Chemical and Biological Activities of a 64-Kilodalton Outer Sheath Protein from *Treponema denticola* Strains

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This study examined the distribution of the major outer sheath proteins (MOSP) in several *Treponema denticola* strains and reports the isolation of a 64-kDa protein from the outer sheath of human clinical isolate *T. denticola* GM-1. The outer sheath was isolated by freeze-thaw procedures, and the distribution of outer sheath proteins was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *T. denticola* GM-1, MS25, SR-5, and three low-passage clinical isolates possessed an MOSP with a relative molecular mass of 60 to 64 kDa. This MOSP was absent in *T. denticola* ATCC 35404 (TD-4) and clinical isolate SR-4. The latter possessed an MOSP of 58 kDa. ¹²⁵I labeling revealed both MOSP to be dissociated forms of higher-molecular-mass oligomeric units between 116 and 162 kDa. Two-dimensional SDS-PAGE confirmed the modifiability of these MOSP. Isoelectric focusing of the 64-kDa MOSP indicated a pI of 6.7. Immunoblots with antiserum to GM-1 whole cells revealed the 64-kDa protein to be immunogenic and not cross-reactive with the MOSP of TD-4 or SR-4, and monospecific antibody to the 64-kDa protein recognized common epitopes on the high-molecular-weight oligomeric protein. These antibodies did not react with any component of TD-4 whole cells in immunoblots or in immunogold electron microscopy. Fab fragments inhibited the adherence of *T. denticola* GM-1 to human gingival fibroblasts by 78% (1:1,600; 0.72 µg of protein per ml), while TD-4 adherence was not inhibited. Amino acid analysis revealed a slightly acidic protein, devoid of cysteine, with 36% hydrophobic residues. Cyanogen bromide fragmentation of the 64-kDa protein revealed that a 42-kDa fragment contained a T-L-D-L-A-L-D segment which was 100% homologous with an integrin alpha subunit of a human leukocyte adhesion glycoprotein p 150,95.

Adherence of a microbe to its host is fundamental to the initiation and progression of many host-parasite interactions (2, 10, 21, 30, 50, 59, 61, 67, 79). In most instances, the adherent organism must first attach to the host surface and then invade its external surfaces to ultimately establish itself in the host's complex environment. There is increasing evidence that the adherence of host-associated spirochetes (i.e., *Treponema*, *Leptospira*, *Borrelia* species) to mammalian cells plays an essential role in the pathogenesis of a large number of spirochetal diseases. Adherence of *Treponema pallidum* to mononuclear phagocytes, epithelial cells, and fibroblastlike cells in the pathogenesis of syphilis has been well documented (6, 9, 23–25, 31, 66). Three distinct *T. pallidum* proteins (89.5, 37, and 32 kDa) are thought to function as cytoadhesins through their specificity for fibronectin (1, 5, 61, 71). Specific adhesive mechanisms have also been suggested for *Borrelia burgdorferi*-host cell interactions in Lyme disease (28, 29, 72) as well as the association of *Leptospira* species with host cells in leptospirosis (22, 51, 52, 65, 73, 74, 76).

The oral treponemes, once considered to be resident opportunists, are assuming increasing importance in the biology of periodontal disease progression. The evidence reported in the literature indicates that it is more than likely that several of these prokaryotes (*Treponema denticola*) play an active and destructive role in host tissue destruction. *T. denticola*, a small oral treponeme, possesses significant proteolytic and cytolytic capabilities almost equal in distribution to *Porphyromonas gingivalis* (34). While *T. denticola* has been shown to adhere to and damage eukaryotic cells (4,

60, 64), and it has been shown that this attachment to specific host cells may be important in the progression of the disease, little is actually known about the mechanisms by which these oral treponemes attach to these cells. We recently reported two possible mechanisms by which human strains of *T. denticola* adhere to human gingival fibroblasts (HGFs) (78): (i) a lectinlike adhesin(s) on the *T. denticola* surface with affinity for galactose and mannose on the HGF surface, and (ii) a serum host factor(s), possibly fibronectin, bridging *T. denticola* and HGFs. Moreover, we observed differences in adherence between *T. denticola* ATCC 35404 and two human clinical isolates. These differences prompted us to analyze surface-associated proteins in these as well as other *T. denticola* strains to locate and characterize potential outer sheath-associated proteins on the *T. denticola* outer sheath which might function in the adherence of these oral spirochetes to HGFs.

This study was undertaken to study the relationship between a specific *T. denticola* surface macromolecule (a 64-kDa protein) and its potential role in mediating host cell surface interactions. These and subsequent data which we will report using the principles of structure-function relations will facilitate identification of important antigenic determinants on the bacterial surface which may be critical to bacterium-host interaction and ultimately to the progression of periodontal disease. This strategy, we believe, represents a cogent approach to eventually generating vaccine candidates to prevent or limit disease progression.

MATERIALS AND METHODS

***T. denticola* strains, growth conditions, and outer sheath isolation.** *T. denticola* GM-1, MS25, SR-4, SR-5, C-1, C-2,

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and C-3 were isolated from human periodontal pockets (78). *T. denticola* ATCC 35404, designated here as strain TD-4, was obtained from the American Type Culture Collection, Rockville, Md. All strains were grown in GM-1 broth (8) without glucose in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) for 5 days as described previously (78). Outer sheaths of representative strains were isolated by repeated freeze-thawing employing a modification of the Masuda and Kawata technique (47). Briefly, cells harvested from 5 liters of GM-1 culture medium were concentrated and washed once in normal saline by using a Pellicon concentrator (0.45- μ m pore size; Millipore Corp., Bedford, Mass.) and centrifuged at $11,000 \times g$ for 20 min at 4°C. The pellet was resuspended in 60 ml of 0.05 M Tris-HCl buffer (pH 7.2) containing 0.02 M $MgCl_2$, DNase I (2 μ g/ml; Sigma Chemical Co., St. Louis, Mo.), 2 mM phenylmethylsulfonyl fluoride (Sigma), 2 mM benzamidine (Sigma), and 2 mM Na-P-tosyl-L-lysine chloromethyl ketone (Sigma). The protease-protected cells were then subjected to 40 freeze-thaw (liquid N_2 -water, 20°C) cycles. The disrupted cells were centrifuged at $8,000 \times g$ for 10 min at 4°C to remove unbroken cells and debris, and the supernatant was centrifuged at $25,000 \times g$ for 30 min at 4°C to obtain the crude outer sheath. After being washed twice with Tris-HCl buffer, the crude outer sheath preparation was suspended in 60 ml of 0.1 M sodium acetate-HCl buffer (pH 3.0), mixed gently for 2 h to remove contaminating flagella, and then centrifuged at $25,000 \times g$ for 30 min at 4°C. The purity of this outer sheath preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis and compared with the protein profiles of this material after sucrose density gradient centrifugation (47) and Sephadex G-100 (fine; Pharmacia, Uppsala, Sweden) column chromatography. Since all three preparations gave similar results and transmission electron microscopy of negatively stained preparations revealed the presence of only outer sheath material, subsequent isolations ended with the removal of contaminating flagella.

