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Tip-Oriented Adherence of *Treponema denticola* to Fibronectin

JULETH R. DAWSON AND RICHARD P. ELLEN*

Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

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The adherence of *Treponema denticola* to ligands on cell surfaces or in basement membranes of periodontal tissues might play an important role in its pathogenicity. A direct microscopic assay was used to examine the binding of *T. denticola* to fibronectin and other protein substrates adsorbed on plastic cover slips. All strains of *T. denticola* that were tested adhered to fibronectin but to different degrees. The strains which bound in high numbers frequently bound by their tips. Type strain ATCC 33520 bound to fibronectin in high numbers (149 ± 11.3 bacteria per microscopic field), with 60% bound by the tips. Strain e' bound in high numbers (140 ± 10.2) and had the highest percentage of tip binding (98%); strain e bound in lowest numbers (39 ± 8.2) and had the lowest percentage of tip binding (15%). Laminin supported binding at a level similar to that of fibronectin, as did fibronectin fragments which contained the cell binding domain peptides, RGDS. Type IV collagen and non-RGDS peptides did not support binding. Binding to fibronectin and laminin was inhibited by the addition of antifibronectin and antilaminin antibodies. By lowering the incubation temperature from 37 to 4°C, the number of cells that attached decreased by 60% and tip binding was reduced by 50%. Pretreatment of the cells with collagen did not affect binding, whereas fibronectin pretreatment enhanced binding by 50% and laminin pretreatment resulted in a decrease of 60%. *T. denticola* adheres by its tips to fibronectin-coated surfaces, which suggests that fibronectin-specific adhesins cluster at the tips.

Spirochetes have been implicated in the etiology and pathogenesis of periodontal diseases (15, 20, 22). Microscopic studies have revealed that they are present in low numbers in healthy gingival crevices but often predominate in pockets associated with periodontal disease (16, 23). Their populations are also markedly decreased after treatment (19). Electron microscopy of periodontal pockets has shown spirochetes at the apical surfaces of subgingival plaque in direct contact with the pocket epithelium and polymorphonuclear leukocytes (18, 26). They are also known to invade the gingival lamina propria in acute necrotizing ulcerative gingivitis (21). Factors contributing to their virulence have become better appreciated in recent years because of the ability to cultivate and maintain some of these fastidious organisms in the laboratory (5, 6, 10).

Treponema denticola is a small spirochete which has been cultivated routinely from periodontal pockets. In vitro, it has been shown to possess certain characteristics which are potential virulence factors. It has the ability to stimulate polymorphonuclear leukocytes (3, 35), to produce endotoxins, and to elaborate chemotactic factors or end products, such as ammonia and putrescine (17), which are potentially cytotoxic for gingival tissues. In addition, *T. denticola* has been reported to suppress fibroblast proliferation (4) and to produce chymotrypsinlike (41) and trypsinlike (27) enzymes, the latter of which has the ability to disrupt cell adhesion by binding to and destroying the collagenase inhibitor α_2-macroglobulin (2, 9, 30, 40, 42). For its colonization to be most efficient and its pathogenic mechanisms to be most damaging in vivo, it would have to bind to host tissues, invade, and interact with specific cells. Therefore, its adherence to host ligands associated with mucous membranes, which are bathed in complex fluids containing both host and bacterial products, would be important for it to express its virulence.

Whereas some bacteria have specialized structures, such as fimbriae, to adhere to oral surfaces or to other bacteria (8), it is not yet evident how oral spirochetes bind. It is known that other spirochetes, like *Treponema pallidum*, *Leptospira* spp. (13, 43), and other intestinal spirochetes, bind by their tips to host cells. In some cases, attachment is mediated by ligands such as fibronectin found in basement membranes and adsorbed on cell surfaces (38). No such data are available for oral spirochetes, although *T. denticola* has been observed to bind to cells (28, 33) and to hydroxyapatite beads coated with saliva, serum, or crevicular fluid (7) by its tips. As previous studies of *T. denticola* surface interactions with beads or cells have been observational in nature, the aim of this study was to use a standard quantitative assay to compare the degree of interaction with fibronectin (Fn)-coated surfaces among different *T. denticola* strains. With this method, tip-associated adherence could be quantified and subsequently mechanisms of adherence might be tested.

