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Interaction of *Treponema denticola* TD-4, GM-1, and MS25 with Human Gingival Fibroblasts

A. WEINBERG AND S. C. HOLT*

Department of Periodontics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7894

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The adherence of *Treponema denticola* GM-1, TD-4, and MS25 to human gingival fibroblasts (HGFs) was studied to serve as an introduction to investigations into the interactions of these oral bacteria with human host cells. Under both aerobic (5% CO_2) and anaerobic (85% N_2 plus 10% H_2 plus 5% CO_2) environments, the interactions with the HGFs were such that strains GM-1 and MS25 were consistently more adherent than strain TD-4. Polyclonal antibodies to GM-1 inhibited GM-1 adherence by 70%, while MS25 and TD-4 showed differing degrees of cross-reactive inhibition, indicative of common but not identical epitopes on the surface of the three *T. denticola* strains. Pretreatment of the three strains with trypsin did not inhibit adherence; proteinase K did, however, inhibit this interaction by 80%. Trypsin pretreatment of the HGFs resulted in increases in adherence of 50 and 86% for GM-1 and MS25, respectively, while a decrease of 41% was noted for TD-4. Exposure of the *T. denticola* strains to sugars and lectin pretreatment of the HGFs implicated adherence mediation by mannose and galactose residues on the HGF surface. Periodate treatment of HGFs resulted in a 50% drop in adherence for GM-1 and MS25, but did not decrease that of TD-4. Addition of fetal bovine serum inhibited adherence of the three strains to differing degrees, with TD-4 being the most susceptible. Addition of purified fibronectin (100 μg/ml) resulted in >50% inhibition in GM-1 and MS25 adherence, while a 25% increase occurred with TD-4. While strain differences were noted in some of the parameters studied, the results indicate two possibilities for *T. denticola*-HGF adherence: a lectinlike adhesin(s) on the *T. denticola* surface with affinity for galactose and mannose on the HGF surface, and a serum host factor(s) bridging *T. denticola* and HGFs.

The primary ecological niche of treponemes in the oral cavity is the gingival crevice area where these microorganisms are established from puberty. Routinely present in small numbers in healthy sites, they increase sharply in sites with periodontal disease (2, 24). Scanning electron microscopic (SEM) investigations have shown that oral treponemes and rods are the principal morphological forms associated with the advancing front of subgingival plaque in sites of rapidly progressive periodontitis (38). While their role in the destruction of oral tissues remains unclear, several investigations have demonstrated a direct interaction between host connective tissue and invading oral treponemes in acute necrotizing ulcerative gingivitis (23, 27). Important to their possible role in the invasion of connective tissue, oral treponemes possess a variety of proteolytic activities including, among others, trypsin- and chymotrypsinlike enzymes (22, 34, 44), fibrinolytic and collagenolytic enzymes (25, 31), iminopeptidases and phospholipase C (26, 41), and enzymes that degrade glycosaminoglycans such as hyaluronic acid and chondroitin-sulfate (13). These enzymes may be playing a role in soft-tissue and bone destruction. Therefore, oral treponemes may be involved in invasiveness of the deeper tissues of the periodontium as well as in the progression of inflammatory periodontal disease. A large number of in vitro observations have shown that oral treponemal extracts inhibit fibroblast proliferation (7), lymphocyte blastogenesis (40), neutrophil degranulation (6), and free-radical production (39), further suggesting that oral treponemes may be able to both inhibit host reparative processes and evade host defenses.

Recently, the first quantitative evidence of a positive relationship between a specific treponemal species, *Treponema denticola*, and severe periodontitis was established (42). Using a monoclonal antibody specific for *T. denticola*, Simonson et al. (42) showed a significant increase in their numbers with disease severity. While this specific increase in *T. denticola* may be due to the accessibility of certain growth requirements supplied in the normal ecological progression of the periodontal disease process, these organisms did represent a significant proportion of the total number of morphological types observed.

The association of the complex oral microbiota with the host requires that there be an interaction either between them and the host tissues or between themselves. In fact, these interactions or adhesive properties are fundamental to the initiation and progression of many infectious diseases (1, 8, 16, 20, 29, 33, 36, 43). The invading bacterium must be able to fix itself to a substrate, multiply, and express its virulence in an environment which would otherwise remove it by bathing the tissue surface with fluids. The inflamed periodontal pocket is an excellent representation of such an interactive environment. While several investigations (4, 10, 35, 37) have shown that oral treponemes interact with or adhere to eucaryotic cells and hard surfaces, the mechanism of this adherence remains unknown. It is therefore the intent of this and subsequent studies to elucidate the mechanism(s) of adherence of *T. denticola* species to human gingival fibroblasts (HGFs) and epithelial cells, two host cells central to the sustained integrity of both periodontal bone and connective tissue.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *T. denticola* GM-1 and MS25 were isolated from human periodontal pockets

* Corresponding author.

(39). *T. denticola* ATCC 35404, designated TD-4, was obtained from the American Type Culture Collection, Rockville, Md. All strains were grown in GM-1 broth (5) for 5 days without glucose in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) in an atmosphere of 85% N_2–10% H_2–5% CO_2 at 37°C. Cultures were harvested when they reached an optical density at 660 nm of 0.270, equivalent to approximately 5×10^8 *T. denticola* per ml (see below). Liquid cultures were maintained by weekly transfers of an approximate 10% inoculum to fresh GM-1 broth. Purity and cell motility were determined by dark-field microscopy. All cultures showed a high degree of motility at the time of harvest.

Radioiodination of bacteria. All strains were radioiodinated by using the technique of Armstrong and Parker (3) with slight modifications. Briefly, *T. denticola* cultures were harvested from 5 ml of broth at 9,000 × *g* for 20 min. The cell pellets were washed twice in phosphate-buffered saline (PBS), pH 7.4, by centrifugation at 9,000 × *g* for 20 min and suspended in 1 ml of PBS. This suspension, consisting of approximately 2.5×10^9 *T. denticola*, was transferred to a 1-dram (ca. 3.7-ml) glass vial, and 0.25 mCi of $Na^{125}I$ (New England Nuclear, Boston, Mass.) was added. The reaction was initiated by the addition of three Iodo-beads (Pierce Chemical Co., Rockford, Ill.) which had been previously rinsed with PBS. The cell suspension was incubated at room temperature with the beads for 5 min with constant stirring. The reaction was terminated by removal of the Iodo-beads, and the radiolabeled bacteria were washed twice and suspended in PBS as described above. Radioactivity of the pooled supernatants, containing unbound ^{125}I, was compared with that in the bacterial cell suspension, containing bound ^{125}I, using a Beckman Gamma 310 counter (Beckman Instruments, Inc., Palo Alto, Calif.). Typical experiments yielded 20 to 40% incorporation of ^{125}I onto whole treponemes.