Extrinsic ^{125}I labeling of bacteria. For autoradiographic analyses and functional assays of the *T. denticola* strains with HGFs, representative *T. denticola* strains were radioiodinated by a modification of the Armstrong and Parker technique (3).

SDS-PAGE, Western blotting (immunoblotting), and IEF. One- and two (2D)-dimensional SDS-PAGE was performed by using the discontinuous gel system of Laemmli (44). Heated and unheated samples (whole cells or isolated outer sheaths) at designated protein concentrations in $1 \times$ treatment buffer (62.5 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol) were electrophoresed through a 4% stacking gel and a 7.5, 10, or 12.5% resolving gel. Protein concentration was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.). Gels were stained with Coomassie brilliant blue R or silver (33) for visualization of protein bands, fixed and dried for autoradiography, or transferred to nitrocellulose (0.45- μ m pore size; Schleicher & Schuell, Inc., Keene, N.H.) for Western blotting.

Western blots were incubated with (i) antiserum against whole-cell proteins of *T. denticola* GM-1 or (ii) monospecific polyclonal rabbit immunoglobulin G (IgG)-enriched fraction directed against a specific outer sheath protein (i.e., the 64-kDa outer sheath protein of *T. denticola*; see below). The appropriate horseradish peroxidase-conjugated secondary antibody was used at a 1:4,000 dilution, and blots were developed with 2 mg of 4-chloro-1-naphthol per ml and

0.03% hydrogen peroxide in 20 mM Tris-HCl (pH 7.5)–0.1 M NaCl. 2D SDS-PAGE was done by the method of Kennell and Holt (41) based on the procedure of Hindahl and Iglewski (32). Briefly, after unheated duplicate samples were run in the first dimension as described above, one lane was cut out of the 1-dimensional slab gel, placed into the $1 \times$ treatment buffer, heated at 100°C for 5 min, juxtaposed horizontally along the top of a 1-cm stacking gel, and electrophoresed through a resolving gel of the same acrylamide concentration as was used in the first dimension. The other duplicate lane was Coomassie brilliant blue stained and kept as a reference for determining heat-modifiable proteins from the second dimension. Isoelectric focusing (IEF) was done by the procedure of O'Farrell (58). Isolated outer sheath preparations (20 to 100 μ g of protein) in lysis buffer containing 9 M urea were applied to one-dimensional tube gels and isoelectric focused. These gels were then resolved in the second dimension through SDS-7.5% polyacrylamide gels (see above).

PAS-silver staining of isolated outer sheaths. After isolated outer sheaths were resolved through SDS-PAGE (see above), gels were fixed and periodic acid-Schiff (PAS)-silver stained by the method of Jay et al. (38). Briefly, gels were fixed overnight by gentle agitation in 50% (vol/vol) methanol in distilled deionized water (ddH_2O). After rehydration for 20 min in ddH_2O , gels were placed in 2% periodate (wt/vol) (Sigma) for 15 min and then washed twice for 2 min each wash with ddH_2O . Concentrated Schiff reagent (fuchsin-sulfite reagent; Sigma) was then added, and the gels were gently agitated in a fume hood until bands turned magenta. After two 2-min washes with ddH_2O , overnight reduction with 2% (wt/vol) sodium metabisulfite (Fisher Scientific Co., Springfield, N.J.) followed to remove unreacted *p*-rosaniline. After several washes with ddH_2O lasting 30 min, gels were stained with silver (33). Bands that stained magenta with PAS, yet were unstained by subsequent silver staining, were suggestive of glycosylated proteins.

Deglycosylation of outer sheath proteins. To further assess the glycosylated characteristic of specific outer sheath proteins, we treated isolated outer sheaths of *T. denticola* GM-1 with trifluoromethanesulfonic acid (TFMS) (40) to remove both O-linked as well as N-linked carbohydrates from the protein backbone. Briefly, an isolated outer sheath preparation was lyophilized to complete dryness in a Reactival (5 ml; Pierce). Completely anhydrous TFMS (Aldrich Chemical Co., Milwaukee, Wis.) was added at a concentration of 60 μ l of TFMS per mg of protein in the Reactival. Anisole (10 μ l; Sigma) was also added to the reaction, which was performed for 2 h on ice. The reacted material was then transferred to a 1.5-ml microcentrifuge tube, and a 9:1 mixture of ether (diethyl; Aldrich)-pyridine (Sigma) was slowly added dropwise to precipitate the mixture. After 1 ml of the ether-pyridine mixture had been added, the contents were centrifuged in the microcentrifuge for 2 min. The pellet, containing protein and the pyridinium salt of the acid, was resuspended in 0.1 M ammonium bicarbonate (NH_4HCO_3) and dialyzed at room temperature against the same overnight. After centrifugation, the sample was analyzed for protein and carbohydrate by SDS-PAGE and PAS-silver staining (see above).

Isolation and purification of 64-kDa outer sheath protein. Isolated outer sheaths (see above) from *T. denticola* GM-1 heated at 100°C for 5 min were electrophoresed through an SDS-7.5% polyacrylamide preparative gel by using a preparative comb with one reference well. The 64-kDa protein was identified, excised, and electroeluted (model 422 Electro-

Eluter; Bio-Rad Laboratories, Richmond, Calif.) at 10 mA per glass tube for 3.5 h in elution buffer minus SDS. Following concentration with a Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.), dialysis against water at 4°C for 2 days, and determination of protein content, the preparation was analyzed by reverse-phase high-pressure liquid chromatography (HPLC). A μ Bondapak C₁₈ column (Waters Chromatography Division, Millipore Corp., Milford, Mass.) was used employing the following HPLC parameters: solvent A was 0.1% trifluoroacetic acid in H₂O; solvent B was 70:30:0.1 acetonitrile-H₂O-trifluoroacetic acid (vol/vol/vol); flow rate was 1 ml/min with a linear gradient of 0 to 60% acetonitrile over 1 h. Protein was detected at 214 nm. Fractions were pooled, lyophilized to remove the acetonitrile, resolved over an SDS-7.5% polyacrylamide gel, and silver stained to determine the 64-kDa peak. Since this method gave only a 14% recovery of the 64-kDa protein after electroelution, a second method was used for recovery of larger amounts of the 64-kDa protein. This latter method employed multiple preparative runs of unheated isolated outer sheath preparations resolved through 7.5% acrylamide SDS-PAGE in the first dimension followed by 2D 7.5% SDS-PAGE as described above. The prominent 64-kDa spot was identified by incubating each gel in cold 0.25 M KCl. Once the opaque spot appeared, it was excised, homogenized in 0.5 ml of H₂O, mixed thoroughly with 0.5 ml of Freund's incomplete adjuvant, and prepared for injection into rabbits (see below).

