INFECTION AND IMMUNITY, Nov. 1991, p. 4230–4237
0019-9567/91/114230-08$02.00/0

Vol. 59, No. 11

Sulfhydryl-Dependent Attachment of *Treponema denticola* to Laminin and Other Proteins

MARKUS HAAPASALO,[1] UMADAT SINGH,[1] BARRY C. McBRIDE,[1] AND VELI-JUKKA UITTO[2]*

Department of Microbiology[1] and Department of Oral Biology,[2] University of British Columbia, Vancouver, British Columbia V6T1Z3, Canada

Received 15 April 1991/Accepted 27 August 1991

Attachment of *Treponema denticola* ATCC 35405 to laminin, a major basement membrane protein, and to other proteins was studied. Microdilution plates were coated with the proteins, and the attachment of *T. denticola* was measured by the enzyme-linked immunosorbent assay technique. Compared with bovine serum albumin (BSA), *T. denticola* had a high affinity to laminin, fibronectin, fibrinogen, and gelatin, as well as to type I and type IV collagens. Attachment to RGD peptide (Gly-Arg-Gly-Asp-Ser, the integrin recognition sequence) was only about 30% of that to laminin and was comparable to attachment to BSA. Tests with laminin fragments obtained through elastase digestion showed that the spirochetes attached well to an A-chain 140-kDa fragment involved in eukaryote cell attachment but did not attach to a 50-kDa fragment that includes the heparin binding site. Pretreatment of *T. denticola* with soluble laminin, fibronectin, gelatin, BSA, or fibrinogen had no effect on the attachment of the bacteria to laminin or fibronectin. A wide variety of compounds were tested for their possible inhibitory actions on the attachment. While most treatments of *T. denticola* ATCC 35405 had little or no effect on the attachment to proteins, sulfhydryl reagents *p*-chloromercuribenzoic acid (pCMBA) and oxidized glutathione inhibited the attachment by 70 to 99%, depending on the protein. When *T. denticola* was first allowed to attach to proteins, addition of pCMBA or oxidized glutathione could no longer reverse the attachment. Heat treatment of the spirochetes also markedly reduced the attachment to laminin, gelatin, and fibrinogen but not to BSA. Mixed glycosidase treatment of the spirochetes inhibited the attachment by 20 to 80%. None of the above treatments of the substrate proteins had any marked effect on the spirochete attachment. The results indicate that *T. denticola* has the capacity to bind to many different kinds of proteins by utilizing specific attachment mechanisms. The binding appears to involve protein SH groups and/or carbohydrate residues on the surface of *T. denticola*.

Oral spirochetes have been implicated as potential etiologic agents of periodontal diseases in humans (1, 18–20). Moore et al. (24) found that *Treponema denticola* was one of the spirochetes most frequently isolated from severely diseased sites in young adults. The gingival crevice area is the principal ecological niche inhabited by treponemes. In healthy sites, treponemes are present in relatively small numbers in supragingival and subgingival plaque, but in sites affected by periodontal disease their number increases dramatically (1, 18). Scanning electron microscopic investigations have shown that oral treponemes and rod-like bacteria are the main morphological forms associated with the advancing front of subgingival plaque in sites of rapidly progressive periodontitis (37). In acute necrotizing ulcerated gingivitis, treponemes appear to invade epithelium and the underlying connective tissue (17, 22). Penetration of gingival tissue by treponemes also occurs in other forms of periodontal disease (11, 36). The first step in the process of tissue invasion is probably an association of the bacteria with the host epithelial cell surface via a receptor-adhesin-like interaction. It is well established for various bacterial pathogens that the initial adhesion of the bacterial cell to host mucosal tissue is a crucial step for the development of an infection (3). It is supposed that charge and hydrophobic interactions as well as more specific receptor-mediated structures binding to epithelial cells may be of importance for adhesion (3). The ability of bacteria to bind to extracellular matrix molecules, such as collagen, fibronectin, and laminin, may also play an important role in the infective process. While some studies have shown that oral treponemes interact with eucaryotic cells and hard surfaces (2, 6, 28, 34), information about the attachment of oral treponemes to extracellular matrix components and about the type and specificity of the attachment is scarce. In this study, we investigated the attachment of *T. denticola* to several proteins, including basement membrane proteins, and studied the character of this attachment.

MATERIALS AND METHODS

Bacterial strain. *T. denticola* ATCC 35405 was obtained lyophilized from the American Type Culture Collection, cultured, and preserved in glycerol at −70°C. For this study, *T. denticola* was grown in a broth medium and maintained by weekly transfers. Broth (100 ml) contained heart infusion broth (1.25 g; Difco Laboratories, Detroit, Mich.), Trypticase (1 g; BBL Microbiology Systems, Cockeysville, Md.), yeast extract (0.25 g; Difco), sodium thioglycolate (0.05 g; Difco), L-cysteine hydrochloride (0.1 g; Sigma Chemical Co., St. Louis, Mo.), L-asparagine (0.025 g; Sigma), glucose (0.2 g; Difco), thiamine pyrophosphate (0.6 mg; Sigma), isobutyric acid (0.001% [vol/vol]; Sigma), DL-2-methylbutyric acid (0.001% [vol/vol]; Sigma), isovaleric acid (0.001% [vol/vol]; Sigma), valeric acid (0.001% [vol/vol]; Sigma), sodium bicarbonate (0.2% [wt/vol]; Fisher), and heat-inactivated horse serum (2 ml; GIBCO, Burlington, Ontario, Canada). Three-day-old cultures were used for the experiments. The purity of the culture was checked each time by phase-contrast microscopy.