Fibroblast cultures. Normal HGF cultures, Gin-1 and Gin-3, were used. Gin-1 was obtained from the American Type Culture Collection and used in the initial stages of experimentation. When sources for Gin-1 were exhausted, Gin-3, an explant culture taken from normal attached gingiva overlying an unerupted second mandibular molar at the UTHSC-SA, was used. Both cultures were grown to confluency in Dulbecco modified Eagle medium (DMEM) containing Ca^{2+} (0.2 mg/ml) and Mg^{2+} (0.2 mg/ml), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml) (GIBCO Laboratories, Grand Island, N.Y.) at 37°C in a 5% CO_2 environment. Cell cultures were split 1:5 weekly after detachment of the cells with trypsin (2.5 g/ml; GIBCO; 1:250 trypsin) in Hanks balanced salts solution (HBSS) without $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, or EDTA. Cells were stored in liquid nitrogen at passages 4 to 8 in DMEM supplemented with 10% (vol/vol) dimethyl sulfoxide. Adherence assays were performed only with HGFs passaged between 8 and 20 times. Gin-1 and Gin-3 cultures behaved identically in the adherence assays.

Adherence assay. The interaction of the *T. denticola* strains with the Gin-1 or Gin-3 HGF cultures was carried out in 96-well polystyrene plates (Corning Glass Works, Corning, N.Y.). Gin-1 or Gin-3 cells, at concentrations of 2×10^4 cells per ml, were seeded to the plates, and the cells were grown in DMEM (100 μl/well) plus 10% FBS (vol/vol) without antibiotic or antimycotic agents. Cells were grown to confluency (approximately 3 days), washed twice with HBSS, and suspended in either HBSS or DMEM. Prior to all conditions tested, confluent monolayer cultures were checked for viability by trypan blue exclusion, and only viability scores of at least 95% were used. Moreover, only confluent cultures were used to minimize nonspecific adsorption of bacteria to the exposed polystyrene surfaces (see Results). Representative wells were trypsinized (see above), and cell number was determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). Results indicated that approximately 3×10^4 cells constituted confluent growth in each well at the start of an assay. ^{125}I-labeled *T. denticola* treponemes (see above) were then added to each well at a ratio of 500 treponemes/1 fibroblast, and plates were allowed to incubate for 1 h at 37°C in a 5% CO_2 environment. Each well was then washed three times with HBSS, the cells were detached with trypsin at 100 μl/well (see above), and the contents of each well were counted with a Beckman Gamma 310 counter. Adherence data are presented as the relative number of bacteria per HGF calculated from the specific activity of the bacteria and the number of HGFs per well.

Effect of divalent cations and EDTA on adherence of *T. denticola* strains to HGFs. The effect of Ca^{2+} (0.2 mg/ml) and Mg^{2+} (0.2 mg/ml) on adherence was determined by their addition to HBSS. Confluently grown HGFs were washed twice with HBSS (minus Ca^{2+}, Mg^{2+}, and FBS) (see above) and suspended in HBSS with Ca^{2+}, Mg^{2+}, or Ca^{2+} and Mg^{2+}. The adherence assay was then followed as described above. Moreover, the addition of EDTA (0.5 and 1.0 mM) to DMEM containing Ca^{2+} (0.2 mg/ml) and Mg^{2+} (0.2 mg/ml) and to HBSS with and without Ca^{2+} and Mg^{2+} was also checked for effects on adherence of the *T. denticola* strains to HGFs.

Antibody production to *T. denticola* GM-1. Five milliliters of *T. denticola* GM-1 was harvested when an optical density at 660 nm of 0.270 was reached; then cultures were centrifuged and washed twice with PBS, pH 7.4. The cell pellet (approximately 2.5×10^9 cells) was suspended in 5 ml of formal-saline for approximately 12 h, washed twice in PBS, and suspended in 0.5 ml of PBS. This suspension was vigorously vortexed with an equal volume of Freund incomplete adjuvant. New Zealand White rabbits (males; approximately 4 kg) were injected at two sites, 0.5 ml intramuscularly and 0.5 ml subcutaneously. The rabbits were prebled on day 0, and the immunization protocol was carried out once per week for 3 weeks. The animals were then rested for 1 week and test bled, and antibody titers to *T. denticola* GM-1 were determined by enzyme-linked immunosorbent assay by the method of Ebersole et al. (11). Antibody titers of 1:819,200 were achieved. The rabbits were bled by cardiac puncture, and the sera were separated and stored at −20°C.

Antibody inhibition. Polyclonal antibodies to *T. denticola* GM-1 (see above) were examined for their ability to affect *T. denticola*-HGF interactions. Various dilutions of heat-inactivated whole-cell antiserum to the GM-1 strain (i.e.; 1:1,600, 1:800, and 1:50) were incubated with the three ^{125}I-labeled *T. denticola* strains in PBS at 37°C for 1 h with constant gentle agitation. Unbound antibody was removed by two washes in PBS by centrifuging at 9,000 × *g* for 20 min, followed by suspension of the bacterial pellets in PBS to their original volume. The bacterial suspensions were then appropriately suspended for the adherence assay (see above). As controls, the three ^{125}I-labeled *T. denticola* strains were also incubated with heat-inactivated preimmune rabbit serum at identical dilutions, as well as with PBS alone.

Protease treatment of *T. denticola*. The effect of trypsin and

proteinase K (PK) on adherence was examined to determine the role of surface proteins in this interaction. Each *T. denticola* strain was intrinsically labeled with [^{3}H]adenine (5 μCi/ml of GM-1 broth; specific activity, 31 Ci/mmol; ICN Biomedicals, Inc., Irvine, Calif.). After a 5-day growth period (see above), cultures were harvested (see above) and treated with trypsin (0.05, 0.1, and 0.5 mg/ml) (type XIII; Sigma Chemical Co., St. Louis, Mo.) or PK (0.1, 0.3, and 0.5 mg/ml) (type XI; Sigma) for 1 h at 37°C with constant gentle agitation. Control bacterial suspensions without the proteases were incubated under identical conditions. Following incubation, the treated cells were washed three times with PBS, and the resulting bacterial pellets were suspended in PBS to their original volumes prior to assaying. The possibility that residual PK was carried over after washing with PBS was eliminated by the incorporation of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma) into the reaction mixture at a final concentration of 1 mM. PMSF was added after the incubation of the ^{125}I-labeled treponemes with PK (0.5 mg/ml) at 37°C for 30 min with constant gentle agitation. PMSF controls consisted of ^{125}I-labeled treponemes with PMSF, incubated at 37°C for 30 min prior to interaction with HGFs, as well as PMSF with PK (0.5 mg/ml) incubated at 37°C for 30 min prior to incubation with the ^{125}I-labeled treponemes.

Enzymatic treatment of HGF. Gin-3 HGFs were exposed to trypsin in an attempt to examine the biochemical characteristics of the receptor-binding molecule on the HGF cell surface. HGFs (2×10^4 per well) were grown to confluency, washed twice with HBSS, and treated for 30 min at 37°C with trypsin (1, 2, and 5 μg/ml) (type XIII; Sigma). The treated and control HGFs were then washed twice with HBSS and suspended in HBSS or DMEM, and adherence was determined as described above. Coulter Counter analysis of representative wells, at the end of each assay, was performed (see above) to determine the number of retained HGFs pretreated with trypsin.