Immunologic reagents. Rabbit monospecific polyclonal antibody directed against the 64-kDa immunogen of *T. denticola* GM-1 was generated by the immunization protocol described previously (78). IgG enriched fractions from whole antiserum to the 64-kDa protein were prepared by ammonium sulfate precipitation. One-third volume of saturated ammonium sulfate was added dropwise to two-thirds volume of the rabbit antiserum to the 64-kDa protein at 4°C. This solution was kept at 4°C overnight, after which it was centrifuged at 12,000 $\times g$ for 15 min. The pellet was resuspended to two-thirds the original volume with phosphate-buffered saline (PBS) and dialyzed against PBS at 4°C for 24 h, and the protein content was determined. Antibody titers to the 64-kDa outer sheath protein, using the 33% ammonium sulfate IgG-enriched fraction against formalinized whole cells of GM-1, were determined by an enzyme-linked immunosorbent assay by the method of Ebersole et al. (18). Antibody titers of 1:102,400 were achieved. Fab fragments from the enriched IgG fraction were generated by papain treatment of the latter (using the ImmunoPure Fab preparation kit, no. 44885; Pierce), and then protein content was determined.

Amino acid analysis and sequencing of 64-kDa antigen. Isolated outer sheath preparations of GM-1 (50 to 100 μ g of protein) were either electrophoresed through SDS-7.5% polyacrylamide gels or focused isoelectrically and then electrophoresed through SDS-polyacrylamide gels in the second dimension as described above. Following transfer to Immobilon P membranes (Millipore) (165 mA, 5 h) by the method of Matsudaira (48), the 64-kDa antigen was visualized with Coomassie brilliant blue and excised from the membranes. Amino acid analyses were done with a Beckman model 7300 analyzer system after hydrolysis of 20 μ g of protein for 24 h at 110°C in vapor-phase 6 N HCl in a Waters Pico Tag hydrolysis workstation (Millipore). Cysteine was determined on samples pretreated with 4-vinyl pyridine (27). Attempts to sequence the N' terminus of the intact 64-kDa protein were unsuccessful, suggesting that a naturally occurring acylated

amino acid at the N' terminus was preventing Edman degradation or that contaminating by-products of urea during the IEF runs (36) or high-temperature heating of the sample during SDS-PAGE analysis (36) were responsible for blockage. Urea contamination was eliminated by purifying urea through Amberlite MB-3 (mixed cation-anion resin) (Sigma). N'-terminus blockage due to heating was eliminated by doing all heating procedures of outer sheath preparations before electrophoresis at 60°C for 5 min. Since these precautions did not help, sequences were determined directly on Pro Blott membranes (Applied Biosystems, Foster City, Calif.) after cyanogen bromide fragmentation, using an Applied Biosystems model 477A pulsed/liquid-phase sequencer coupled to an on-line HPLC model 120A analyzer.

Fibroblast cultures. Normal HGF cultures, designated Gin-3, were used as described previously (78). Adherence assays were performed only with HGFs passaged between 6 and 13 times.

Adherence assay. All adherence assays were performed according to previously described methods (78), with the exception that confluent monolayers were suspended only in Dulbecco modified Eagle medium for the duration of each assay. ¹²⁵I-labeled *T. denticola* GM-1 and TD-4 (see above) were incubated with various protein concentrations of either the enriched IgG fraction of the rabbit anti-64-kDa protein antigen or Fab fragments of this fraction (see above) at 37°C for 1 h with constant gentle agitation. After removal of unbound antibody fragments, adherence assays followed as described previously (78).

Immunoelectron microscopy. Samples for transmission electron microscopy were prepared by the method of Kinder and Holt (42) and Mclean and Nakane (49). *T. denticola* GM-1 or TD-4, grown for 4 days, was washed twice in PBS (pH 7.2) and resuspended in 1 ml of the same buffer. Specimens for negative staining were prepared by taking a 10- μ l sample of the bacterial suspensions mounted on colloidal membrane-coated nickel grids which had been pretreated with 4% bovine serum albumin for 30 min and incubating them with 10 μ l of preimmune rabbit serum, rabbit antisera against *Eubacterium saburreum* or *Wolinella recta* ATCC 33238 (as negative controls), or the monospecific polyclonal rabbit 64-kDa protein antigen-specific antiserum. All four antisera were prepared at a dilution of 1:100. The grids were washed twice in PBS and subsequently reacted with anti-rabbit IgG-gold complexes (particle size, 10 nm; E-Y Laboratories, Inc., San Mateo, Calif.) for 30 min at room temperature. Following an additional two washes in distilled water, the cells were negatively stained with 1% (wt/vol) phosphotungstic acid and observed in a JEOL 1200 transmission electron microscope at 60 kV. Immunostaining of thin sections, following routine fixation, dehydration, and embedding (31) of the bacterial samples incubated with the respective primary and gold-complexed secondary antibodies (see above), was done by the method of Berryman and Rodewald (7).

RESULTS

SDS-PAGE analysis of whole cells of *T. denticola*. Coomassie brilliant blue-stained SDS-PAGE polypeptide profiles of sonicated and boiled whole cells of the human *T. denticola* strains used in this study are shown in Fig. 1. Although large numbers of polypeptides were common to all strains, major differences were evident in TD-4 (Fig. 1, lane 3) and clinical isolate SR-4 (not shown) compared with the other *T. denticola* strains examined. The major 60- to 64-kDa

FIG. 1. SDS-PAGE of sonicated whole cells of *T. denticola* strains. Lanes: 1, GM-1; 2, SR-5; 3, TD-4; 4, MS25; 5, C-1; 6, C-2; 7, C-3; 8, Bio-Rad low-molecular-weight standards ($\times 10^3$). Arrow indicates 64-kDa band calculated by linear regression of the molecular weight standards. All samples were heated at 100°C for 5 min. We used a 12.5% acrylamide gel which was stained with Coomassie brilliant blue R-250 (0.025%). There is 20 μ g of protein per lane.

polypeptide found in *T. denticola* GM-1, SR-5, and MS25 (Fig. 1, lanes 1, 2, and 4) was also present in the fresh human clinical *T. denticola* isolates C1 to C3, even when cells were passaged only four times in vitro (Fig. 1, lanes 5 to 7). The 64-kDa protein was absent in *T. denticola* SR-4 and TD-4. These strains did, however, possess a major polypeptide at 58 kDa (Fig. 1, lane 3). Interestingly, *T. denticola* MS25 possessed both the 64- and 58-kDa polypeptide bands (Fig. 1, lane 4).