MATERIALS AND METHODS

Treponema strains, culture conditions, and reagents. *Treponema denticola* ATCC 33520 and *T. denticola* a, b, c, d, e, and e' were generously provided by E. Chan, McGill University, Montreal, Quebec, Canada. Stocks were maintained by growth in spirochete medium (described below) supplemented with 0.3% Noble agar and subcultured once every 3 weeks. For experiments, the bacteria were grown in spirochete medium (containing, per liter, 12.5 g of brain heart infusion broth, 10.0 g of trypticase, 2.5 g of yeast extract, 0.5 g of sodium thioglycolate, 1.0 g of L-cysteine hydrochloride, 0.25 g of L-asparagine, 2.0 g of glucose, 2.0 ml of a volatile fatty acid mixture, 20.0 ml of rabbit serum, 20.0 ml of 10% sodium bicarbonate, and 3.0 ml of 0.2% cocarboxylase) 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 10 min at 800 × *g* and washed three times with and resuspended in phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.6 mM Na_2HPO_4, and 1.5 mM

* Corresponding author.

TABLE 1. Adherence of *T. denticola* strains to Fn-coated cover slips

T. denticola strain	Mean no. of bacteria attached/field[a]		% Tip binding to Fn
	Fn[b]	BSA	
ATCC 33520	104 ± 10.6	8 ± 1.2	50
Strain a	88 ± 10.2	6 ± 1.0	42
ATCC 33520	115 ± 18.1	11 ± 2.1	40
Strain b	122 ± 16.3	24 ± 2.7	37
ATCC 33520	139 ± 12.1	12 ± 5.6	56
Strain c	144 ± 9.8	6 ± 2.2	50
Strain d	106 ± 27.2	7 ± 1.9	51
ATCC 33520	149 ± 11.3	6 ± 1.3	60
Strain e	39 ± 8.2	4 ± 0.3	15
Strain e'	140 ± 10.2	13 ± 2.1	98

[a] Mean ± standard error of 9 samples.
[b] Fn values were significantly different from values for the BSA control ($P < 0.001$).

KH_2PO_4 (pH 7.2) (27) to a final concentration of 2×10^8 cells per ml, as determined by microscopic counts using a Petroff-Hauser counting chamber. Human Fn, type IV collagen, laminin, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, Mo.; Fn fragments (RGDS peptides) and non-RGDS peptides were obtained from Telios Pharmaceutical Inc., San Diego, Calif.; rabbit antiserum to human Fn and preimmune serum were obtained from Calbiochem, La Jolla, Calif. Antilaminin was kindly supplied by Jaro Sodek, University of Toronto, Toronto, Ontario, Canada.

Attachment of *T. denticola* to protein-coated cover slips. The method of attaching *T. denticola* to protein-coated cover slips was based on that used by Petersen et al. (29) in their study of *T. pallidum*. Forty microliters of the protein, i.e., Fn, collagen, laminin, or peptides (1 mg/ml), was evenly spread on plastic cover slips and allowed to dry at 37°C. The choice of this concentration was based on the work of Petersen et al. (29) and our own observations that twofold dilutions below 0.25 mg/ml yielded fewer bacteria attached. Control cover slips were coated with 3% BSA (wt/vol). The cover slips were then washed in PBS to remove unbound protein, after which 80 μl of BSA (3% [wt/vol]) was added as a blocking agent to decrease nonspecific binding. The cover slips were then placed in small petri dishes, coated side up. To the dried, coated cover slips, 0.5 ml of a washed *T. denticola* cell suspension was added, after which they were incubated for 2 h at 37°C. Following incubation, the cover slips were rinsed by dipping 30 times in PBS. The number of cells attached to each cover slip was counted by dark-field microscopy, using an Olympus System Series BH microscope at ×1,000 magnification. For each cover slip, three randomly selected fields were counted, and samples were done in triplicate. Each experiment was run at least three times. When comparing *T. denticola* strains, type strain ATCC 33520 was used as a standard in each experiment, so the data from experiments conducted on different days could be analyzed.

Effect of temperature on attachment of *T. denticola* to Fn-coated cover slips. *T. denticola* Fn attachment assays were run as described above but in the cold, either at 4°C for 2 h or at 4°C overnight. The cells were then washed and examined by dark-field microscopy as usual.

Effect of anti-Fn and antilaminin on the adherence of *T. denticola*. Plastic cover slips were coated with Fn and blocked with BSA as described above, but prior to addition of *T. denticola* cells, some cover slips were exposed to a 1/10 dilution of rabbit anti-Fn; an equal number of cover slips were exposed to a 1/10 dilution of preimmune rabbit serum. Similar experiments were conducted with the Anti-Fn, or a 1/20 dilution of antilaminin on laminin-coated cover slips. Controls consisted of cover slips coated with BSA only.