Effect of lectins on adherence of *T. denticola* strains to HGFs. The effect of lectins on adherence was assessed by incubating confluent layers of HGFs with concanavalin A (Con A; type IV), *Bauhinia purpuraea, Bandeiraea simplicifolia,* or *Ricinus communis* (Sigma) diluted in DMEM to a 50- or 100-μg/ml final concentration for 1 h at 37°C before the addition of bacteria. Adherence assays followed as described above.

Periodate oxidation of HGFs. HGFs were incubated with 10 mM sodium metaperiodate (Sigma) for 10 min at room temperature by the method of Ofek et al. (32) to determine the presence or absence of oxidizable sugar moieties which could be involved in the adherence of *T. denticola* to HGFs. After two washes with HBSS, the experimental and control HGFs were assayed for *T. denticola*-HGF adherence (see above).

Sugar inhibition. To determine whether a sugar moiety, and, if so, what type, on the surface of the HGF was involved in the adherence of *T. denticola* GM-1 to HGFs, treponemal cell suspensions (2.5×10^9 cells per ml) were incubated with equal volumes of D-glucose (D-Glu), D-galactose (D-Gal) (J. T. Baker Inc., Phillipsburg, N.J.), D-mannose (D-Man; Fisher Scientific Co., Fair Lawn, N.J.), and *N*-acetyl-D-galactosamine (D-GalNAc; Sigma) at concentrations of 5, 10, 25, and 50 mM. The sugar-*T. denticola* suspensions were mixed by occasional gentle agitation for 1 h at 37°C, after which they were incubated directly with the HGFs for 1 h at 37°C (see above). The extent of *T. denticola* adherence was determined as described above.

Role of FBS and FN. The role of FBS and freshly isolated bovine fibronectin (FN) in *T. denticola*-HGF adherence was examined. Heat-inactivated (56°C, 30 min) FBS was added at 1, 5, or 10% (vol/vol) to confluent HGFs. *T. denticola* adherence was then determined as described above.

Purified FN was prepared from bovine serum by gelatin-affinity chromatography by the method of Miekka et al. (30). Briefly, cyanogen bromide-activated Sepharose CL4B (Pharmacia Inc., Piscataway, N.J.) was mixed with gelatin (Bloom 300, 2 mg/ml) for 12 h at 4°C. After washing, the gel was mixed with 10% bovine serum which had previously been diluted in 0.1 M imidazole in normal saline, pH 6.8. This mixture was then packed into a polypropylene column (2.5-cm inner diameter), washed with 10 mM sodium citrate (pH 7.2), and then slowly percolated with 3 M urea–50 mM sodium citrate in PBS, pH 5.5. Column fractions (5 ml) were collected, and protein was detected by UV measurements at 280 nm. The protein-rich fractions were combined and dialyzed against 0.1 M imidazole in normal saline, pH 6.8, for 3 days and stored at 4°C. Protein content was determined by using the BCA protein assay (Pierce Chemical Co., Rockford, Ill). To determine the purity of the eluted FN, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (21) as described by Miekka et al. (30). Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of the recovered FN revealed one distinct band at approximately 230 kilodaltons, characteristic of reduced FN. Prior to *T. denticola*-HGF adherence assays with the purified FN, the latter was dialyzed against normal saline, pH 6.8, for 48 h to remove imidazole. The biological activity of the isolated FN was determined by the cell attachment assay of Klebe (personal communication) based on the method of Engvall and Ruaslahti (12). Purified FN in HBSS or DMEM was added at 30, 60, and 100 μg/ml to washed HGFs and assayed for *T. denticola* adherence (see above).

AbFN studies. The effect of blocking FN binding sites located on the HGF surface on the adherence of ^{125}I-labeled *T. denticola* to HGFs was assessed with rabbit anti-human fibronectin (AbFN) (immunoglobulin G fraction; Cappel, Organon Teknika Corp., West Chester, Pa.), diluted 1:5. AbFN was incubated with prewashed HGFs for 1 h at 37°C prior to the addition of the ^{125}I-labeled *T. denticola* strains. To demonstrate that the AbFN was actually binding to FN on the HGF surface, we used an immunofluorescence assay with fluoresceinisothiocyanate-labeled goat anti-rabbit immunoglobulin. HGF cells (2×10^4 per well) were grown in Lab-Tek multichamber slides (Miles Scientific, Naperville, Ill.) for 24 h. After washing each well three times with HBSS, 0.1 ml of ice-cold acetone per well was added for 1 min. Following removal of the acetone, the wells were air dried, and 100 μl of serial dilutions (ranging from 1:50 to 1:6,400) of AbFN per well was added; wells were incubated with the fixed cells for 1 h at room temperature. After three washes with HBSS, 100 μl of fluorescein isothiocyanate-antibody per well was added at 1:200 and wells were incubated in the dark for 1 h at room temperature. Slides were then prepared for viewing under UV light. As a control, identical dilutions of rabbit antibody to *Bacteroides intermedius* instead of AbFN were used. While the latter antibody was negative, the presence of AbFN produced intense fluorescence (peaking at 1:800).

To explain some of the results arising from the AbFN studies (see Results), the AbFN was treated with papain (using the ImmunoPure Fab preparation kit, no. 44885;

TABLE 1. Adherence of *T. denticola* GM-1, TD-4, and MS25 to HGFs under aerobic and anaerobic conditions[a]

Strain	Medium	Relative no. of bacterial cells attached per HGF ± SD[b]	
		Aerobic	Anaerobic
GM-1	DMEM	51 ± 4	55 ± 4
	HBSS	103 ± 9	112 ± 10
TD-4	DMEM	27 ± 3	32 ± 3
	HBSS	63 ± 6	58 ± 6
MS25	DMEM	31 ± 3	41 ± 5
	HBSS	92 ± 10	102 ± 9

[a] ^{125}I-labeled *T. denticola* GM-1, TD-4, or MS25 was added to confluent HGFs at a ratio of 500 treponemes/1 fibroblast, in either DMEM or HBSS, and incubated for 1 h at 37°C in either aerobic or anaerobic conditions.

[b] Mean of eight experiments; $P > 0.05$ for aerobic versus anaerobic conditions by the *t* test for two samples.

Pierce Co.) prior to its incubation with prewashed HGFs at the same titer as for untreated AbFN as described above.

Electron microscopy. Samples for both transmission electron microscopy and SEM were prepared by the method of Kinder and Holt (18).

Statistical analysis. When applicable, data were analyzed by the Student *t* test for two samples, the test for polynomial trends, and the Student Neuman-Keul's procedure (19, 28). Analysis of variance, using unique sums of squares, was conducted for the lectin data. Statgraphics software (STSC, Inc.) was the source for this analysis. In all analyses, a value of $P < 0.05$ was accepted as significant.