SDS-PAGE-silver staining of the isolated outer sheath of *T. denticola* GM-1 examined before and after heating at 70°C

FIG. 2. SDS-PAGE of isolated outer sheath of *T. denticola* GM-1. Lanes: 1, unheated sample; 2, heated at 70°C for 5 min. Arrow indicates 64-kDa doublet (7.5% acrylamide gel; silver stained; 15 μ g of protein per lane). Numbers on left show molecular weight standards ($\times 10^3$).

FIG. 3. 2D SDS-PAGE of isolated outer sheath of *T. denticola* GM-1. Sample was electrophoresed in the first dimension without prior heat treatment and in the second dimension after heating at 70°C for 5 min. Both dimensions used 7.5% acrylamide gels stained with 0.025% Coomassie brilliant blue R-250 (25 μ g of protein electrophoresed in the first dimension). Numbers on left show molecular weight standards ($\times 10^3$).

(Fig. 2, lanes 1 and 2, respectively) revealed significant differences in the distribution of the other sheath polypeptides. The unheated outer sheath preparation separated in SDS-PAGE as a ladderlike polypeptide with the major polypeptides in the relative molecular size migration range between 116 and 162 kDa (Fig. 2, lane 1). We estimate the polypeptide ladder to consist of at least six individual polypeptide bands separated by approximately 7 to 8 kDa. Non-heat-modified polypeptides were resolved at 70, 95, and 97 kDa when the outer sheath was heated at 100°C for 5 min. All other high-molecular-weight bands which appeared as non-heat modified at 70°C for 5 min were modified at 100°C for 5 min. Heating of the outer sheath at 70°C for 5 min resulted in the modification of the ladderlike polypeptides to a major outer sheath protein at a relative molecular migration of 64 kDa (Fig. 2, lane 2, arrow). While it would appear that a doublet resolves at the 64-kDa region (Fig. 2, lane 2), reverse-phase HPLC and IEF 2D analysis results indicate the presence of only one polypeptide (see below and Discussion). Moreover, PAS-silver-stained gels suggested that the 64-kDa region was composed of a large glycosylated lower band (see Fig. 11, lane 1, large arrow) and a smaller nonglycosylated upper band (see Fig. 11, lane 1, small arrow) (see below). 2D SDS-PAGE analysis of the outer sheaths confirmed the migration of the high-molecular-mass polypeptides to the 64-kDa region upon heating (Fig. 3). While the heat-modifiable association of the 64-kDa polypeptide with higher-molecular-mass moieties was also seen in Sarkosyl-insoluble preparations (Fig. 4, compare lanes 1 and 2), Sarkosyl did not separate the higher-molecular-mass polypeptides into distinct bands (Fig. 4, lane 1). Compared with *T. denticola* GM-1 outer sheaths run unheated through SDS-7.5% polyacrylamide gels (Fig. 2, lane 1), the Sarkosyl-insoluble polypeptides did not resolve in the gel as a ladderlike polypeptide but appeared as an aggregate in an area between 112 and 120 kDa (Fig. 4, lane 1). This aggregate did not separate into distinct bands even when allowed to run further down the gel (data not shown).

Identification of the 64-kDa polypeptide on the surface of *T. denticola*. Surface-exposed proteins of *T. denticola* GM-1 and TD-4 were identified by extrinsic 125 I labeling with Iodo-beads (Fig. 5 and 6). Kinetics of 125 I labeling of whole cells and then Sarkosyl extraction of outer sheaths of *T. denticola* GM-1 revealed that the 64-kDa polypeptide appeared within 30 s of exposure to 125 I (Fig. 5A and B, lanes 1) and increased its surface labeling during the 5-min labeling experiment (Fig. 5A and B, lanes 2 to 4). Autoradiographs of

FIG. 4. SDS-PAGE of 2% Na-sarcosinate-insoluble fraction of whole cells of *T. denticola* GM-1. Lanes: 1, unheated; 2, heated at 70°C for 5 min (7.5% acrylamide gel; stained with 0.025% Coomassie brilliant blue R-250; 30 μ g of protein per lane). Numbers on left show molecular weight standards ($\times 10^3$).

outer sheaths of *T. denticola* GM-1 and TD-4 which had previously been isolated from 125 I-labeled whole cells of the respective strains were similar to the SDS-PAGE protein profiles of the isolated outer sheaths in the absence of 125 I (compare Fig. 2, lane 1, with Fig. 6, lanes 1 and 3, respectively). The characteristic ladder-like polypeptide pattern in the high-molecular-mass region was apparent in the 125 I-labeled outer sheath, which, upon heating, migrated to the 64-kDa area for strain GM-1 and the 58-kDa area for strain TD-4 (Fig. 6, lanes 2 and 4, respectively). However, unheated outer sheath of strain TD-4 included the presence of a high-molecular-size mass labeling at 200 kDa (Fig. 6, lane 3) which was absent in the GM-1 strain (Fig. 6, lane 1). 2D SDS-PAGE of heated and 125 I-labeled outer sheath prepara-

FIG. 6. Autoradiograph of isolated outer sheaths of *T. denticola* GM-1 and TD-4. Whole cells were extrinsically labeled with 125 I and then freeze-thawed multiple times to isolate the outer sheaths. Lanes: 1 and 3, GM-1 and TD-4 outer sheath, respectively, unheated; 2 and 4, GM-1 and TD-4 outer sheath, respectively, heated at 70°C for 5 min (15 μ g of protein per lane). The 64- and 58-kDa proteins are labeled. Numbers on left show molecular weight standards ($\times 10^3$).

tions revealed that this 200-kDa polypeptide in TD-4 migrated to 45 kDa. No polypeptide was observed at a comparable relative molecular mass in GM-1 (data not shown).

Western immunoblot analysis. Immunoblots employing antiserum to *T. denticola* GM-1 whole cells blotted against whole cells of *T. denticola* GM-1, TD-4, and MS25 are shown in Fig. 7. Immunoblotting against *T. denticola* GM-1

FIG. 5. Autoradiograph of 125 I-labeled whole cells of *T. denticola* GM-1 after SDS-PAGE. (A) Untreated after labeling; (B) 2% Na-sarcosinate-insoluble fraction after labeling. Lanes: 1, 30-s labeling; 2, 1-min labeling; 3, 3-min labeling; 4, 5-min labeling. All samples were heated at 70°C for 5 min before electrophoresis through a 12.5% acrylamide gel (20 μ g of protein per lane). Numbers in middle show molecular weight ($\times 10^3$).