Effect of precoating *T. denticola* cells with proteins. Washed *T. denticola* cells were preincubated with 1 mg of the appropriate protein (laminin, collagen, or Fn) per ml for 2 h at 37°C. The cells were washed and resuspended in PBS, adjusted to the required concentration, and then added to cover slips previously coated with Fn.

Statistical evaluation. Data were analyzed by Student's *t* test for unpaired data to determine statistically significant differences.

RESULTS

Attachment of *T. denticola* to protein-coated cover slips. All strains of *T. denticola* bound to human Fn (Table 1). The number attached did not vary much for six of the strains, with strain e being the exception. The number of strain e cells bound was approximately 75% lower than that of standard strain ATCC 33520 ($P < 0.002$). Of the strain e cells bound to Fn, only 15% were bound by their tips. In contrast, the percentage of tip-bound cells ranged from 37 (strain b) to 98% (strain e') for the other strains. Anti-Fn inhibited the attachment of ATCC 33520 to Fn by 77%, whereas the preimmune serum did not affect its attachment (data not shown). There was no significant difference between the samples that were exposed to the preimmune serum and those which were left untreated.

T. denticola did not bind well to type IV collagen (Table 2). There was no difference between binding to collagen and binding to the BSA control. This pattern was similar for strains e and e'. The mean number of cells that attached when cover slips were coated with laminin was similar to

TABLE 2. Adherence of *T. denticola* to the basement membrane protein type IV collagen and laminin

T. denticola strain	Mean no. of bacteria attached/field[a]		% Tip binding to collagen	Mean no. of bacteria attached/field[a]		% Tip binding to laminin
	Collagen IV[b]	BSA		Laminin[c]	BSA	
ATCC 33520	35 ± 5.3	34 ± 7.9	10	150 ± 6.0	39 ± 9.8	50
Strain e	15 ± 2.7	11 ± 4.0	1	49 ± 9.3	5 ± 2.1	25
Strain e'	38 ± 9.7	10 ± 1.7	30	98 ± 8.4	9 ± 2.2	90

[a] Mean ± standard error of 9 samples.
[b] Values for collagen were not statistically different.
[c] Values for laminin were significantly different from those for BSA ($P < 0.001$).

TABLE 3. Adherence of *T. denticola* ATCC 33520 to Fn fragments and to control peptides

Substrate on cover slip (mg/ml)	Mean no. of bacteria attached/field[a]	% Tip binding
Fn (0.5)[b]	79 ± 6.0	41
BSA (3.0)	5 ± 1.9	48
GRGDSP (1.0)	84 ± 10.1	73
GRGDSPK (1.0)	68 ± 7.6	70
Fn (0.5)[b]	84 ± 10.1	50
BSA (3.0%)	5 ± 2.4	60
GRGESP (1.0)	5 ± 2.0	42
GRADSP (1.0)	10 ± 4.5	53

[a] Mean ± standard error of 9 samples.
[b] Fn and RGDS peptides differed significantly from BSA ($P < 0.001$) in adherence, whereas non-RGDS peptides were not significantly different from BSA.

that for Fn. Strain e bound poorly with fewer cells bound by their tips. As found with Fn-coated surfaces, strain e' yielded a greater percentage of cells bound by their tips to laminin than did strain ATCC 33520. Treatment of the laminin-coated cover slips with antilaminin inhibited attachment of ATCC 33520 by 80%, and treatment with anti-Fn resulted in a 35% decrease in attachment.

Additional experiments were conducted by preparing cover slip coatings with a combination of Fn and laminin. When their combined concentration was held to 1.0 mg/ml, the attachment was similar to that for 1.0 mg of Fn per ml alone. Attachment to coatings prepared from solutions containing 1 mg of each protein per ml was almost twice as much as with coatings from 1.0 mg of Fn per ml alone. These data suggest that the total receptor availability for the Fn and laminin combination was additive. Two fragments of Fn containing the cell-binding domain with the sequence RGDS (GRGDSP and GRGDSPK) both supported *T. denticola* ATCC 33520 attachment at a level of 84 ± 6.9 and 68 ± 7.6 cells per field, respectively (Table 3). This was similar to that for the intact Fn molecule (79 ± 6.0 cells per field). In contrast, the non-RGDS control peptides (GRGESP and GRADSP) showed a level of attachment similar to that of the negative control, BSA.