RESULTS

Adherence of *T. denticola* strains to HGFs. The adherence of *T. denticola* GM-1, TD-4, and MS25 to HGFs, under aerobic and anaerobic conditions, in the presence of DMEM and HBSS is seen in Table 1. In the presence of DMEM, strain GM-1 showed the maximum adherence to the HGFs under both aerobic (5% CO_2) and anaerobic (85% N_2–10% CO_2–5% H_2) conditions, while strain TD-4 showed the lowest. However, when adherence was examined in HBSS, strains GM-1 and TD-4 adhered to the HGF cultures at least two times (for strain MS25, three times) more than that which occurred in DMEM. The cellular confluency, however, was retained better in DMEM than in HBSS for the duration of each assay. SEM revealed that, after 1-h incubation in HBSS, the HGFs lost their confluency, exposing considerably more cellular surface area (Fig. 1E). This was not observed in DMEM. Therefore, unless otherwise indicated, the results presented are from assays in DMEM, under aerobic conditions, using *T. denticola* GM-1. Generally, results in aerobic conditions were not significantly different from those in anaerobic conditions ($P > 0.05$). Where major differences between the strains were found, they are indicated.

Electron microscopy of adherence of *T. denticola* strains to HGFs. Both transmission electron microscopy and SEM studies showed that *T. denticola* GM-1, TD-4, and MS25 adhered primarily to HGF surface villi and fibroblast trabeculations (Fig. 1A to D, F, and G). This adherence was not exclusively tip associated, as noted for *T. pallidum* (14), but appeared to be random at points on the treponemal surface (Fig. 1F to H). When assays were conducted in strict anaerobic conditions and processed for SEM examination, no difference in binding was noted.

Effect of divalent cations on adherence of *T. denticola* to HGFs. There was no appreciable difference in adherence when assays were run in HBSS with or without Ca^{2+}, Mg^{2+}, or Ca^{2+} and Mg^{2+}. Moreover, EDTA had no effect on adherence of any of the *T. denticola* strains to HGFs when added to either DMEM or HBSS.

Trypsin and PK activity on *T. denticola*-HGF interactions. (i) The role of *T. denticola* GM-1 surface proteins in the adherence to HGFs was examined after exposure to various concentrations of trypsin and PK, prior to incubation with the HGFs (Table 2). To avoid possible proteolysis of treponemal surface proteins radiolabeled with ^{125}I, thereby resulting in false-positive interpretations, we intrinsically labeled each *T. denticola* strain with [^{3}H]adenine (see above). At the trypsin concentrations selected, there was no inhibition of adherence to HGFs with the strains tested. PK, however, was inhibitory to GM-1–HGF interactions, with 0.5 mg/ml inhibiting this adherence by 80% (Table 2). The effect of the protease inhibitor PMSF on GM-1–HGF interaction was also examined (Table 2). Blocking of PK activity with the protease inhibitor PMSF resulted in GM-1–HGF interaction comparable to that of the control ($P > 0.05$). Assays which included PMSF only after incubation of the GM-1 strain with PK (0.5 mg/ml) resulted in a comparable inhibition of the GM-1–HGF interaction as seen when PK was added to the treponemes followed by washing with PBS.

(ii) The effect of trypsin on HGF surface proteins and subsequent effect on *T. denticola* GM-1–HGF interaction was examined after exposure of confluent HGFs to various concentrations of trypsin, prior to incubation with *T. denticola* GM-1 (Table 3). Exposure of the HGFs to trypsin (1 and 2 μg/ml) in DMEM for 30 min prior to incubation with *T. denticola*-GM-1 resulted in almost no change in the GM-1–HGF interaction compared with the control. At the highest trypsin concentration used (5 μg/ml), there was a 50% increase in adherence. In comparison, HGFs pretreated with trypsin at this concentration followed by interaction with *T. denticola* MS25 resulted in an 86% increase in adherence when compared with the control (Table 3). In contrast to strains GM-1 and MS25, strain TD-4 showed a decrease in adherence of 34 and 41% when exposed to HGFs previously treated with 1 and 5 μg of trypsin per ml, respectively (Table 3). Coulter Counter analysis of confluent HGF layers pretreated with 1, 2, and 5 μg of trypsin per ml assayed without the addition of treponemes in DMEM showed losses of 3.6, 6.2, and 10.0% fibroblasts, respectively.

Effect of AbGM-1 on adherence of *T. denticola* to HGFs. Exposure of *T. denticola* GM-1 to GM-1 whole-cell antibody (AbGM-1) resulted in a significant inhibition of *T. denticola*-HGF interactions (Table 4). At a dilution of 1:1,600, the AbGM-1 reduced adherence of strain GM-1 to HGFs by 67%. At higher concentrations (i.e., 1:800 or 1:50), inhibition of GM-1 adherence to the HGFs increased to 77 and 90%, respectively. In comparison, at the highest concentration (1:50), the AbGM-1 only reduced adherence of strains TD-4 and MS25 to HGFs by 54 and 29%, respectively (Table 4).

Periodate oxidation of HGFs and effect on adherence of *T. denticola*. Exposure of the HGFs to sodium metaperiodate and subsequent examination of the interaction of these fibroblasts with *T. denticola* GM-1, TD-4, and MS25 is seen in Fig. 2. The adherence of both *T. denticola* GM-1 and MS25 to periodate-exposed HGFs was significantly affected by the activity of this carbohydrate-oxidizing agent. Adherence of strain GM-1 to fibroblasts pretreated with 10 mM periodate for at least 1 min resulted in an approximate 50% inhibition of adherence (Fig. 2), while strain MS25 showed a