FIG. 7. Western blot analysis of whole cells of *T. denticola* strains. Whole-cell sonicates of the respective strains were heated at 100°C for 5 min, resolved through SDS-PAGE (12.5% acrylamide), transferred to nitrocellulose, and reacted with rabbit antiserum to whole cells of GM-1 (1:400). (A and B) Lanes: A and B, 1, GM-1; A, 2, TD-4; A, 3, MS25; B, 2, SR-4. A, 30 μ g of protein per lane; B, 20 μ g of protein per lane. Arrows indicate major differences in reactivity of strains TD-4 and SR-4 when compared with strains GM-1 and MS25. Numbers to left of each panel show molecular weight standards ($\times 10^3$).

FIG. 8. Western blot analysis of isolated outer sheath of *T. denticola* GM-1 and TD-4. Unheated outer sheath preparations of the respective strains were resolved through SDS-PAGE (7.5% acrylamide), transferred to nitrocellulose, and reacted with rabbit antiserum to the 64-kDa protein of GM-1 (1:400). Lanes: 1, isolated outer sheath, GM-1; 2, isolated outer sheath, TD-4 (30 μ g of protein per lane). Numbers on left show molecular weight standards ($\times 10^3$).

revealed significant immunoreactive cells with highly immunogenic polypeptides occurring at approximately 12, 33, 39, 47, 64, 65, 66, and 80 kDa (Fig. 7A, lane 1). Almost identical polypeptides were immunoreactive in *T. denticola* MS25 when immunoblotted against *T. denticola* GM-1 antiserum (Fig. 7A, lane 3). While there was immunologic cross-reactivity of the *T. denticola* GM-1 antiserum with strains TD-4 and SR-4, the *T. denticola* GM-1 antiserum did not cross-react with the 58-kDa major outer sheath protein of these strains (Fig. 7A and B, lanes 2). Moreover, there was no reactivity at 12 kDa in either TD-4 or SR-4 when immunoblotted against antiserum to GM-1 (Fig. 7A and B, lanes 2), suggesting the absence of that immunogenic polypeptide in these two *T. denticola* strains. Immunoblots with antiserum prepared against the 64-kDa protein from *T. denticola* GM-1 did not cross-react with any component of the isolated outer sheath of TD-4 (Fig. 8, lane 2). This antiserum was, however, highly specific for the high-molecular-weight ladderized polypeptide in unheated GM-1 outer sheath preparations (Fig. 8, lane 1). IgG-enriched anti-64-kDa protein fractions as well as Fab fragments from the IgG-enriched fractions showed specificity to the 64-kDa band when reacted with heated GM-1 whole cells (Fig. 9).

Immunogold labeling of *T. denticola*. Immunogold labeling of whole cells and ultrathin sections of *T. denticola* GM-1 and whole cells of TD-4 employing IgG-enriched anti-64-kDa protein serum is shown in Fig. 10. The negatively stained *T. denticola* GM-1-anti-64-kDa protein-gold conjugate showed specific labeling of the outer sheath as well as isolated outer sheath vesicles (Fig. 10A). The immunogold-IgG-anti-64-kDa protein conjugate was randomly distributed over the *T. denticola* GM-1 surface. The gold label also adhered to what we consider to be fragments (vesicles?) of the outer sheath (Fig. 10A, arrow and inset). Little to no label was observed at the tips of the *T. denticola* GM-1 cells. There was also essentially no labeling of the outer sheath of strain TD-4 at the anti-64-kDa protein antiserum and immunogold dilution

FIG. 9. Western blot analysis of whole cells of *T. denticola* GM-1. Whole-cell sonicates of GM-1 were heated at 100°C for 5 min, resolved through SDS-PAGE (7.5% acrylamide), transferred to nitrocellulose, and reacted with either the IgG-enriched fraction to the 64-kDa outer sheath protein of GM-1 (1:1,600) (lane 1) or Fab fragments generated from the IgG-enriched fraction to the 64-kDa protein (1:1,600) (lane 2). Lanes 1 and 2 were taken from two separate blots run under identical conditions (25 μ g of protein per lane). Numbers on left show molecular weight standards ($\times 10^3$).

studied (Fig. 10B). Thin sections of similar immunogold-labeled cells (Fig. 10C) revealed that the *T. denticola* GM-1 immunogold labeled identical to that of the negatively stained preparations. The anti-64-kDa protein serum-gold label was associated exclusively with the outer sheath, with essentially no label being found within the cytoplasmic region of the cell (Fig. 10C). Note in Fig. 10C that the gold label adhered to the "ruptured" (Fig. 10C, large arrow) outer sheath as well as to the sheath which was juxtaposed to the protoplasmic cylinder (Fig. 10C, double arrows).

Other characteristics of isolated 64-kDa protein. As described above, PAS-silver-stained gels of isolated outer sheath preparations of *T. denticola* GM-1 suggested that the 64-kDa region was composed of a large glycosylated lower band (Fig. 11, lane 1, large arrow; lane 2) and a smaller nonglycosylated upper band (Fig. 11, lane 1, small arrow; lane 2). In fact, it would appear that the large glycosylated band is a heat-modified product of the first two glycopeptide bands composing the lower segment of the ladderized high-molecular-weight polypeptide (Fig. 11, lane 3, asterisk). Furthermore, deglycosylated outer sheath preparations resolved in SDS-PAGE without the characteristic glycosylated large lower band in the 64-kDa region (Fig. 12, lanes 3 and 4, arrow, compared with lane 2, arrow), suggesting that acid hydrolysis of glycosidic bonds by TFMS removed carbohydrates from the protein backbone.

Analysis of the electroeluted 64-kDa polypeptide by reverse-phase HPLC revealed it to be a hydrophobic protein eluting from the HPLC column at 48% acetonitrile (Fig. 13, arrow). SDS-PAGE and silver staining revealed this peak to consist of one polypeptide band which migrated with a relative molecular mass of 64 kDa (Fig. 13, inset). The other peaks, after collecting and concentrating, did not resolve into any bands upon SDS-PAGE and silver staining.

IEF and immunoblotting with the IgG-enriched anti-64-kDa protein fraction indicated that the 64-kDa major outer sheath protein had a pI of 6.7 (Fig. 14).