* Corresponding author.

Antiserum against *T. denticola*. A New Zealand White rabbit was immunized with whole cells of *T. denticola* ATCC 35405. One milligram of cells was injected intramuscularly with complete Freund adjuvant. Subsequent intramuscular injections without adjuvant were performed after 1, 2, 3, 5, and 7 weeks. The rabbit was then bled via the marginal ear vein 1 week after the last booster. The specificity of the antiserum was determined by enzyme-linked immunosorbent assay (ELISA) (see below), with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:3,000; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The highest dilution to give an optical density at 405 nm (OD_{405}) of >0.3 (2550 EIA Reader, Bio-Rad, Ontario, Canada) was 1:12,800. A control (no bacteria) and *Fusobacterium nucleatum* ESF4 used as a reference gave values <0.15 at all antiserum dilutions tested (1:100 to 1:25,600).

Epithelial cells. Epithelial cell rests of Malassez were isolated from porcine periodontal ligament as described by Brunette et al. (5). Briefly, periodontal ligament tissue was scraped from freshly extracted teeth of young slaughterhouse pigs and cultured in alpha minimal essential medium (Flow Laboratories, McLean, Va.) supplemented with antibiotics and 15% fetal calf serum. The epithelial cells that grew out of the explants were separated from fibroblasts by a selective trypsinization technique (30). In culture, these cells exhibit a homogeneous epithelial cell population with a cytokeratin pattern (K 4, 5, 6, 14, 16, 19) typical of basal cell-like, undifferentiated, hyperproliferative characteristics (31). These cells have been found to secrete fibronectin, laminin, type IV collagen, hyaluronic acid, and matrix metalloproteinases as their major extracellular products (23, 38, 44). The cells of the third to fifth passage were utilized in the assays. In order to investigate whether *T. denticola* has affinity to the epithelial cell surface or to extracellular elements, epithelial cells were cultured for various times in the presence of living *T. denticola* cells. Attachment of *T. denticola*, as well as the morphological changes in the epithelial cells, was monitored by a scanning electron microscope (Cambridge Stereoscan, Cambridge, United Kingdom) (10).

Attachment of *T. denticola* to proteins. *T. denticola* was collected from liquid culture by centrifugation (10,000 × *g*), washed once in phosphate-buffered saline (PBS), and adjusted to an OD_{660} of 0.2 (Varian DMS 100 spectrophotometer; Varian Techtron Pty Ltd., Mulgrave, Victoria, Australia) in PBS corresponding to 5×10^8 cells/ml as determined with a Petroff-Hausser bacteria counter (Hausser & Son, Philadelphia, Pa.). The following proteins were used to study the attachment: laminin (Sigma), laminin fragments obtained through elastase digestion (see below), collagen type I (ICN Biochemicals, Cleveland, Ohio), collagen type IV (Sigma), gelatin (Bio-Rad), fibronectin (Sigma), fibrinogen (Sigma), and bovine serum albumin (BSA) (Sigma). In addition, attachment of *T. denticola* to RGD peptide (Gly-Arg-Gly-Asp-Ser; Sigma), the integrin recognition sequence (33, 35), was studied. Attachment was measured by ELISA (see below). All proteins and the RGD peptide were used in a concentration of 1 mg/ml in PBS except laminin, for which a concentration of 0.1 mg/ml was used, because preliminary experiments showed that the saturation for laminin was obtained at a concentration below 0.1 mg/ml. The intactness of the protein films in the wells before and after washings with Tween-PBS and PBS was demonstrated in control experiments in which the film was visualized either by biotin-streptavidin reaction or by antiprotein antibodies and secondary antibodies conjugated to alkaline phosphatase. After incubation, an insoluble color substrate for alkaline phosphatase was added to the wells, which were studied under a stereo microscope with a magnification of ×30. Controls included wells with no protein. The results of *T. denticola* attachment to proteins are calculated from three parallel tests done at the same time, and they are representative of experiments done on different occasions.

ELISA. Microdilution plates (Corning Glass Works, Corning, N.Y.) were coated with 100 μl of the protein or RGD peptide solutions for 12 h at 20°C. After being washed (all washes were done twice with 0.05% Tween 20 in PBS and once with PBS), the wells were blocked with 200 μl of BSA (1 mg/ml) for 2 h, washed, incubated for 1 h with 100 μl of *T. denticola* ATCC 35405 (OD_{660} of 0.2 in PBS), and washed again. *T. denticola* antiserum (100 μl, 1:1,000 in PBS, 1 h) was then added, and the wells were washed. Then, 100 μl of goat anti-rabbit antibody (1:3,000 in PBS) conjugated with alkaline phosphatase (Bethesda Research Laboratories) was added, and the wells were incubated for 1 h. Chromogenic substrate tablets for alkaline phosphatase (*p*-nitrophenylphosphate; Sigma) were dissolved in substrate buffer (1 mg/ml) and used in aliquots of 100 μl per well. Color development was monitored at 405 nm (2550 EIA Reader; Bio-Rad).