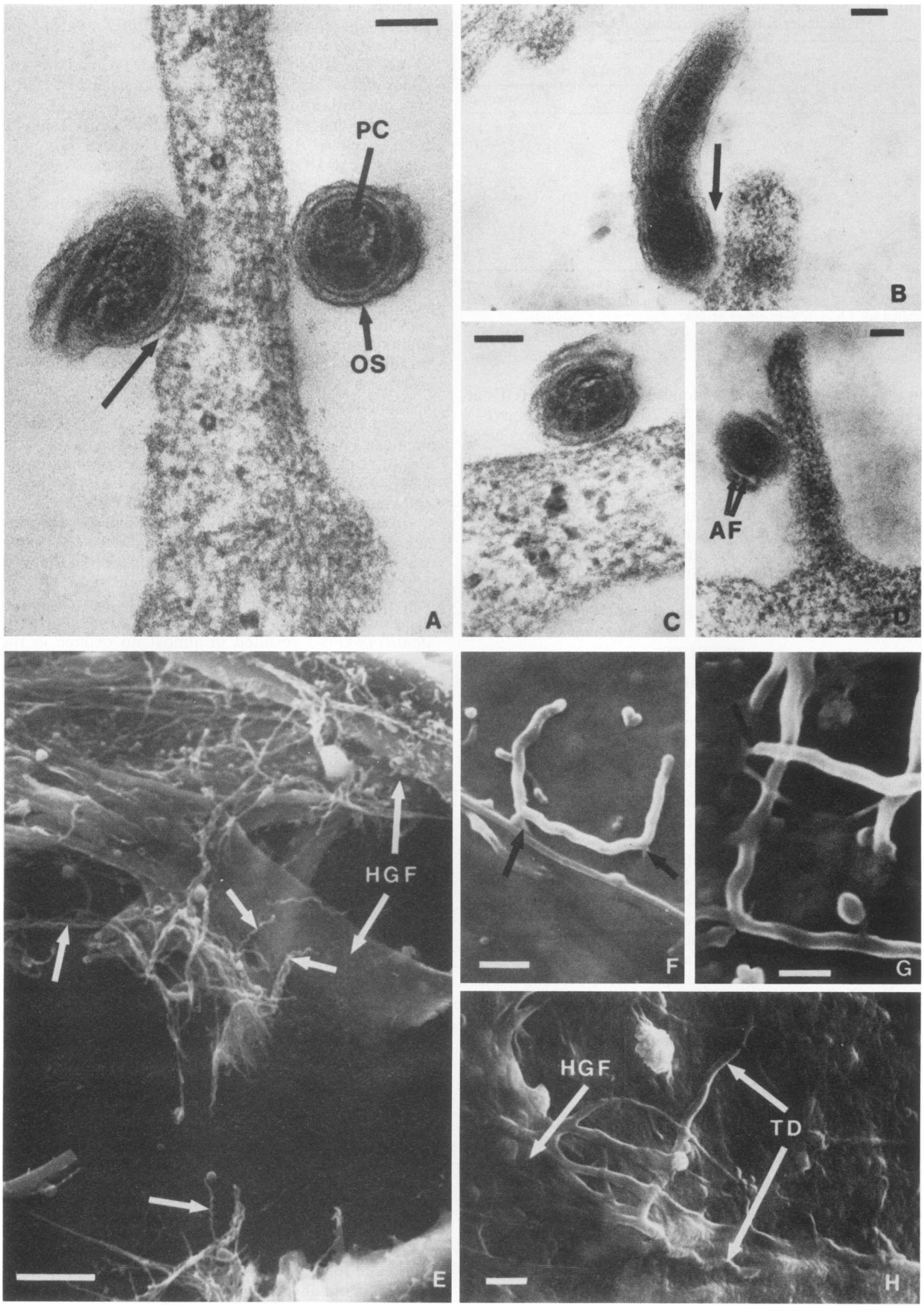
PC
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TABLE 2. Effect of protease treatment of bacterial cells on adherence of *T. denticola* GM-1 to HGFs[a]

Treatment	Relative no. of bacterial cells attached per HGF ± SD[b]	Relative adherence (%) ± SD
None	54 ± 6	100 ± 11
Trypsin (mg/ml)		
0.05	56 ± 6	104 ± 11
0.10	55 ± 7	102 ± 13
0.50	52 ± 5	96 ± 9
PK (mg/ml)		
0.1	46 ± 4	85 ± 7
0.3	22 ± 4	41 ± 7
0.5	11 ± 3	20 ± 5
PMSF (1 mM)[c]	53 ± 6	98 ± 11
PK (0.5 mg/ml) + PMSF[d]	52 ± 4	96 ± 7
[PK (0.5 mg/ml) + treponemes] + PMSF[e]	13 ± 2	24 ± 4

[a] Results for strains TD-4 and MS25 were similar to those for strain GM-1. [^{3}H]adenine-labeled *T. denticola* GM-1 was treated with trypsin or PK for 1 h at 37°C. Treated bacteria were washed three times with PBS and suspended to original volumes, after which adherence to HGFs was determined as described in the text.

[b] Mean of quadruplicate determinations.

[c] PMSF was added to [^{3}H]adenine-labeled *T. denticola* GM-1 and incubated for 30 min at 37°C. Adherence assays with HGFs followed as described in the text. $P > 0.05$ for PMSF-treated bacteria versus control by the *t* test for two samples.

[d] PK was incubated with PMSF (1 mM) for 30 min at 37°C, after which [^{3}H]adenine-labeled *T. denticola* GM-1 was added and incubated for 1 h at 37°C. Adherence with HGFs followed as described in the text. $P > 0.05$ for results with treated bacteria versus control by the *t* test for two samples.

[e] [^{3}H]adenine-labeled bacteria were treated with PK for 1 h at 37°C, after which PMSF (1 mM) was added and incubated for 30 min at 37°C. Adherence with HGFs was determined as described in the text.

TABLE 3. Effect of trypsin treatment of HGFs on adherence of *T. denticola* GM-1, TD-4, and MS25[a]

Strain	Trypsin (μg/ml)	Relative no. of bacterial cells attached per HGF ± SD[b]	Relative adherence (%) ± SD
GM-1	None	50 ± 7	100 ± 14
	1	45 ± 9	90 ± 18
	2	54 ± 3	108 ± 6
	5	75 ± 6	150 ± 12
MS25	None	33 ± 5	100 ± 15
	1	31 ± 1	94 ± 3
	2	42 ± 3	127 ± 9
	5	61 ± 1	185 ± 3
TD-4	None	32 ± 7	100 ± 22
	1	21 ± 4	66 ± 13
	2	20 ± 2	63 ± 6
	5	19 ± 4	59 ± 13

[a] Confluent HGFs were treated with trypsin for 30 min at 37°C, after which adherence with the three *T. denticola* strains was determined as described in the text.

[b] Mean of eight determinations.

similar but more linear inhibition over the time periods studied (1 to 5 min). Interestingly, strain TD-4 reacted very differently from GM-1 and MS25 with the periodate-exposed HGFs. Exposure of the HGFs to periodate for 2 min resulted in a 32% increase in TD-4–HGF interactions.

Role of sugars and lectins in *T. denticola* GM-1–HGF interaction. (i) The effects of D-Glu, D-Gal, D-Man, and D-GalNAc on *T. denticola* GM-1–HGF interactions are seen in Table 5. D-Glu had very little effect on this interaction, with 50 mM resulting in only a 20% inhibition of adherence. In contrast, D-Gal, D-Man, and D-GalNAc resulted in a >60% reduction in GM-1–HGF interaction. A distinct dose-response inhibition was evidenced with these three sugars, a result not seen with D-Glu (Table 5).

