remaining on HGF surface, represented by ELISA values (means ± standard deviations), when *T. denticola* cells were pretreated with PMSF or APMSF. Td cells, *T. denticola* not treated with enzyme inhibitors. α-MEM, *T. denticola*-free suspension medium.

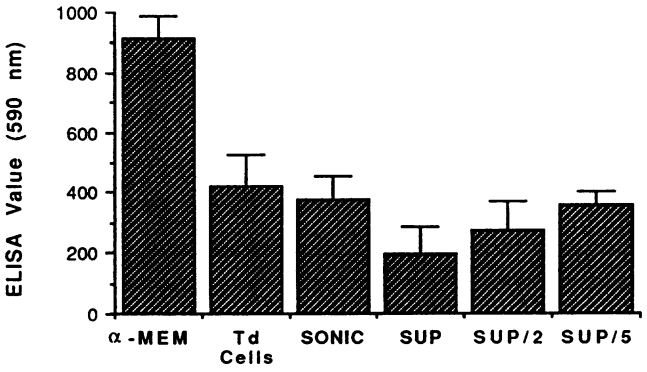

FIG. 4. Amount of immunoreactive Fn remaining on HGF surface, represented by ELISA values (means ± standard deviations), comparing challenge by whole *T. denticola* cells (Td cells) with challenge by bacterial sonicate (sonic), bacterial culture supernatant (sup), and supernatant diluted 1/2 (sup/2) and 1/5 (sup/5). α-MEM, control HGF challenged with α-MEM alone.

extracts of *T. denticola* ($P < 0.001$; Fig. 4). The extracts were prepared by sonicating a suspension of 10^9 bacteria per ml on ice for 2 min at the low setting (Kontes sonicator; Vineland, N.J.) and by diluting the sonicate 1/10 in α-MEM. Similarly, *T. denticola* culture supernatants yielded concentration-dependent degradation of HGF Fn when diluted fivefold in α-MEM ($P < 0.001$). PMSF significantly reduced ($P < 0.01$) but did not fully inhibit HGF Fn degradation by both the sonicated extract and the supernatant, as it had in experiments with *T. denticola* cells (data not shown). With PMSF, the amount of Fn remaining on cells challenged with sonicates and supernatants was approximately 70% of that in α-MEM controls ($P < 0.001$). These preparations would be expected to contain proteases other than the PMSF-inhibitable chymotrypsinlike protease that is the major surface-associated activity on intact *T. denticola* cells.

Evidence for the degradation of endogenous HGF Fn was also documented by immunofluorescence microscopy of the HGF at the beginning of and at various times during the in vitro infection assay. HGF were grown for 2 days on glass coverslips in wells of 24-well culture plates. After exposure to *T. denticola* for 1 h, the coverslips were rinsed, fixed by incubating at 25°C for 60 min in 3.7% formaldehyde, and washed twice in buffer (1). Three hundred microliters of specific anti-Fn antibody was added to each well. The dishes were incubated at 37°C for 30 min, followed by two washes in PBS and Tween 20. Three hundred microliters of 1/50 rhodamine-conjugated goat anti-rabbit gamma globulin was added, and the dishes were incubated for 30 min, followed by two washes. The coverslips were inverted onto a drop of mounting fluid, and the edges were sealed with nail polish. The samples were examined by using 530- to 560-nm excitation filters and a 580-nm barrier filter on a Leitz Dialux microscope. A control experiment using HGF grown short-term in serum-free medium confirmed that the immunoreactive Fn was endogenous and not acquired from serum.