Enzymatic digestion and collection of laminin fragments. Laminin (0.5 ml at a concentration of 1 mg/ml) was incubated with human pancreatic elastase (type IV; Sigma) for 12 h at 20°C at a protein:enzyme ratio of 100:1. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. The laminin fragments were separated with fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) by gel filtration (Superose 12 HR10/30 column, 0.75 ml/min) in 0.05 M Tris–0.15 M NaCl buffer at pH 7.4 and collected in fractions of 0.5 ml. The molecular weights of the laminin fragments were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini Protean II; Bio-Rad) with nonreducing sample buffer. The affinity of the fragments to heparin was tested by incubation of the elastase digest at 25°C for 1 h in PBS with heparin-Sepharose affinity chromatography material (Pharmacia) and by analyzing the soluble (unattached) proteins with SDS-PAGE (15).

Inhibition of attachment. To obtain information about the type and specificity of the attachment of *T. denticola* to proteins, the proteins in the ELISA wells or *T. denticola* cells (OD_{660} of 0.2) or both were pretreated for 1 h with one of the following substances: D-galactose (100 mM; Sigma), mannose (100 mM; Sigma), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (1 mM; Sigma), PMSF (1 mM; Sigma), proteinase K (100 μg/ml; Bethesda Research Laboratories), mixed glycosidase (100 μg/ml; Miles Laboratories Inc., Elkhart, Ind.), *p*-chloromercuribenzoic acid (pCMBA) (2 mM; Sigma), cysteine (10 mM; Sigma), oxidized glutathione (10 mM; ICN Pharmaceuticals, Cleveland, Ohio), reduced glutathione (10 mM; Nutritional Biochemicals Corp., Cleveland, Ohio), *N*-ethylmaleimide (10 mM; Sigma), and antiserum raised against a chymotrypsin-like enzyme isolated from *T. denticola* (12) (diluted 1:10 in PBS). All treatments were done at 20°C, except proteinase K and mixed glycosidase (37°C) and *T. denticola*-chymotrypsin antiserum (4°C). As pCMBA is soluble only in alkaline conditions (pH <8.4), the possibility that inhibition was due to high pH and not to pCMBA was tested with control experiments in PBS at pH 8.4 without pCMBA. These experiments showed that higher pH had no effect on the attachment of *T. denticola*. After the

treatments, the bacteria or protein was washed with PBS. In some experiments, the attachment was assayed by adding the test substance together with *T. denticola* to the ELISA well coated with protein.

Because some of the compounds tested were able to effectively inhibit the attachment of *T. denticola* to proteins, additional experiments were done to see whether the attachment was reversible. *T. denticola* cells were first attached to the proteins as described above and, following this, 10 mM β-mercaptoethanol, 10 mM oxidized glutathione, 10 mM cysteine, or 2 mM pCMBA (final concentration) was added to the wells and incubated for an additional 5, 10, and 30 min. Three parallel tests were done for each experiment.

Protein specificity of the binding site(s) of *T. denticola*. The protein specificity of the site(s) mediating the attachment of *T. denticola* was studied by incubating *T. denticola* (OD_{660} of 0.2) with 1-mg/ml solutions of laminin, fibronectin, BSA, fibrinogen, or gelatin in PBS in polypropylene microcentrifuge tubes. After 1 h, the excess protein was washed off, the *T. denticola* cells were transferred into ELISA wells coated with either laminin or fibronectin, and the number of attached *T. denticola* cells was measured as described above. In some experiments, after the incubation of *T. denticola* with BSA, fibrinogen or gelatin, laminin (100 μg/ml) was added to the centrifuge tubes and the attachment of *T. denticola* to laminin in solution was measured. After the unattached laminin was washed off, the *T. denticola* cells were resuspended to the original volume in PBS, and incubated with antilaminin antibodies (Sigma) diluted 1:200 in PBS and then with goat anti-rabbit immunoglobulin G (Bethesda Research Laboratories) with alkaline phosphatase substrate as described above. When the color intensity reached a suitable level, the supernatant obtained by centrifugation was transferred to microdilution plate wells (100 μl per well) and the color intensity was measured with an ELISA reader at 405 nm (2550 EIA Reader).

Effect of heat on attachment. *T. denticola* cells were heated in a water bath at 60, 70, 80, or 90°C for 10 min, and attachment to laminin, gelatin, BSA, and fibrinogen was measured. In other experiments, the proteins were heated at 90°C for 10 min before the microdilution plates were coated.

Effect of pH and ionic strength. Freshly harvested *T. denticola* cells were adjusted to an OD_{660} of 0.2 in 10 mM citrate buffer at pH 8.5, 7.0, or 5.5. Sodium chloride concentrations used at each pH were 0, 0.2, and 0.5 M. Attachment of bacteria to proteins was measured as described above.