(ii) To implicate a lectinlike adhesin on the surface of *T. denticola* as the mediator of adherence to HGFs, a variety of plant lectins known to be specific for D-Gal, D-Man, and D-GalNAc were screened as potential inhibitors of *T. denticola* attachment (Table 6). There appeared to be *T. denticola* strain specificity relevant to lectin activity for the treponemal strains studied. *Bandeiraea simplicifolia* inhibited the GM-1 and TD-4 strains by 34 and 47%, respectively (at 100 μg/ml), while not inhibiting the MS25 strain. Strain MS25, however, was maximally inhibited by *R. communis* (50% at 100 μg/ml). In the case of strain TD-4, the predominant inhibition was manifested with ConA and *R. communis* (>60% at 100 μg/ml), while for GM-1 maximum inhibition occurred with ConA (46% at 100 μg/ml). Minimal inhibition was evidenced with the *Bauhinia purpurea* lectin.

Effect of FBS on adherence of *T. denticola* to HGFs. The effect of 56°C heat-inactivated FBS at final concentrations between 1 and 10% on the *T. denticola*-HGF interaction is seen in Fig. 3. All of the *T. denticola* strains were affected by the FBS concentrations in a dose-dependent fashion. Note that at all FBS concentrations, strain TD-4 was affected in its interaction with the HGFs to a greater degree than strains GM-1 and MS25 ($P < 0.05$).

Effect of FN on adherence of *T. denticola* to HGFs. (i) Since FN has been shown to be involved in the adherence of *T. pallidum* to eucaryotic cells (36), we examined the role of purified FN in these *T. denticola*-HGF interactions (Fig. 4). FN significantly affected both GM-1 and MS25 adherence to the HGFs, resulting in an inhibition of approximately 60% at 100 μg/ml in both strains. However, FN had an opposite effect on strain TD-4-HGF interaction, with a 30% increase in adherence at the same FN concentration (Fig. 4).

(ii) The addition of AbFN (1:5) to the HGFs increased the adherence of strain GM-1 to HGFs by approximately 96%

FIG. 1. Electron micrographs of *T. denticola* adherence to HGFs. (A to D) Transmission electron microscopy of thin sections showing attachment of strain GM-1 (A) or strain TD-4 (B to D) to HGF villi. Indentations of the villi occur at points of attachment with the bacteria (arrows, panels A and B). (E) SEM of adherence of strain GM-1 to HGFs in HBSS. There is disruption in HGF confluency and adherence of bacteria to the smooth, raised surface of an HGF. This surface differs from the confluent surface, rich in villi, seen in the upper HGF. (F to H) SEM of *T. denticola* adherence to HGF surface structures. (F) Strain MS25 adhering along its body to HGF microvilli (arrows). (G) Tip-oriented (upper arrow) and body-oriented (lower arrow) adherence of strain GM-1 to HGF trabecular surface structures. (H) Surface trabecular HGF interaction with strain GM-1. Note the apparent embodiment of the treponeme to the HGF surface (lower arrow). PC, Protoplasmic cylinder; OS, outer sheath; AF, axial fibrils; TD, *T. denticola*. Bars: (A to D) 0.1 μm; (E) 5 μm; (F to H) 0.5 μm.

TABLE 4. Effect of *T. denticola* AbGM-1 on adherence of *T. denticola* GM-1, TD-4, and MS25 to HGFs[a]

Strain	AbGM-1 dilution	Relative no. of bacterial cells attached per HGF ± SD[b]	Relative adherence (%) ± SD
GM-1	0[c]	30 ± 5	100 ± 17
	1:1,600	10 ± 3	33 ± 10
	1:800	7 ± 3	23 ± 10
	1:50	3 ± 1	10 ± 3
TD-4	0	26 ± 4	100 ± 15
	1:1,600	15 ± 3	58 ± 11
	1:800	15 ± 2	58 ± 8
	1:50	12 ± 1	46 ± 4
MS25	0	28 ± 4	100 ± 14
	1:1,600	26 ± 3	93 ± 11
	1:800	22 ± 1	79 ± 3
	1:50	20 ± 1	71 ± 3

[a] ^{125}I-labeled *T. denticola* GM-1, TD-4, and MS25 were incubated with AbGM-1 at the given dilutions for 1 h at 37°C, after which adherence with HGFs was determined as described in the text.
[b] Mean of quadruplicate determinations. $P < 0.05$ for any titer when comparing any two strains by the t test for two samples.
[c] 0 = preimmune serum.

(Table 7). This dramatic increase was not seen with strain MS25; moreover, strain TD-4 showed a decrease of 35% (Table 7). Fab fractions from papain-treated AbFN resulted in adherence levels of strain GM-1 to HGFs similar to those of the control (data not shown). Therefore, the increase in adherence observed for strain GM-1 was apparently due to Fc portions of the immunoglobulin G molecules bridging between FN on the surface of HGFs and FN on the surface of the treponemes (the source for the latter being from the 1.7% normal rabbit serum added to the liquid medium used to grow *T. denticola*).

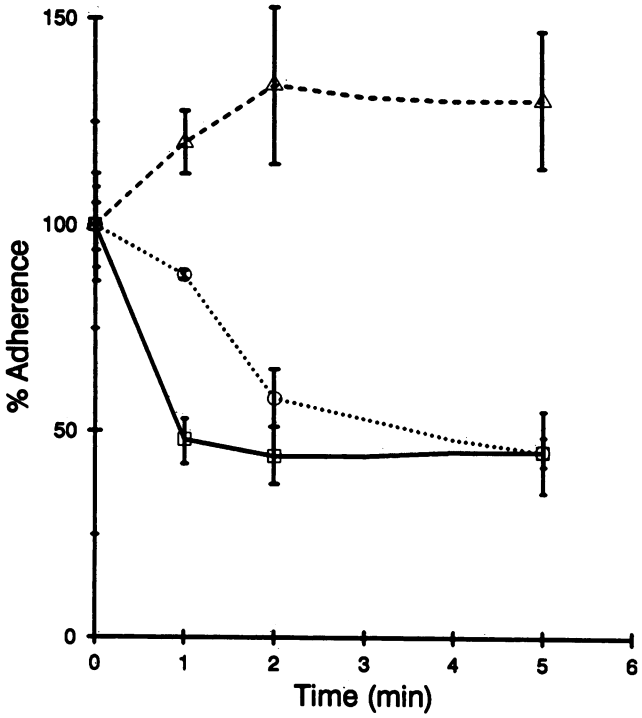

FIG. 2. Effect of periodate oxidation of HGFs on adherence of *T. denticola* GM-1, TD-4, and MS25. HGFs were incubated with 10 mM $NaIO_4$ for 1, 2, and 5 min at room temperature and assayed for *T. denticola* adherence as described in the text. Results represent the mean of four determinations ± standard deviation. Symbols: □, GM-1; △, TD-4; ○, MS25.