Amino acid analysis and sequencing of the 64-kDa major

FIG. 10. Immunoelectron micrographs of *T. denticola* GM-1 and TD-4. (A) Negative stain of an intact GM-1 treponeme showing immunogold labeling of the outer sheath. Arrow and inset, immunogold labeling of isolated vesicles of GM-1. (B) Negative stain of an intact TD-4 treponeme showing no immunogold labeling of the outer sheath. (C) Transmission electron microscopy of thin section of GM-1 treponeme showing immunogold labeling associated with the outer sheath. Essentially no label was found within the cytoplasmic region of the cell. Arrow, gold label adhering to the "ruptured" outer sheath. Double arrow, gold label adhering to the outer sheath juxtaposed to the protoplasmic cylinder. Bars: A, inset, C, 0.1 μm ; B, 0.5 μm .

outer sheath protein. The amino acid composition of the 64-kDa polypeptide from *T. denticola* GM-1 was determined after three isolation methods. These included (i) resolution of isolated outer sheath proteins through 7.5% SDS-PAGE and then excision of the 64-kDa band after transfer onto an Immobilon P membrane; (ii) IEF of isolated outer sheath proteins and then 2D 7.5% acrylamide SDS-PAGE and

excision of the 64-kDa spot; or (iii) reverse-phase HPLC isolation of the 64-kDa polypeptide. The three methods gave identical amino acid analysis profiles of the 64-kDa polypeptide. Glycine, alanine, and asparagine plus aspartic acid were the major amino acids (14.2, 12.15, and 13.8%, respectively) (Table 1). Combined, they accounted for 40.15% of the amino acid content of the 64-kDa polypeptide. The other

FIG. 11. PAS-silver-stained SDS-polyacrylamide gel of isolated outer sheath of *T. denticola* GM-1. Outer sheath preparations resolved through SDS-PAGE (7.5% acrylamide) were fixed and PAS-silver stained. Lane 1, outer sheath heated at 100°C for 5 min. Large arrow, glycosylated lower band of 64-kDa protein. Small arrow, nonglycosylated upper band of 64-kDa protein. Lane 2, outer sheath heated at 70°C for 5 min. Lane 3, unheated outer sheath. Asterisk, first two glycopeptide bands composing the lower segment of the ladderized high-molecular-weight polypeptide (20 μ g of protein per lane). Numbers on left show molecular weight standards ($\times 10^3$).

hydrophobic amino acids (Phe, Ile, Leu, Met, Val, Pro) plus alanine revealed the 64-kDa polypeptide to consist of more than 36% hydrophobic amino acids on a per amino acid basis. Tryptophan, another hydrophobic amino acid, was not determined, and cysteine was not found in samples pretreated with 4-vinyl pyridine.

All attempts at sequencing the 64-kDa protein from the N' terminus were unsuccessful. It appeared to be blocked to

FIG. 12. PAS-silver-stained SDS-polyacrylamide gel of isolated outer sheath of *T. denticola* GM-1. Outer sheath preparations, both untreated (lanes 1 and 2) and treated with TFMS (lanes 3 and 4), were resolved through SDS-PAGE (7.5% acrylamide) and then fixed and PAS-silver stained. Lanes 1 and 3, unheated outer sheath. Lanes 2 and 4, outer sheath heated at 70°C for 5 min. Arrow, lane 2, glycosylated lower band of 64-kDa protein. Arrow, lanes 3 and 4, lack of glycosylated lower band of 64-kDa protein. (20 μ g of protein per lane). Numbers in center show molecular weight standards ($\times 10^3$).

FIG. 13. Isolation of 64-kDa outer sheath protein by reverse-phase HPLC. Following electrophoresis of the outer sheath of *T. denticola* GM-1 through SDS-PAGE (7.5% acrylamide) and identification, excision, electroelution, dialysis, and concentration of the 64-kDa protein, 85 μ g of protein was placed on a Waters μ Bondapak C₁₈ column and chromatographed as described in Materials and Methods. Arrow, 64-kDa fraction eluting at 48% acetonitrile. Inset, 64-kDa fraction resolved through SDS-PAGE (7.5% acrylamide) and silver stained (25 μ g of protein per lane).

Edman degradation even after it was isolated either by gel electroelution and reverse-phase HPLC or by IEF with urea pretreated with Amberlite MB-3 (see Materials and Methods). Cyanogen bromide fragmentation of the 64-kDa polypeptide did result in successful N'-terminal amino acid sequences of four cleavage products (Table 2). One frag-

FIG. 14. Western blot of *T. denticola* GM-1 outer sheath showing a pI of 6.7 for the 64-kDa protein. A total of 50 μ g of protein was resolved in first dimension by IEF, and this was followed by 2D SDS-PAGE (12.5% acrylamide). The protein was then transferred onto nitrocellulose and reacted with the IgG fraction of rabbit anti-64-kDa protein (1:1,000). Numbers on left show molecular weight standards ($\times 10^3$).

TABLE 1. Amino acid analysis of *T. denticola* 64-kDa major outer sheath protein

Amino acid	% (ranked)	%
Glycine	14.20	
Asparagine + aspartic acid	13.80	
Alanine	12.15	
Glutamine + glutamic acid	8.90	
Lysine	7.30	
Leucine	7.10	
Valine	7.05	
Threonine	6.60	
Serine	5.85	
Phenylalanine	4.10	
Tyrosine	3.70	
Isoleucine	2.80	
Proline	2.05	
Histidine	1.70	
Arginine	1.55	
Methionine	1.20	
Hydrophobic residues		
Alanine		12.15
Valine		7.05
Leucine		7.10
Isoleucine		2.80
Proline		2.05
Phenylalanine		4.10
Tryptophan		ND ^a
Methionine		1.20
Hydrophilic residues		
Glycine		14.2
Serine		5.85
Threonine		6.60
Cysteine		— ^b
Tyrosine		3.70
Asparagine		13.70 ^c
Glutamine		8.90 ^d
Positively charged residues		
Lysine		7.30
Arginine		1.55
Histidine		1.70
Negatively charged residues		
Aspartic acid		13.80 ^c
Glutamic acid		8.90 ^d
Hydrophobic residues		36.45 ^e
Negatively charged residues		22.70 ^e

^a ND, not determined.^b —, cysteine was not found in samples pretreated with 4-vinyl pyridine.^c Combination of aspartic acid and asparagine.^d Combination of glutamic acid and glutamine.^e % of total.

ment, which resolved at 42 kDa, showed 62.5% homology with a 16-amino-acid fragment from an integrin alpha-subunit of human leukocyte adhesion glycoprotein p 150,95 (16). The amino acid sequence of the fragment from the alpha-subunit was T-L-D-L-A-L-D-P-G-R-L-S-P-R-A-T. Note that the first seven amino acids, T-L-D-L-A-L-D, show 100% homology with the first seven amino acids of the 42-kDa fragment (Table 2). The cyanogen bromide fragments which resolved at 34 and 21 kDa showed 67% homology to each other through their first 12 amino acids (Table 2).