Statistical evaluation. Student's *t* test for unpaired data was used to determine the statistically significant differences.

RESULTS

Scanning electron microscopy of the cultured epithelial cells in the presence of *T. denticola* revealed that although numerous bacteria were initially attached to the cells, only a few spirochetes were found attached to epithelial cells after incubations of 8 h or longer. Most of the *T. denticola* cells in these cultures appeared to be bound to material left behind by migrating epithelial cells (Fig. 1). We have earlier found that fibronectin, type IV collagen, and laminin are major components of this material (44).

The attachment of *T. denticola* ATCC 35405 to basement membrane proteins laminin, fibronectin, and collagen type IV as well as to collagen type I, gelatin, fibrinogen, and RGD peptide (Gly-Arg-Gly-Asp-Ser) was studied by ELISA with BSA as a reference. *T. denticola* showed increased attachment to all proteins compared with BSA (Fig. 2). Attachment to laminin was most prominent, but the differences from fibrinogen, gelatin, fibronectin, and collagen types I and IV were quite small. In contrast, attachment to RGD peptide was comparable to that obtained with BSA.

The time required for optimum binding of *T. denticola* to laminin was evaluated. The binding of bacteria was relatively rapid and was completed within 20 min of incubation at 20°C. The attachment site in the laminin molecule was studied by incubating the spirochetes with the major peptides derived from elastase digestion. The 140-kDa peptide, having no affinity to heparin, showed binding of *T. denticola* as strong as that of intact laminin. The spirochetes had markedly less affinity to the 50-kDa peptide (Fig. 2).

The protein specificity of *T. denticola* binding sites was studied by incubating *T. denticola* with laminin, fibronectin, BSA, fibrinogen, or gelatin in solution and measuring, thereafter, the attachment of *T. denticola* to laminin and fibronectin coated on the ELISA wells. Pretreatment of the spirochetes with the proteins had no effect on the attachment of *T. denticola* to laminin or fibronectin. In some experiments, after the incubation of *T. denticola* with soluble substrate proteins, laminin (100 μg/ml) was added to the centrifuge tubes and the attachment of laminin to *T. denticola* in solution was measured. No inhibition in attachment of soluble laminin to *T. denticola* was observed.

Heat treatment of *T. denticola* at 70°C or higher for 10 min reduced the attachment to laminin by more than 70% and to fibrinogen by 40 to 50%. The effect of heat on the attachment to gelatin was less pronounced, and no reduction in the attachment to BSA was obtained (Fig. 3). Heat treatment of the proteins had no effect.

Varying the ionic strength (NaCl concentration of 0, 0.2, or 0.5 M) and pH (5.5, 7.0, or 8.5) had no effect on the attachment to gelatin and BSA. High ionic strength (0.5 M) reduced the attachment to laminin at all three pH values, but the reduction was only 10 to 16%, although it was statistically significant ($P < 0.05$). Similar small but statistically significant ($P < 0.05$) reductions were measured also for laminin and fibrinogen at pH 5.5 with a NaCl concentration of 0 M.

To obtain information about the nature of the attachment of *T. denticola*, the spirochetes or the substrate proteins were preincubated for 1 h with several potential inhibitors of the attachment. Figure 4 shows that a strong effect was obtained with the sulfhydryl reagent pCMBA. The number of attached *T. denticola* cells was reduced by 80 to 90% for each protein. Mixed glycosidase reduced the attachment to laminin and fibrinogen by about 50% and to gelatin by 20%. BSA was different from the other three proteins in that mixed glycosidase inhibited the attachment as effectively as pCMBA. PMSF appeared to somewhat inhibit the attachment to laminin but increased the attachment to fibrinogen. No inhibition of attachment was obtained by adding D-galactose, mannose, or RGD peptide to the *T. denticola* incubations (data not shown). None of the compounds tested had an effect on the attachment of *T. denticola* when only the proteins on the ELISA plate were treated, and the results from concomitant treatment of the spirochetes and the proteins were not different from those of treating only the *T. denticola* cells.

Since the sulfhydryl reagent pCMBA caused the most pronounced effect on the attachment of *T. denticola* to proteins, the effects of other compounds reacting with SH or disulfide groups of proteins were tested. Cysteine, reduced