Immunofluorescence microscopy was consistent with the results of the quantitative assays. Typically, almost all control HGF and HGF exposed to PMSF-pretreated *T. denticola* had thick bands of immunoreactive Fn distributed over the entire cell surface (Fig. 5). In contrast, *T. denticola*-challenged HGF monolayers and single cells and HGF exposed to APMSF-treated *T. denticola* showed a time-dependent diminution of labelled surface Fn. The remaining fluorescence was associated with scattered, fragmented bands. Exposure of HGF to sonicates and diluted spent growth medium of *T. denticola* or to chymotrypsin (Sigma) yielded similar fragmentation of immunoreactive Fn bands (data not shown). These findings indicate that the distribution of endogenous HGF Fn on the plasma membrane is disrupted following exposure to *T. denticola* and the extracts and that the degradation is attributable in part to active chymotrypsinlike activity. Uitto and coworkers have reported a similar pattern of immunoreactive Fn degradation on HGF challenged with crude extracts and a purified protease from *Porphyromonas gingivalis*; the proteolytic activity in some way stimulated the HGF to secrete endogenous collagenase and plasminogen activator (16).

HGF were also studied by fluorescence microscopy to determine whether exposure to sonicates and spent medium would affect the distribution of F-actin, as we had reported for HGF exposed to whole *T. denticola* cells (1). HGF were challenged for 1 h, fixed and permeabilized (1), and stained with rhodamine-phalloidin. With whole *T. denticola* cells, many HGF shrank considerably, and their F-actin staining pattern changed from a well-defined array of stress fibers seen in control cells to a brightly fluorescing, ill-defined mass around the nucleus. In contrast, HGF challenged with chymotrypsin equivalent in SAAPPNA-degrading activity to 10^9 *T. denticola* cells per ml, with sonicates, and with spent medium diluted more than 1:1 in α-MEM showed few signs of F-actin rearrangement, even though bands of surface Fn were fragmented. Undiluted spent medium caused disruption of F-actin stress fibers.

There are a few ways by which the adhesion of *T. denticola* and the degradation of endogenous Fn might be of significance to cytoskeletal and other cytopathic reactions of HGF. Fn binds to integrins, which are known to transmit signals across the plasma membrane to cytoskeletal proteins. Therefore, *T. denticola* adhesion to or cross-linking of Fn molecules or degradation of Fn by *T. denticola* might activate an initial step in a signalling cascade resulting in F-actin rearrangement, distortions of cell morphology, and eventual detachment of HGF from the substratum matrix. *T. denticola* synthesizes several polypeptides with plasma Fn binding properties (17). Weinberg and Holt have reported a 64-kDa outer sheath protein with some sequence homology to an integrin alpha subunit (19); this surface-accessible molecule might conceivably have the capacity to bind *T. denticola* to HGF integrins by bridging via endogenous Fn. Alternatively, *T. denticola* might adhere to HGF via other avid mechanisms that would allow concomitant adhesion to and degradation of Fn by either its cell-associated or its soluble proteases.

Optimum fibroblast spreading and actin microfilament organization in cultured cells evidently depend on complementary peptide sequences located more than 20 kDa apart on the Fn molecule (11). It is conceivable that fragmentation of endogenous HGF Fn by *T. denticola*, as demonstrated in this study, might disrupt matrix-encoded signals to the cytoskeleton and ultimately account for the reorganization of F-actin and cell detachment. Our observed PMSF inhibition of both Fn degradation and HGF detachment (1) tends to support this contention. Yet the reduced ability of sonicated extracts, diluted spent medium, and purchased chymotrypsin to alter F-actin distribution concomitantly with Fn degradation suggests that adhesion or some other activity of intact *T. denticola* cells is significant for cytoskeletal stimulation. We have also demonstrated rapid HGF actin and intracellular calcium responses to stretch activation, when the HGF plasma membrane was physically stretched or expanded by exposure to anisosmotic solutions (2, 13). Thus it is also feasible that a