Effect of temperature on attachment of *T. denticola* to Fn. Incubation at 4°C for 2 h decreased the mean number of cells that attached by 60% and also decreased the number that attached by their tips from 70 to 20% (Table 4). However, when the incubation period at 4°C was extended overnight, the mean number that attached per field approached that

TABLE 4. Effect of temperature on adherence of *T. denticola* ATCC 33520 to Fn

Experimental conditions	Mean no. of bacteria attached/field[a]		% Tip binding to Fn
	Fn[b]	BSA	
37°C, 2 h (control)	135 ± 9.8	19 ± 5.0	70
4°C, 2 h[c]	34 ± 8.0	7 ± 1.5	20
37°C, 2 h (control)	131 ± 11.1	20 ± 6.9	80
4°C, 24 h	103 ± 7.3	7 ± 1.9	40

[a] Mean ± standard error of 9 samples.
[b] Fn values differed significantly from BSA values ($P < 0.001$).
[c] Values for 4°C at 2 h differed significantly from the control ($P < 0.002$). A control was included in each experimental run, under standard assay conditions.

TABLE 5. Effect of pretreatment with Fn on the adherence of *T. denticola* ATCC 33520 to Fn

Fn concn (μg/ml)	Mean no. of bacteria attached/field[a]		% Tip binding to Fn
	Fn[b]	BSA	
0	155 ± 12.1	5 ± 1.2	49
50	160 ± 10.9	9 ± 2.1	52
250	210 ± 22.3	7 ± 1.0	40
1,000	236 ± 20.8	8 ± 0.9	29

[a] Mean ± standard error.
[b] Fn values differed significantly from BSA values ($P < 0.001$), and pretreatment at 1 mg/ml differed from no pretreatment ($P < 0.002$).

observed under the standard conditions of 37°C for 2 h, but the percent bound by their tips remained significantly lower.

Effect of precoating *T. denticola* with proteins or peptides on the attachment to Fn. Precoating the cells with Fn enhanced binding by 52% (Table 5). Precoating with laminin had an inhibitory effect (~50%), and with collagen there was no effect (Table 6).

DISCUSSION

This investigation provides evidence that *T. denticola* binds to plastic-adsorbed Fn, most often by its tips. All strains of *T. denticola* that were tested adhered to Fn but each to a different degree. One strain, strain e, which had a low percentage of tip binding, yielded low numbers adhering to Fn; all other strains attached in high numbers. Among these, strain e' had a very high proportion of cells attached by their tips. The interaction of *T. denticola* with Fn is apparently specific, since it was inhibited by the addition of anti-Fn but not preimmune serum. Attachment to type IV collagen was markedly less than that to Fn or to laminin, which supported a comparable number of attached cells to Fn. In vivo, laminin, like Fn, might have the opportunity to mediate *T. denticola* attachment at the basement membrane. Other invasive bacteria, such as *Streptococcus pyogenes* (37) and *Staphylococcus aureus* (24), have been found to adhere to laminin. The amino acid sequence in some domains of laminin is similar to that of Fn; thus, these two proteins might share some specific recognition sequences for *T. denticola* adhesins, accounting for the reduction of attachment to laminin by anti-Fn observed in this study.

Piershchbacher and co-workers (31, 32) identified a certain sequence of amino acids which they called RGDS peptide within the Fn molecule and demonstrated that eucaryotic cells bind to this region. Subsequently, Thomas et al. (38)

TABLE 6. Effect of pretreatment with collagen and laminin on the adherence of *T. denticola* ATCC 33520 to Fn

Treatment	Mean no. of bacteria attached/field[a]		% Tip binding to Fn
	Fn	BSA	
None (control)	135 ± 21.4	6 ± 3.1	52
Laminin (1.0 mg/ml)[b]	54 ± 9.8	8 ± 1.7	47
None (control)	83 ± 13.4	8 ± 2.1	54
Collagen (1.0 mg/ml)[b]	79 ± 11.9	10 ± 2.9	50

[a] Mean ± standard error.
[b] Cells pretreated with laminin differed significantly from those that were untreated ($P < 0.002$), but cells that had been pretreated with collagen did not differ from the controls.