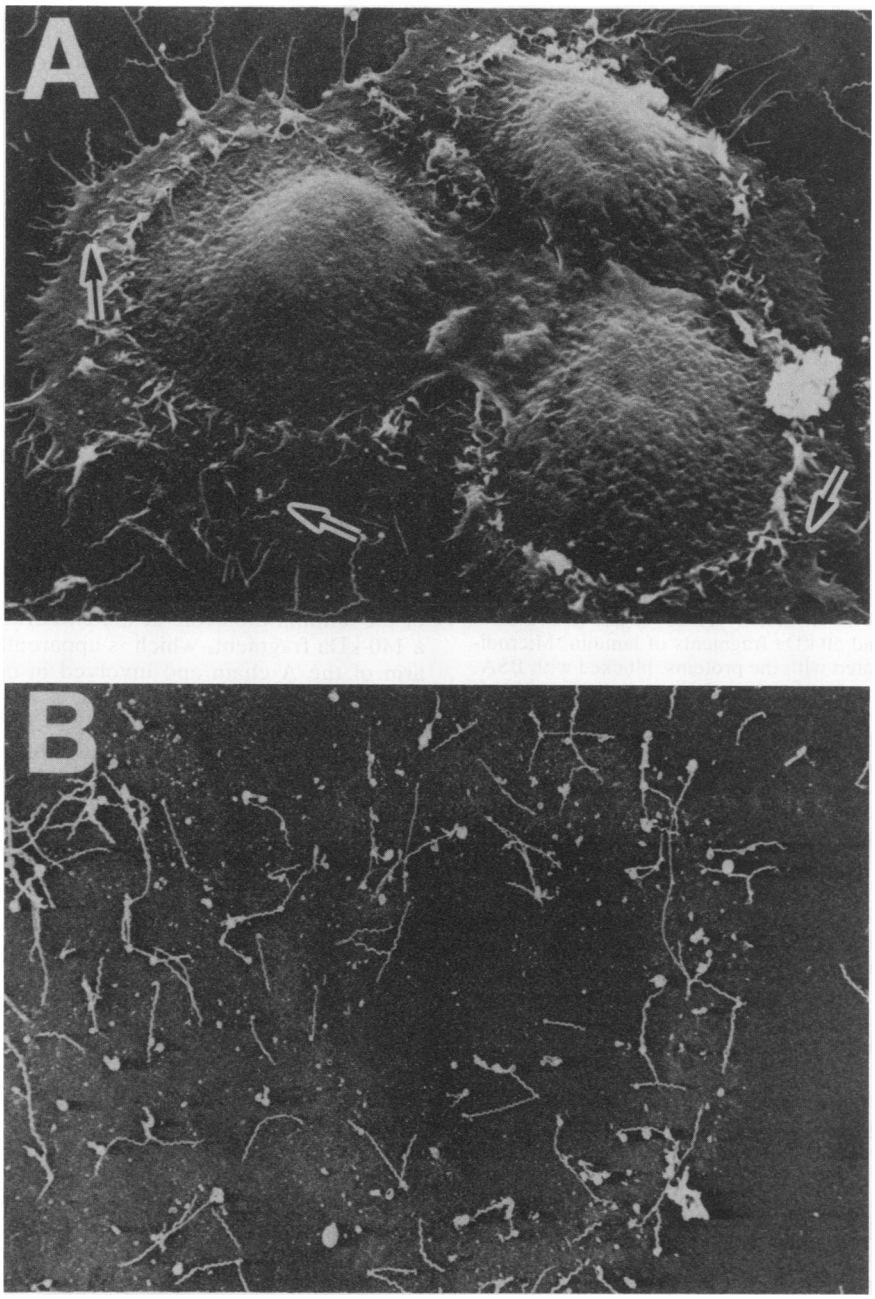

FIG. 1. Scanning electron microscopy of the attachment of *T. denticola* to an epithelial cell culture. Porcine periodontal ligament epithelial cells were cultured on a plastic plate to about 50% confluency. Living *T. denticola* was then added to the culture in a ratio of approximately 200 bacteria per epithelial cell. After 20 h, the culture was fixed and processed for electron microscopy. Note that only a few spirochetes are located in the epithelial cell margins (arrows in panel A) and that most spirochetes are attached to the material left behind by migrating epithelial cells (B).

glutathione, and *N*-ethylmaleimide inhibited the attachment to BSA but not markedly to other proteins. Oxidized glutathione strongly inhibited the attachment to all proteins (Fig. 5). Inhibition with oxidized glutathione and pCMBA could be obtained only when *T. denticola* was treated before attachment to proteins. When the spirochete was allowed to attach first, adding oxidized glutathione or pCMBA could not reverse the attachment, nor could β-mercaptoethanol or cysteine. Preincubation of the substrate proteins with SH or disulfide reagents had no effect on the attachment of *T. denticola*.

DISCUSSION

A prerequisite for tissue invasion by bacteria is the penetration of the epithelium and then the basement membrane. The latter serves as an effective barrier for diffusion of substances into the connective tissue (41, 42). It is supposed