TABLE 5. Inhibition of adherence of *T. denticola* GM-1 to HGFs by carbohydrates[a]

Carbohydrate	Concn (mM)	Relative no. of bacterial cells attached per HGF ± SD[b]	Relative adherence (%) ± SD
None		40 ± 4	100 ± 9
D-Glu	5	36 ± 5	90 ± 13
	10	43 ± 6	107 ± 15
	25	39 ± 4	97 ± 10
	50	33 ± 3	83 ± 7
D-Gal	5	37 ± 4	93 ± 10
	10	33 ± 3	83 ± 7
	25	19 ± 0.5	47 ± 1
	50	16 ± 2	40 ± 5
None		44 ± 0.5	100 ± 1
D-Man	5	43 ± 1	98 ± 2
	10	26 ± 0.9	59 ± 2
	25	23 ± 1	52 ± 2
	50	16 ± 2	36 ± 5
D-GalNAc	5	39 ± 3	89 ± 7
	10	24 ± 1	55 ± 2
	25	23 ± 0.9	52 ± 2
	50	16 ± 2	36 ± 5

[a] Results for strains TD-4 and MS25 were similar to those for strain GM-1. ^{125}I-labeled *T. denticola* GM-1 were incubated with the given sugars for 1 h at 37°C, after which adherence with HGFs was determined as described in the text.
[b] Mean of quadruplicate determinations. $P < 0.05$, using the test for polynomial trends showing that D-Glu results deviate from the linear trend.

DISCUSSION

Of the three strains tested, *T. denticola* GM-1 showed the greatest ability to adhere to HGFs in the presence of DMEM (Table 1). While there was an almost doubling of *T. denticola*-HGF adherence in HBSS, the latter result was probably due to the exposure of more HGF surface area for binding. HGFs, in HBSS, lost confluency with exposure of considerably more cellular surface area. SEM showed that all three treponemal strains adhered avidly to these newly exposed surfaces as well as to HGF anchoring trabeculations in the presence of HBSS. These structures and surfaces were not exposed to the treponemes under DMEM conditions. The increase in HGF surface area would therefore explain the concomitant increase in treponemal adherence.

To assess the possible role of treponemal surface proteins as mediators of adherence, we tested polyclonal antibodies to GM-1 whole cells for their ability to inhibit attachment. Strain GM-1 was inhibited by almost 70% at an antibody dilution of 1:1,600, while the other two strains showed differing degrees of cross-reactive inhibition (Table 4). Moreover, protease treatment of the three strains suggest the involvement of surface-exposed proteins in the adherence to HGFs. Since these *T. denticola* surface proteins are trypsin resistant, and since a trypsinlike enzyme has been reported

TABLE 6. Inhibition of *T. denticola* attachment to HGFs by plant lectins[a]

Lectin	Concn (μg/ml)	Lectin specificity	% Inhibition of strain[b]:		
			GM-1	MS25	TD-4
Bauhinia purpurea	50	β-D-Gal(1→3)-D-GalNAc	0	0	0
	100		26	4	0
Bandeiraea simplicifolia	50	α-D-Gal; α-D-GalNAc	20	0	29
	100		34	7	47
ConA	50	α-D-Man; α-D-Glu	28	17	50
	100		46	24	61
Ricinus communis	50	β-D-Gal	28	33	55
	100		32	50	66

[a] Confluent HGFs were preincubated with lectins for 1 h at 37°C, after which adherence for the three strains was determined as described in the text.
[b] Calculated from means of eight determinations. Standard deviations never exceeded 12% for any mean. $P < 0.05$ when comparing the inhibition of a strain by one lectin to another lectin by analysis of variance, using unique sums of squares. $P < 0.05$ when comparing the inhibition of a strain by a lectin to another strain by the same lectin by analysis of variance, using unique sums of squares.

for *T. denticola* (22, 34), it is essential that they be nonreactive to this protease. Adherence involving these proteins would not be possible. Moreover, trypsinlike enzymes probably occur in the crevicular milieu, and trypsin-sensitive receptors on the *T. denticola* surface would not be ecologically sensible for in vivo interactions.

Pretreatment of HGFs with low concentrations of trypsin and subsequent interaction with *T. denticola* revealed an increase in adherence with strains GM-1 and MS25, while a decrease was evidenced for the TD-4 strain (Table 3). These findings suggest a proteolytic "unmasking" of HGF surface receptors (i.e., specific sugar receptors or cryptitopes) (16), with an avidity for the treponemal adhesins. *Bacteroides gingivalis* and *Pseudomonas aeruginosa* have been shown to adhere much more readily to trypsin-treated epithelial cells (16). Our observations that FN may be involved in *T. denticola*-HGF interactions, and the response of the trypsin-pretreated HGFs, suggest the exposure of specific sugar moieties. Since the glycoprotein FN was present on the fibroblast surface and has been shown to contain a major

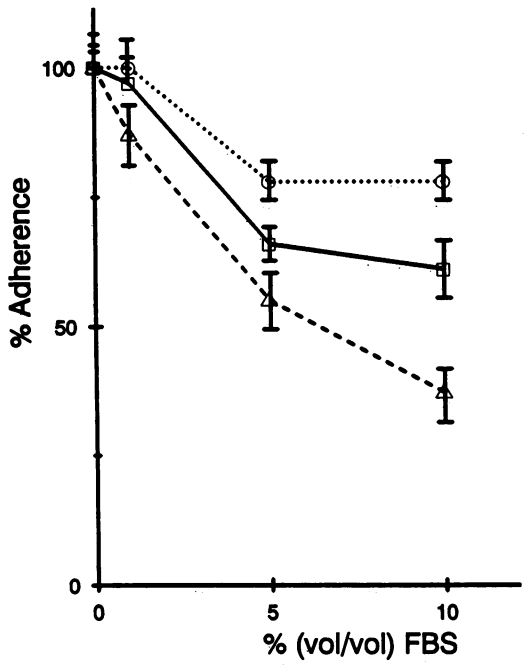

FIG. 3. Effect of FBS on adherence of *T. denticola* GM-1, TD-4, and MS25 to HGFs. Heat-inactivated FBS was added at 1, 5, or 10% (vol/vol) to confluent HGFs. *T. denticola* adherence was determined for each strain as described in the text. Results represent the mean of four determinations ± standard deviation. $P < 0.05$ when comparing results for one strain with those for another by the Student Neuman-Keul's procedure. Symbols: □, GM-1; △, TD-4; ○, MS25.