Adherence assays with the anti-64-kDa protein antibody. Preliminary investigations into the role of the 64-kDa outer sheath protein of *T. denticola* GM-1 in interactions with HGFs were made by determining the effect antibodies and Fab fragments generated against this protein would have on normal *T. denticola*-HGF interactions (Table 3). IgG-enriched anti-64-kDa protein fraction (5.6 µg of protein per ml) preincubated with ¹²⁵I-labeled *T. denticola* GM-1 resulted in a 50% inhibition of adherence to HGFs (Table 3). An IgG-enriched fraction of preimmune *T. denticola* GM-1 serum had no effect on the *T. denticola*-HGF interaction (Table 3), nor did the IgG-enriched anti-64-kDa protein fraction have an inhibitory effect on adherence of *T. denticola* TD-4 to HGFs (Table 3). Fab fragments from the IgG-enriched anti-64-kDa protein fraction were significantly more efficient in their ability to inhibit the adherence of *T. denticola* GM-1 to HGFs than the enriched IgG fraction. For example, Fab fragments at a protein concentration of 0.72 µg of protein per ml after incubation with *T. denticola* GM-1 inhibited adherence to HGFs by 78% (data not shown).

DISCUSSION

This investigation extends previously reported studies into the characterization and antigenic analyses of the outer sheath of *T. denticola* as well as providing evidence for a possible role of the 64-kDa outer sheath protein of *T. denticola* GM-1 as an adhesin to human gingival fibroblasts. An earlier study (47) of a human oral treponeme (designated *Treponema* sp. strain E-21), using a multiple freeze-thaw technique which enriched for outer sheath, reported the sheath to possess a major polypeptide at 62 kDa. We are unsure whether it is identical to the 64-kDa protein isolated from our *T. denticola* strains. The use of the freeze-thaw technique permits a much clearer and more representative chemical analysis and protein profile analysis of the outer sheath than the use of detergents since detergents have been reported to solubilize both inner and outer membrane proteins of gram-negative bacteria (20). Na-sarcosinate treatment, for example, resulted in a Sarkosyl-insoluble *T. denticola* GM-1 fraction which, while enriching for outer sheath-associated proteins, resulted in a different protein profile compared with freeze-thaw-isolated outer sheaths of this strain (compare Fig. 2, lane 2, with Fig. 4, lane 2). It is more

TABLE 2. Amino acid sequence of *T. denticola* 64-kDa major outer sheath protein after CnBr fragmentation

Mol wt fragment	Amino acid sequence ^a
42,000.....	<u>T L D L A L D L G E A X I N A T</u>
34,000.....	<u>V N F G L A F K S E P I G D A P N A</u>
30,000.....	<u>T N F G G A D G V T P L E Y X D</u>
21,000.....	<u>V N F L G A V K D E P I K K G T R F Y R K V A</u>
Alpha-subunit of p 150,95 ^b	<u>T L D L A L D P G R L S P R A T</u>

^a Underlined amino acids show homology.^b From reference 16.

TABLE 3. Effect of IgG fraction of anti-64-kDa protein on adherence of *T. denticola* to HGFs^a

Condition	Relative no. of <i>T. denticola</i> /HGF $\pm$ SD ^b	% Relative adherence $\pm$ SD ^b	% Relative inhibition
GM-1 + HGFs (no serum)	22 $\pm$ 2	100 $\pm$ 9	0
GM-1 preimmune ^c (1:1,600)	23 $\pm$ 4	105 $\pm$ 18	0
GM-1 + immune ^d (1:1,600)	11 $\pm$ 1	50 $\pm$ 4.5	50
TD-4 + HGFs (no serum)	20 $\pm$ 2	100 $\pm$ 9	0
TD-4 + immune ^d (1:1,600)	20 $\pm$ 0.8	100 $\pm$ 1	0

^a Passage 15.^b Mean of eight determinations $\pm$ standard deviation.^c Results for strain TD-4 were similar to those for strain GM-1.^d Protein concentration at the given dilution: 1:1,600 = 5.6 μ g/ml.

than likely that Sarkosyl extraction resulted in a selective solubilization of some of the oligomeric units of the higher-molecular-weight outer sheath polypeptides which resulted in the formation of polypeptide aggregates. Cockayne et al. (15) used Triton X-100 fractions of *T. denticola* ATCC 33520 to characterize outer sheath-associated antigens. They demonstrated a 54-kDa outer sheath-associated antigen which appeared to be a breakdown product of a larger, heat-modifiable polypeptide. Beyond suggesting an oligomeric nature to this major surface antigen, the authors did not show or describe the profile of the high-molecular-weight polypeptide. It is possible that Triton X-100 resulted in the aggregation of the high-molecular-weight polypeptides similar to our observations of the Sarkosyl-insoluble fractions of *T. denticola* GM-1 through 7.5% acrylamide SDS-PAGE (Fig. 4). Our observations do, however, indicate that both the 64-kDa major outer sheath protein of *T. denticola* GM-1 and the 58-kDa major outer sheath protein of *T. denticola* TD-4 originated from larger, heat-modifiable, oligomeric polypeptide structures. The presence of heat-modifiable outer sheath polypeptides has also been reported in *Spirochaeta aurantia* (43), in which the major outer sheath protein was a 90-kDa polypeptide which upon heating, migrated to 36.5 kDa. In addition to being smaller than the 64-kDa major outer sheath protein of *T. denticola* GM-1, the *S. aurantia* 36.5-kDa polypeptide was an acidic protein with a pI of 3.4 and displayed porin activity. The authors suggested that the 36.5-kDa polypeptide was in fact the porin monomeric unit. Since most of the pore-forming porin proteins have monomeric molecular masses ranging from 28 to 47 kDa as well as being very acidic (46, 56), it is more than likely that the *T. denticola* major outer sheath protein at 64 kDa (pI 6.7) is not a porin but functions in the adherence of *T. denticola* to HGFs.

A high-molecular-mass, disulfide-bonded oligomeric polypeptide, the 4D antigen, with a monomeric unit of 19.4 kDa, has also been described for *T. pallidum* (19, 77). However, as noted by others (15) and now corroborated by us, the high-molecular-weight oligomeric polypeptide of *T. denticola* does not appear to be maintained by disulfide bonds. We found no difference in migration of the ladderized polypeptide in the presence or absence of β -mercaptoethanol, and amino acid analyses, after protection of cysteine residues with 4-vinyl pyridine, did not detect any cysteine. Moreover, reducing agents did not result in the dissociation of the high-molecular-mass polypeptide to the 64-kDa region.