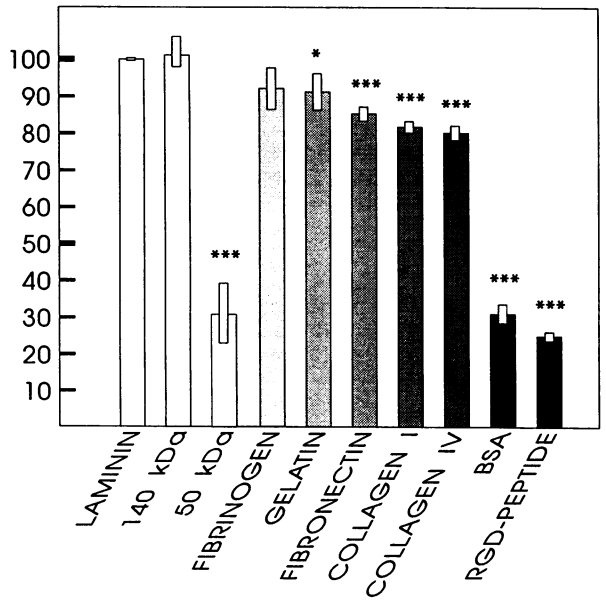

FIG. 2. Attachment of *T. denticola* ATCC 35405 to proteins, RGD peptide, and 140- and 50-kDa fragments of laminin. Microdilution plate wells were coated with the proteins, blocked with BSA, and *T. denticola* (OD_{660} = 0.2) was allowed to attach to the proteins for 1 h. Attachment was measured by ELISA with rabbit antiserum against *T. denticola* and then with goat anti-rabbit antibodies conjugated with alkaline phosphatase. Three parallel experiments were performed. Values are given relative to laminin, which is adjusted to 100. Insert bars show ± standard deviation. Statistical evaluations by Student's *t* test for unpaired data are as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

that there are two rate-limiting steps to invasion, the adherence of the organism to the barrier and penetration through the barrier, which may involve the enzymatic degradation of its constituents (9, 21, 26, 27, 43). The purpose of this study was to investigate the interaction of *T. denticola* with epithelial cells as well as the adherence of the bacteria to extracellular matrix proteins. Special emphasis was directed to laminin, a major glycoprotein of basement membranes (4, 39, 40).

Scanning electron microscopic observations were in accordance with earlier studies showing the attachment of *T. denticola* to epithelial cells (28, 34). The morphological changes observed included impairment of cell surface texture and loss of cell-cell contacts (unpublished data). Interestingly, following initial attachment to epithelial cells, most spirochetes appeared to be detached from the cells in later stages of the culture and bound to the intercellular material on the plate.

Our results suggest that *T. denticola* adheres well to the matrix proteins laminin and fibronectin. This is in accordance with the recent report by Dawson and Ellen, although they found that attachment to fibronectin was slightly better than attachment to laminin (7). However, in our study, the attachment of *T. denticola* to another basement membrane component, type IV collagen, was quite close to that observed with fibronectin and showed no statistically significant difference, whereas in the former study the attachment to type IV collagen was low and comparable to that to BSA (7). Dawson and Ellen employed direct microscopic counting to determine the number of *T. denticola* cells attached to plastic coverslips coated with the proteins, while in our study microdilution plate wells were used and the attachment was measured by ELISA. Differences in the experimental design may explain the different results with type IV collagen. No previous comparative data about the attachment of *T. denticola* to type I collagen are available. In our study, *T. denticola* also showed strong affinity to native and denatured type I collagen as well as to fibrinogen.

Since *T. denticola* showed a strong adhesion to laminin, a major constituent of basement membranes, the nature of this attachment was studied further. Laminin is a cross-shaped molecule which consists of three large polypeptide chains, designated B1, B2, and A. The carbohydrate component constitutes about 13% of the molecule. It has been shown that different parts of the laminin molecule serve different functions, including mitogenicity, promotion of neurite outgrowth, heparin binding, and cell attachment (4, 8, 25, 39, 40). However, no data about the binding sites of laminin for bacteria are available at present. Peptides from the different functional domains of laminin can be isolated after digestion with specific proteases, such as elastase (4, 29). In this study, we purified two major elastase digestion products of laminin by using fast protein liquid chromatography. A 50-kDa fragment corresponding to the heparin-binding part of the laminin molecule at the end of the A chain (4, 8) and a 140-kDa fragment, which is apparently also from the long arm of the A chain and involved in cell attachment, were isolated (4, 32, 39). Interestingly, *T. denticola* showed markedly greater attachment to the 140-kDa fragment than to the 50-kDa fragment. Some studies have suggested that some bacteria, including *Treponema pallidum*, utilize Arg-Gly-Asp (RGD) sequences for attachment, the same sequence recognized by several integrins of mammalian cells (13, 35). In our study, the RGD peptide did not inhibit the attachment of *T. denticola* to laminin, gelatin, and BSA, nor did the bacteria attach well to the peptide coating the ELISA well.

In order to characterize the nature of the attachment of *T. denticola* to proteins, several compounds with different types of activities were studied for their possible inhibitory effects on the attachment. It has been recently suggested that a protease of *Porphyromonas gingivalis* may be involved in the attachment of *P. gingivalis* to fibrinogen (16). Since the enzyme inhibitors PMSF and TLCK and the antibody to the *T. denticola* chymotrypsin-like enzyme, which have been found to inhibit *T. denticola* proteases (12), had little or no inhibitory effect on the attachment, it appears that the proteases are not directly involved in the spirochete binding. The finding that PMSF increased attachment to fibrinogen and possibly to BSA suggests that, in some cases, the proteases may degrade the ligand and could release the spirochetes following attachment. Treatment of *T. denticola* with proteinase K, which degrades a wide variety of proteins, had only a minor effect on the attachment to laminin and no effect on attachment to other proteins. The negative result may mean that the receptor(s) is not a protein or that the protein is part of shielded structures as often seen with bacterial and eucaryotic cell surface molecules. Contrary to proteinase K, mixed glycosidase reduced the attachment to laminin and fibrinogen by about 50% and to BSA by more than 80%. This, together with the observations that heating or treatment of the spirochetes with a sulfhydryl reagent, *p*-chloromercuribenzoic acid, strongly inhibited the attachment, suggests that the adhesin may be a glycoprotein. Galactose and mannose actively inhibit lectin-mediated binding of many other bacteria (14). We tested the effect of 100 mM galactose and mannose on the attachment of *T. denticola* to the proteins, but no inhibition was detected.