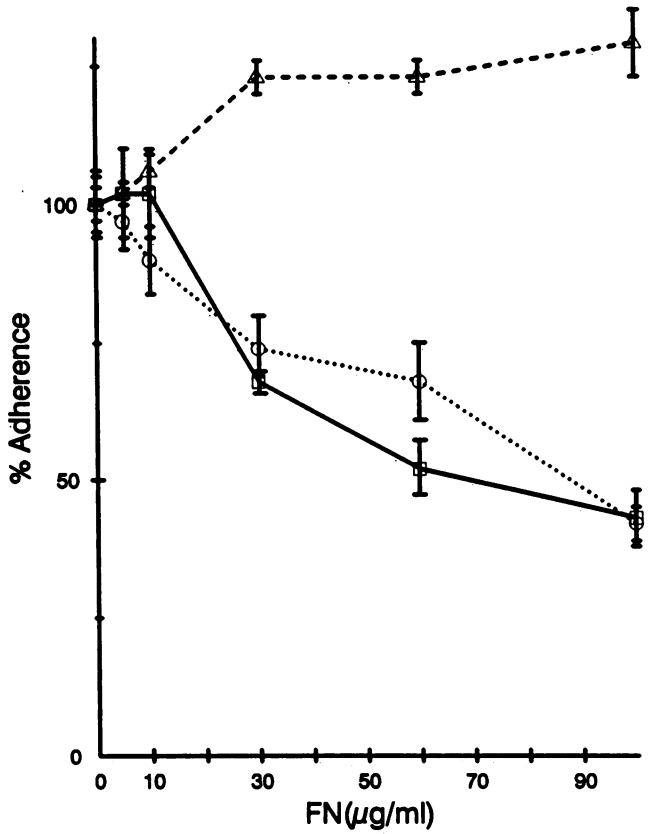

FIG. 4. Effect of FN on adherence of *T. denticola* GM-1, TD-4, and MS25 to HGFs. Purified FN was added to confluent HGFs in the given concentrations, after which *T. denticola* adherence for each strain was determined as described in the text. Results represent the mean of four determinations ± standard deviation. Symbols: □, GM-1; △, TD-4; ○, MS25.

TABLE 7. Effect of AbFN on adherence of *T. denticola* to HGFs[a]

Strain	Treatment	Relative no. of bacterial cells attached per HGF ± SD[b]	Relative adherence (%) ± SD
GM-1	None	51 ± 3	100 ± 6
	AbFN (1:5)	100 ± 7	196 ± 14
MS25	None	33 ± 4	100 ± 12
	AbFN (1:5)	40 ± 6	121 ± 18
TD-4	None	26 ± 3	100 ± 6
	AbFN (1:5)	17 ± 3	65 ± 6

[a] Confluent HGFs were incubated with AbFN (1:5) for 1 h at 37°C, after which adherence assays were conducted for the strains GM-1, TD-4, and MS25 as described in the text.

[b] Mean of eight determinations. $P < 0.05$ when comparing results of one strain with those of another by the Student Neuman-Keul's procedure.

oligosaccharide unit with two terminal β-D-galactose residues (9, 15), it is consistent with our observations of galactose residues being involved in adherence to *T. denticola*. Cleavage of FN during the course of hydrolysis by trypsin may be facilitating exposure of this sugar residue to *T. denticola* and increasing its adherence to HGFs.

Normal, heat-inactivated FBS resulted in a dose-response inhibition in the *T. denticola* strains tested (Fig. 3), and the differences observed in the inhibition patterns for the three strains were significant. These results suggest that a serum factor(s) binds to either the treponemes or the HGFs, causing an inhibition in adherence. If *T. denticola* has an equal affinity for a serum factor which is also expressed on the HGF surface, then addition of this factor would competitively inhibit the treponemes from adhering to the HGFs. The addition of purified fibronectin, at increasing concentrations, simultaneously with treponemes caused a dose-response inhibition for strains GM-1 and MS25 while increasing adherence for strain TD-4 (Fig. 4), further supporting *T. denticola* strain differences for adherence to HGFs. TD-4 may be demonstrating an additional mechanism of adherence through the mediation of free FN. AbFN blocked adherence of strain TD-4 to HGFs by 35%, suggesting a direct involvement of FN with the TD-4 strain in adherence to HGFs.

The plant lectins used in this study further defined the sugar residues on the HGF surface. Lectins from ConA (with specificity for α-D-mannose and α-Dglucose) and *R. communis* (with specificity for β-D-galactose) were most effective inhibitors of attachment for all three strains (Table 6). Moreover, the carbohydrate inhibition studies (Table 5) showed a linear dose response for D-Gal, D-Man, and D-GalNAc, but not for D-Glu. This, therefore, would eliminate the possibility that ConA inhibition of adherence could be due to the presence of glucose residues on the HGF surface and supports an α-D-mannose involvement. Differences in the degree to which the three *T. denticola* strains were inhibited by each of the lectins tested also indicated a sugar specificity. *Bandeiraea simplicifolia*, with a primary specificity for α-D-galactose and a secondary specificity for α-D-GalNAc (17), inhibited the GM-1 and TD-4 strains but not the MS25 strain (Table 6). Since *R. communis*, with a β-D-galactose specificity, inhibited the MS25 strain by 50%, it is more than likely that β-D-galactose is specifically involved in *T. denticola* MS25 interaction with HGFs. Moreover, the TD-4 and GM-1 strains were inhibited by >30% by those lectins with a primary specificity for α- or β-D-galactose and α-D-mannose. Therefore, taken collectively, these observations indicate that HGF receptors for *T. denticola* adherence possess mannose and galactose moieties.

Periodate oxidation of HGFs (Fig. 2) also suggests a sugar residue involvement in HGF-*T. denticola* interactions. The absence of inhibition of strain TD-4 and its slight positive effect on adherence could be due to the short periods of periodate oxidation (1 to 5 min). It is possible that there was only partial cleavage of 1,2-dihydroxyl groups of the HGF surface sugar molecules, leaving others intact and retaining intact terminal sugars for adherence. It is also possible that strain TD-4 may recognize a greater variety of terminal sugars than strains GM-1 and MS25, thereby retaining adherence at control levels even after 5 min of periodate treatment.

The findings of the present investigation on *T. denticola* adherence to mannose and galactose residues on HGFs suggest that these bacteria may adhere to other cell surfaces possessing these same sugar moieties. Indeed, *T. denticola* has been shown to adhere to epithelial cells (4, 35, 37) as well as being toxic to them (4, 37). In one case, morphological damage and detachment of epithelial cells occurred after bacterial attachment during 24- and 48-h incubations (37). A recent study (V.-J. Uitto, D. Grenier, and B. C. McBride, J. Dent. Res. **68**:894, abstr. no. 223, 1989) has shown that, when porcine periodontal ligament epithelial cells were cultured in the presence of live *T. denticola*, epithelial cell growth was inhibited and morphological damage occurred. This damage was described as the appearance of perinuclear vacuoles and formation of a hole in the epithelial cell membrane. Moreover, it has recently been shown that a chymotrypsinlike enzyme, isolated from *T. denticola* (44), resides on its external surface (D. Grenier, B. C. McBride, and V.-J. Uitto, J. Dent. Res. **68**:894, abstr. no. 222, 1989) and is capable of both inhibiting growth and causing morphological damage to epithelial cells, as do intact *T. denticola* (Uitto et al., J. Dent. Res. **68**:894, 1989).

The observations reported here, when taken together with other findings, suggest the possibility of a dynamic and fluid interaction between the host and the resident microbiota. This interaction further suggests the presence of extracellular enzymatic activity in the crevicular milieu, originating from various microorganisms, which have the ability to chemically modify the cells, exposing receptors for bacteria-cell interactions. Once these bacteria attach to host cells, surface-bound enzymes are activated, causing disruption of host cell integrity.

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