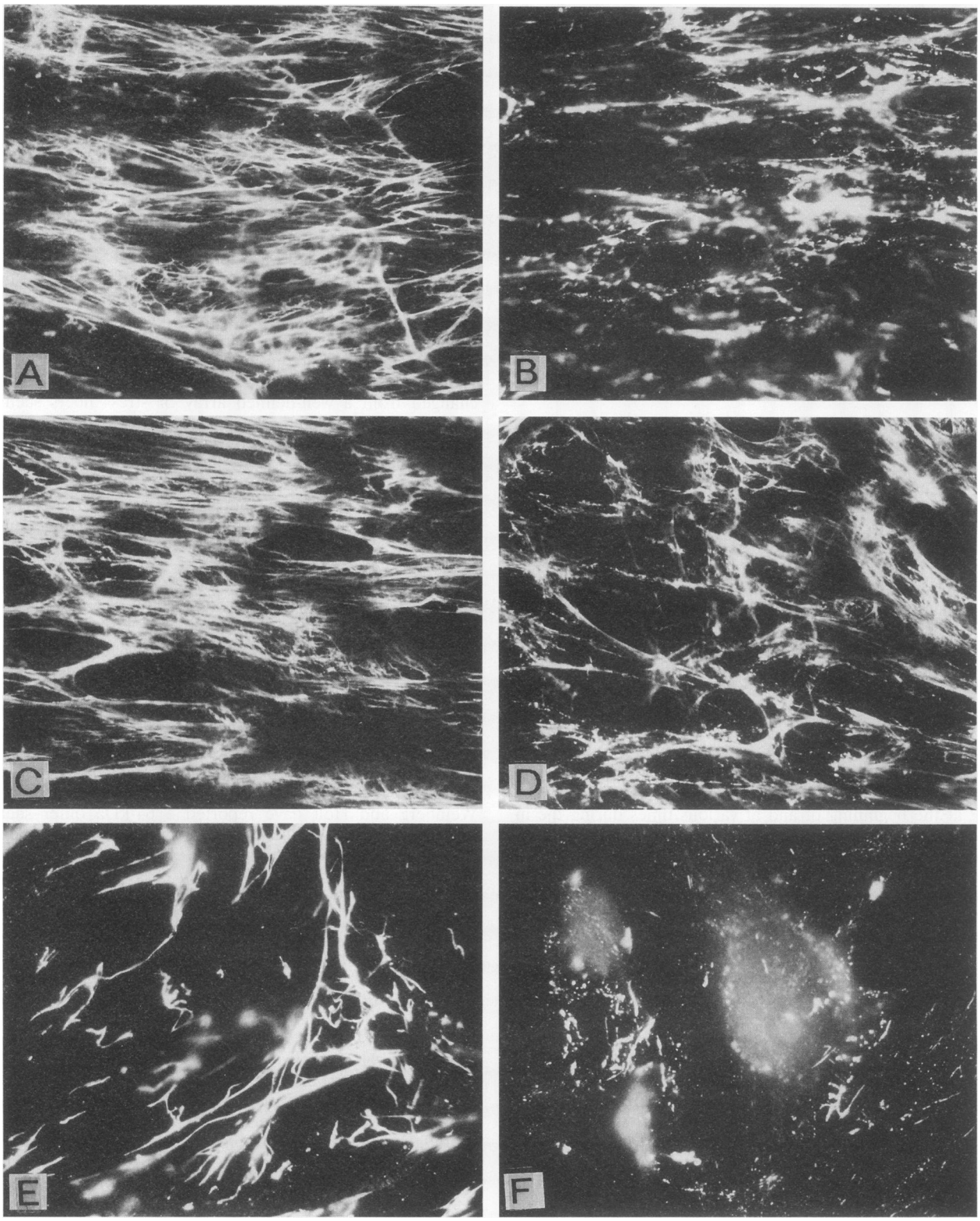

FIG. 5. Immunofluorescence micrographs of typical control and *T. denticola*-challenged HGF. Immunoreactive Fn appears as bright, intact bands on the surface of HGF that have not been exposed to *T. denticola* (A and E) and on HGF exposed to PMSF-pretreated *T. denticola* (C). The Fn label appears less intense, and fragments of bands are clearly visible as punctate fluorescence on HGF exposed to *T. denticola* (B and F) and to APMSF-pretreated *T. denticola* (D). Experiments were run with either confluent monolayers of HGF (A to D) or noncontacting HGF (E and F). Magnification, ×432.

sinuous bacterium like *T. denticola*, which has the capacity to cluster Fn-reactive adhesins toward its adherent pole (5) while flexing or rotating its free end, might exert uneven tractional forces and thereby physically distort the plasma membrane.