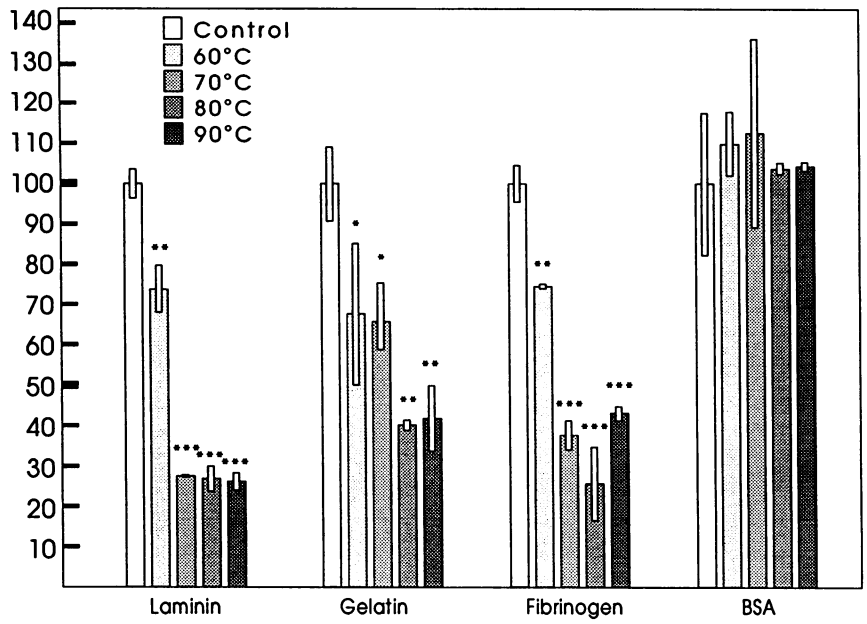

FIG. 3. Effect of heating *T. denticola* ATCC 35405 on the attachment to laminin, fibrinogen, gelatin, and BSA. After being heated for 10 min, the bacteria were cooled and allowed to attach to proteins as described for Fig. 2. The control value for each protein without heating was adjusted to 100. Statistical significance was calculated separately for each protein. Insert bars and asterisks are as defined for Fig. 2.

To further characterize the specificity of the attachment, other disulfide-sulfhydryl reagents were tested. While cysteine, reduced glutathione, and *N*-ethylmaleimide had little or no effect on the attachment to laminin, gelatin, and fibrinogen, oxidized glutathione proved to be the most efficient inhibitor used in our experiments. Oxidized glutathione reacts with the SH groups and results in disulfide bridge formation between the adjacent residues, while organomer-

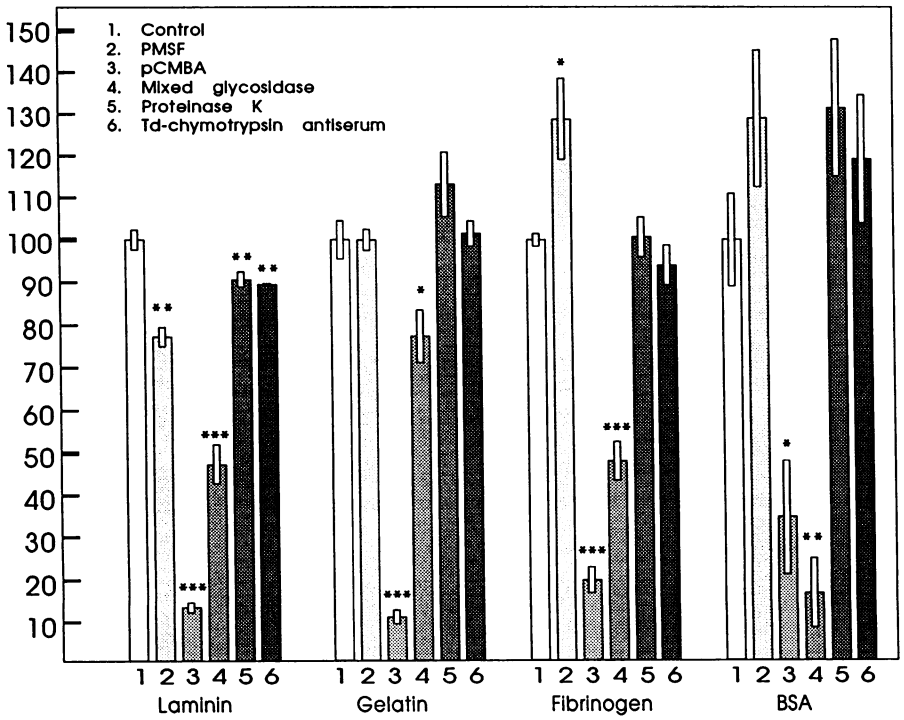

FIG. 4. Effect on the attachment of *T. denticola* ATCC 35405 by PMSF (1 mM), pCMBA (2 mM), mixed glycosidase (100 μg/ml), proteinase K (100 μg/ml), and antiserum (1:10 in PBS) against a chymotrypsin-like enzyme isolated from *T. denticola*. The bacterial cells were incubated in the presence of the substances for 1 h, washed, and allowed to attach to proteins as described for Fig. 2. The control value for each protein with no inhibitor was adjusted to 100. Statistical significance was calculated separately for each protein. Insert bars and asterisks are as defined for Fig. 2.