showed that *T. pallidum* also attached to Fn within a binding domain containing the same sequence of amino acids: arginine, glycine, aspartic acid, and serine. Our results seem to indicate that *T. denticola* binds to the same region, since coating cover slips with RGDS peptides supported attachment at a level similar to that of whole Fn, whereas the non-RGDS peptides did not. Without the previous studies on *T. pallidum*, one might have expected that *T. denticola* would adhere to domains within the Fn molecule that have been identified as regions to which bacteria bind. For example, *Escherichia coli* and *S. aureus* have been shown to adhere to Fn at different sites within the N-terminal region but not in the eucaryotic cell attachment domain (11, 36). Therefore, it appears that *T. denticola* possesses adhesins which are similar in recognition specificity, but not necessarily composition or structure, to those on eucaryotic cells. It is possible that *T. denticola* either synthesizes these adhesins or adsorbs them from the complex serum-containing growth medium used in our experiments and perhaps from host sources during invasion. There is evidence for acquisition of host proteins by *T. pallidum*, which is cultivated in an in vivo testicular system (1). However, it is unlikely that *T. denticola* adsorbed significant amounts of immunoreactive Fn from our culture medium. Subsequent experiments using dot blot and Western blot (immunoblot) analyses to identify Fn-reactive proteins have yielded no positive results for control whole-cell or outer membrane preparations when exongenous Fn was omitted prior to probing with anti-Fn. A few medium proteins can be detected in the preparations, but they appear to be distinct from major bacterial Fn-binding proteins (R. P. Ellen and D. A. Grove, unpublished data).

Pretreatment of the bacteria with proteins had several different effects on attachment. Type IV collagen did not appear to affect binding to Fn, so it can be assumed that *T. denticola* does not have complementary receptors. Laminin pretreatment resulted in a 60% decrease in the number of cells adhering to Fn, which would tend to support the idea that both laminin and Fn share common recognition sequences for *T. denticola* attachment. It is also possible that the sites for laminin and Fn interaction are in close proximity, resulting in decreased adherence due to steric hindrance. This could explain why anti-Fn caused a small but significant decrease in the attachment of spirochetes to laminin. Pretreatment with Fn enhanced attachment instead of inhibiting it. It is possible that since the Fn molecule contains domains for binding to other proteins, such as heparin, gelatin, and collagen (34), it is also able to bind multivalently to Fn. This Fn-Fn interaction, or bridging, would enable more bacteria to appear to be attached to the Fn coating on the cover slips, thereby causing an apparent enhancement in our assay.

As discussed by Gibbons (12), it it is also possible that conformational changes in soluble Fn occur when it adsorbs to a solid support. For example, in the case of *Streptococcus sanguis* adherence, the binding domain on Fn is apparently cryptic while in solution, but it becomes exposed by a conformational change when Fn binds to gelatin-coated plastic (25). Thus, different types of receptor domains might be exposed when Fn is in solution and when Fn is immobilized, especially by drying onto a surface as was done in our experiments. It is also conceivable that the bacterial cells could possess different classes of adhesins which recognize different conformations of ligands, explaining why *T. denticola* binds soluble Fn and still can recognize and bind immobilized Fn. It is significant to mention that preliminary experiments with soluble Fn have shown no differences in Fn adsorption between strains e and e′, yet strain e′ adheres in greater numbers than strain e to gingival fibroblasts. This is similar to what has been shown for Fn-coated cover slips in this study (R. P. Ellen, D. A. Grove and M. Song, unpublished data). Our observation of increased attachment following protein coating of bacteria is similar to that seen with *T. pallidum* (39) and *S. aureus* (14) when they were pretreated with Fn and then exposed to solid-phase Fn.

The fact that *T. denticola* is able to bind by its tips suggests that adhesins might be synthesized, clustered, and enriched at the tips. This phenomenon could also be due to lateral migration of the adhesins to the tip through the outer membrane of the bacterium when an appropriate ligand substrate is presented or when this motile microorganism moves toward it. Although this question was not examined extensively, the observed reduction in attachment in the cold may have resulted either from a reduction in spirochete motility delaying the opportunity for contact or from decreased mobility of the bacterial adhesins toward the tip of the bacteria. Therefore, even after 24 h of incubation in the cold, although the number of bacteria that attached was close to the level for the controls, the proportion that were bound by their tips was still 50% less.

Presumably, if the bacteria were suspended in a solution of Fn, as in the crevicular fluid of periodontal pockets, the adhesins might be located evenly throughout the entire surface and not necessarily clustered at the tips. Adsorption of Fn from crevicular fluid might even enhance their adherence to the pocket lining, analogous to observations in our experiments. As these microorganisms are often associated with surfaces, the clustering of adhesins, perhaps even different adhesins, at their tips would be important only when directional attachment was required, such as when Fn is immoblized on a cover slip or in vivo when bacteria have to bind to or invade the tissues lining a periodontal pocket. This may then contribute to the pathogenicity of *T. denticola*.

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