Functionally, several of the *T. denticola* outer sheath

proteins are probably involved in adherence to mammalian cells. Several reports suggest that host fibronectin functions to mediate adherence between some *Treponema* species and mammalian cells (6, 17, 61, 69, 70). Our previous results (78) indicate that fibronectin might be of importance in *T. denticola*-HGF interactions. Anti-human fibronectin resulted in significant inhibition of *T. denticola* TD-4 adherence to HGFs but not of strains GM-1 or MS25. *T. denticola* TD-4 appears to be more fibronectin dependent in its interaction with the HGFs than *T. denticola* GM-1 and MS25. Dawson and Ellen (17) have observed that pretreatment of *T. denticola* ATCC 33520 with soluble fibronectin enhanced attachment to fibronectin-coated coverslips instead of inhibiting it. We demonstrated that of the three *T. denticola* strains tested, only TD-4 increased its adherence to HGFs with the addition of soluble fibronectin (78). Interestingly, a major outer sheath antigen in the molecular mass range of 53 to 58 kDa in *T. denticola* ATCC 33520 has been demonstrated through fractionation by detergent extraction (37) or with polyclonal and monoclonal antibodies (75). Whether the 53- to 58-kDa polypeptide is similar to the 58-kDa polypeptide reported by us for *T. denticola* TD-4 as well as clinical isolate SR-4 is unclear. Since pretreatment of HGFs with low concentrations of trypsin resulted in specific inhibition of *T. denticola* TD-4 adherence to the HGFs (78), and since strains GM-1 and MS25 demonstrated increases in adherence to the HGFs of more than 50% after this proteolytic digestion, it is possible that cleavage of cell surface fibronectin by trypsin could have disrupted its RGD-binding domain (62, 63), resulting in diminished adherence of strain TD-4 to HGFs, while exposing the core terminal β -D-galactose residues (11, 26), as well as other cryptic cell surface receptors (30) for increased adherence of GM-1 and MS25. *T. denticola* strains which contain a 58-kDa major outer sheath protein might represent fibronectin-binding treponemes, while those with major outer sheath proteins other than 58 kDa (i.e., 64 kDa) might mediate adherence directly with carbohydrate receptors on host cell surfaces. However, we have no direct proof that this specific interaction occurs. Immunogold localization of the 64-kDa polypeptide in *T. denticola* GM-1 did demonstrate an even distribution of the anti-64-kDa protein IgG-gold label over the treponeme surface (Fig. 10A), with little to no labeling at the treponeme poles. Since both *T. pallidum* and *T. denticola* have been shown to bind by their tips (9, 14, 17, 31, 76), and since those strains of *T. denticola* which bind more avidly to fibronectin also bind with a greater percentage by tip orientation (17), it is possible that the treponemal adhesins with avidity for fibronectin are either clustered at the tips and/or migrate to the tips when encountering fibronectin. The fact that we found virtually no tip labeling using the anti-64-kDa polypeptide might indicate that this polypeptide is not located at the treponemal pole and is not fibronectin mediated in its adherence to HGFs. Moreover, when ³⁵S-labeled *T. denticola* GM-1 whole cells were solubilized by using 1% Zwittergent, the 64-kDa polypeptide was found not to bind to fibronectin-coated Sepharose beads (73a).

It is also possible that adherence of *T. denticola* GM-1 to host cells occurs through nonspecific hydrophobic interactions. Since the 64-kDa polypeptide is hydrophobic, adherence of *T. denticola* GM-1 to host cells may be mediated, in part, by these nonspecific forces. We are, however, uncertain as to where the hydrophobic domains reside in the intact 64-kDa polypeptide, or if they are part of the active sites for adherence.

There have been very few studies of the molecular genet-

ics of outer sheath proteins of the pathogenic treponemes. The 47-kDa major outer membrane immunogen of *T. pallidum* has been cloned and partially sequenced (35). Aside from similarity in location (39), N'-terminal blockage (35), and the characteristic migration as doublets on SDS-PAGE (57), there appear to be very few similarities of the 47-kDa *T. pallidum* polypeptide and the *T. denticola* 64-kDa polypeptide studied by us (12, 13). The 47-kDa *T. pallidum* antigen was never described as originating from higher-molecular-weight oligomeric units, and Southern hybridization has indicated the absence of a homologous 47-kDa immunogen gene in *T. denticola* (57).

Most recently, a gene encoding for a 53-kDa immunogenic protein of *T. denticola* Johnson was cloned and sequenced (54). A signal peptide sequence which was homologous to that of bacterial lipoprotein indicated that this protein was membrane bound. However, while the deduced amino acid sequence showed no homology with our sequence data for the 64-kDa protein of *T. denticola* GM-1, Southern hybridization analysis revealed that the gene was conserved in *T. denticola* E-21, a strain found to have a major outer membrane protein at 62 to 64 kDa (47). This suggests that while the 53-kDa membrane-associated protein is common to *T. denticola* strains, it is different from the 64-kDa protein.

Our preliminary results suggest that the 64-kDa major surface protein is glycosylated. This differs with the finding that the 47-kDa major outer membrane immunogen of *T. pallidum* is a proteolipid (12). Surface glycoproteins have been identified in prokaryotes (45, 53, 55), and some have been associated with adhesion and aggregation activity (25a, 45). Most recently, the major outer membrane protein of *Chlamydia trachomatis* was found to be glycosylated and able to adhere to HeLa cell components (68). However, since the precedent for glycoproteins in gram-negative microorganisms is weak, our preliminary finding, suggesting a carbohydrate association with the 64-kDa *T. denticola* major outer sheath protein, requires rigid gas-liquid chromatographic and mass spectroscopic analyses. If indeed a carbohydrate association is determined with this protein, it would be interesting to assess whether the glycosylated moiety is involved in the formation of a conformational epitope and/or in the active site of the adhesin.

The homology between a 16-amino-acid segment of the integrin alpha-subunit of human leukocyte adhesion glycoprotein p 150,9 (16) and the cyanogen bromide-generated 42-kDa fragment from the 64-kDa protein of *T. denticola* is interesting; however, lengthier sequence analysis are required before any firm associations between these two proteins can be made. At present, we are using monoclonal antibodies to the alpha-subunit segment to assess its ability to block *T. denticola* GM-1 adherence to HGFs.

The fact that the anti-64-kDa protein antibody from *T. denticola* GM-1 had a significant inhibitory effect on the adherence of *T. denticola* to HGFs suggests that the 64-kDa outer sheath polypeptide is functioning in attachment of *T. denticola* to HGFs.

Since Fab fragments were at least eight times more effective in the inhibition of *T. denticola* GM-1-HGF interactions than the IgG-enriched fraction, it is more than likely that a binding of the Fab fragment to the 64-kDa outer sheath polypeptide occurred rather than a steric inhibition of *T. denticola* GM-1-HGF interactions. However, since specific antibody directed against a structure covering much of the surface of an organism might, in itself, inhibit adherence, it will more than likely require genetic approaches to demonstrate that the gene expressing the 64-kDa protein does in

fact express an antigen which mediates adherence to host cells. Moreover, we still have no understanding of the in vivo role the 64-kDa protein may have in *T. denticola*-mediated oral infections.

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