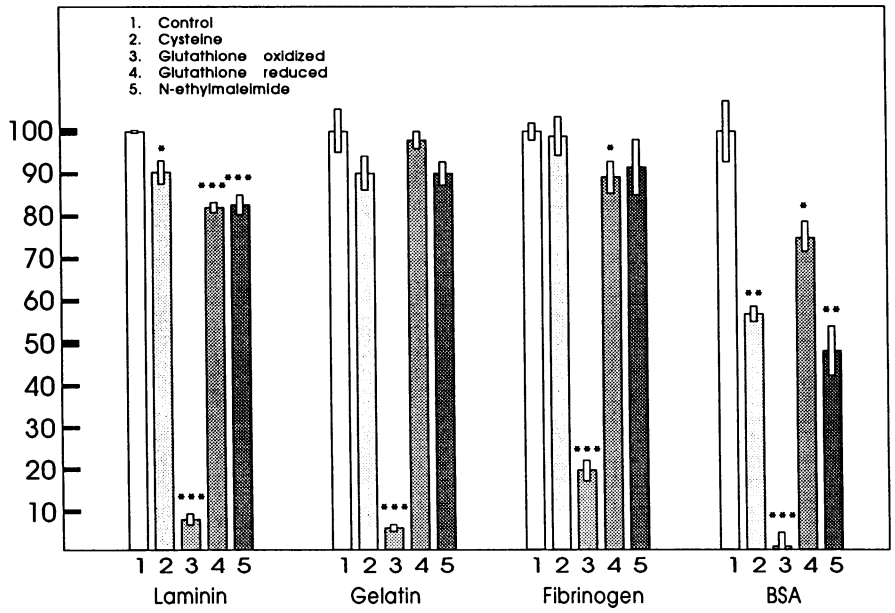

FIG. 5. Effect on the attachment of *T. denticola* ATCC 35405 by cysteine (10 mM), oxidized and reduced glutathione (10 mM), and *N*-ethylmaleimide (10 mM). *T. denticola* was incubated with the reagents for 1 h, washed, and allowed to attach to proteins as described for Fig. 2. Values for each protein with no inhibitor were adjusted to 100. Statistical significance was calculated separately for each protein. Insert bars and asterisks are as defined for Fig. 2.

curials, such as pCMBA, block the protein's SH groups by replacing the H atom. Effective inhibition of *T. denticola* attachment by these two compounds strongly suggests that SH groups of *T. denticola* surface proteins are important in the attachment to proteins. On the other hand, treatment of the substrate proteins with compounds reducing the disulfide groups to SH groups had no effect on the attachment. Type I collagen does not even have any disulfide groups. This suggests that the attachment of *T. denticola* to proteins is not a sulfhydryl-disulfide exchange reaction. It is possible that the SH groups are not directly involved in the attachment but that conformational changes of a *T. denticola* surface protein(s) induced by the pCMBA-oxidized glutathione-SH interaction affect the actual binding site in *T. denticola*.

Attachment of *T. denticola* to the proteins studied shows that it has unusual protein binding capacity. Laminin, collagen types I and IV, fibronectin, fibrinogen, and serum albumin differ markedly in their properties, such as molecular weight, primary structure, molecular conformation, net charge, carbohydrate content, and the numbers of disulfide bonds and free thiol groups. Important differences in the attachment of *T. denticola* to these proteins were found. Blocking of the SH groups had less effect on the attachment to fibrinogen, and heat treatment reduced the attachment to laminin more than the attachment to gelatin or fibrinogen, while no effect was obtained with BSA. Further, compared with attachment to other proteins, attachment to laminin seemed to be more sensitive to increased salt concentration. In addition, both cysteine and *N*-ethylmaleimide inhibited the attachment of *T. denticola* to BSA but not to other proteins. These differences suggest that *T. denticola* has several specific mechanisms of attachment to proteins. This view is further supported by the finding that pretreatment of *T. denticola* with soluble laminin, fibronectin, BSA, fibrinogen, or gelatin did not inhibit the attachment to laminin and fibronectin. The binding mechanisms to distinct proteins appear to differ in their dependency on sulfhydryl groups, electrostatic properties, and carbohydrate composition of the receptor molecules. Isolation and characterization of these receptors will give us important information about the virulence determinants of *T. denticola* and hopefully provide us means to develop effective measures to prevent its invasion into periodontal tissues.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Council of Canada and the Canadian Bacterial Disease Network.

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