This investigation was supported by grant MT-5619 and grant MA-8903 from the Medical Research Council of Canada.

REFERENCES

1. **Baehni, P. C., M. Song, C. A. G. McCulloch, and R. P. Ellen.** 1992. *Treponema denticola* induces actin rearrangement and detachment of human gingival fibroblasts. Infect. Immun. **60:**3360–3368.
2. **Bibby, K. J., and C. A. G. McCulloch.** Regulation of cell volume and intracellular calcium in attached human fibroblasts responding to anisosmotic buffers. Am. J. Physiol., in press.
3. **Boehringer, H., N. S. Taichman, and B. J. Shenker.** 1984. Suppression of fibroblast proliferation by oral spirochetes. Infect. Immun. **45:**155–159.
4. **Dawson, J. R., and R. P. Ellen.** 1990. Tip-oriented adherence of *Treponema denticola* to fibronectin. Infect. Immun. **58:**3924–3928.
5. **Dawson, J. R., and R. P. Ellen.** 1994. Clustering of fibronectin adhesins toward *Treponema denticola* tips upon contact with immobilized fibronectin. Infect. Immun. **62:**2214–2221.
6. **Grenier, D., V.-J. Uitto, and B. C. McBride.** 1990. Cellular location of a *Treponema denticola* chymotrypsinlike protease and importance of the protease in migration through the basement membrane. Infect. Immun. **58:**347–351.
7. **Haapasalo, M., U. Singh, B. C. McBride, and V.-J. Uitto.** 1991. Sulfhydryl-dependent attachment of *Treponema denticola* to laminin and other proteins. Infect. Immun. **59:**4230–4237.
8. **Listgarten, M. A., and D. W. Lewis.** 1967. The distribution of spirochetes in the lesion of acute necrotizing ulcerative gingivitis: an electron microscopic and statistical survey. J. Periodontol. **38:**379–386.
9. **Loesche, W. J.** 1988. The role of spirochetes in periodontal disease. Adv. Dent. Res. **2:**275–283.
10. **Mikx, F. H. M., F. Jacobs, and C. Satumalay.** 1992. Cell-bound peptidase activities of *Treponema denticola* ATCC 33520 in continuous culture. J. Gen. Microbiol. **138:**1837–1842.
11. **Obara, M., M. S. Kang, and K. M. Yamada.** 1988. Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. Cell **53:**649–657.
12. **Ohta, K., K. K. Makinen, and W. J. Loesche.** 1986. Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect. Immun. **53:**213–220.
13. **Pender, N., and C. A. G. McCulloch.** 1991. Quantitation of actin polymerization in two human fibroblast subtypes responding to mechanical stretching. J. Cell Sci. **100:**187–193.
14. **Riviere, G. R., K. S. Weisz, L. G. Simonson, and S. A. Lukehart.** 1991. Pathogen-related spirochetes identified within gingival tissue from patients with acute necrotizing ulcerative gingivitis. Infect. Immun. **59:**2653–2657.
15. **Uitto, V.-J., D. Grenier, E. C. S. Chan, and B. C. McBride.** 1988. Isolation of a chymotrypsinlike enzyme from *Treponema denticola*. Infect. Immun. **56:**2717–2722.
16. **Uitto, V.-J., H. Larjava, J. Heino, and T. Sorsa.** 1989. A protease of *Bacteroides gingivalis* degrades cell surface and matrix glycoproteins of cultured gingival fibroblasts and induces secretion of collagenase and plasminogen activator. Infect. Immun. **57:**213–218.
17. **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.
18. **Weinberg, A., and S. C. Holt.** 1990. Interaction of *Treponema denticola* TD-4, GM-1, and MS25 with human gingival fibroblasts. Infect. Immun. **58:**1720–1729.
19. **Weinberg, A., and S. C. Holt.** 1991. Chemical and biological activities of a 64-kilodalton outer sheath protein from *Treponema denticola* strains. J. Bacteriol. **173:**6935–6947.