Receptors for *Treponema pallidum* Attachment to the Surface and Matrix Proteins of Cultured Human Dermal Microvascular Endothelial Cells

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Pathogenicity of *Treponema pallidum* may depend upon the binding of *Treponema pallidum* to matrix proteins, especially to fibronectin. Infectious organism or cell to matrix interactions are mediated by a family of adhesion molecule receptors known as integrins. Once in the host, the pathogenic *Treponema pallidum* dum adheres to the vascular endothelium and readily penetrates surrounding tissues. Fibronectin plays an important role in the mediation of the attachment of *Treponema pallidum* to host cells, including endothelial cells. We found that the binding of *Treponema pallidum* to human dermal microvascular endothelial cells and to a glass surface coated with fibronectin is inhibited by the presence of arginine-glycine- aspartic acid (RGD), and analysis of the surface receptor revealed an antigenic similarity to an integrin molecule, namely $\alpha5$. This ability to adhere to host endothelium and fibronectin is quite unique to *T. pallidum* among the treponemes, and may be a key pathogenic factor.

Key Words: *T. pallidum*, RGD, $\alpha5$ integrin

INTRODUCTION

Numerous studies have suggested that the adherence of *Treponema pallidum* to host cells is an early event in the pathogenesis of syphilis, and that this is mediated by tip-like structures on virulent spirochetes.[1-5] The attachment of pathogenic

treponemes to cultured cells, and the failure of nonpathogenic treponemes to attach suggest that attachment is an important step in the treponemal pathogenesis. For *T. pallidum* to disseminate and cause systemic infection, it must first traverse the endothelial cells lining the vasculature. Indeed, spirochetes are often found on the outside of inflamed blood vessels.[6] The incubation of virulent *T. pallidum* with human umbilical vein endothelial cell (HUVEC) monolayers demonstrates that *T. pallidum* attaches to endothelial cells and traverses them by penetrating intercellular junctions, while non-pathogenic treponemes or heat-killed *T. pallidum* are incapable of either cytoadherence or monolayers penetration.[7,8] The observations that host fibronectin avidly binds to freshly extracted *T. pallidum* and that antibodies to fibronectin block treponemal attachment to cultured cells suggest that fibronectin might have a key role in the adherence of treponemes to host cells. In contrast, the avirulent Reiter treponeme fails to bind fibronectin and other plasma proteins, thus supporting the idea that host protein binding may be an exclusive property of virulent spirochetes.[2,3,9-12]

Treatment with the synthetic peptide gly-arg-gly-asp-ser-pro-cys (GRGDSPC), in which the active site sequence arg-gly-asp-ser (RGDS) is specifically competed with ^{125}I-labeled cell-binding domain, results in diminished *T. pallidum* attachment to HEp-2 and HT1080 cell monolayers. This suggests that *T. pallidum* cytoadherence requires recognition of the RGDS sequence that is also known to be important for eukaryotic cell-fibronectin binding,[13,14] thus proving its significance in

the life cycle of *T. pallidum*.

Integrins are heterodimeric, transmembrane cell adhesion receptors for fibronectin and other extracellular matrix molecules. However, little is known of integrin classes that affect the adherence of *T. pallidum* to endothelial cells.

In this study, we characterized the receptor proteins required for *T. pallidum* attachment to the surface and fibronectin of cultured human dermal microvascular endothelial cells (HDMECs) by using RGD peptide and monoclonal antibody to the integrins.

MATERIALS AND METHODS

Isolation and culture of HDMECs

HDMECs were isolated from human neonatal foreskins by trypsinization and Percoll (Sigma Chemical Co., St. Louis, MO, USA) gradient centrifugation, as described previously.[15] Cells were cultured in endothelial basal media (Clonetics Corps., San Diego, CA, USA) containing 5 ng/ml epidermal growth factor (Clonetics), 1 μg/ml hydrocortisone acetate (Sigma), 5×10^{-5} M dibutyryl cyclic AMP (Sigma), 2×10^{-9} M glutamine (Sigma), 100 U/ml penicillin (Gibco Laboratories, Grand Island, NY, USA), 100 μg/ml streptomycin (Gibco), 250 μg/ml amphotericin B (Sigma), and 30% human serum. The experiments were conducted with endothelial cells at passages 2-6.

Isolation and culture of treponemes

T. pallidum Nichols strain (CDC, Atlanta, GA, USA) was maintained and passaged by the intratesticular inoculation of adult male New Zealand White rabbits. Ten days after inoculation, the rabbits were euthanized and the testes were aseptically removed. Each pair of testes was minced with a sterile scissors and organisms were extracted using 10 ml of extraction medium per testis for 10 minutes on a rotary shaker. Heat-inactivated normal rabbit serum (Gibco) containing 10 ml of normal saline was used as an extraction medium. Gross debris and whole cells were removed from the extract by two centrifugations at 1,000 × g for 5 minutes, and then 8 ml

of the supernatant was gently overlayed onto a 16 ml cushion of 43% Percoll (Pharmacia Biotech., Uppsala, Sweden). In-situ density gradients were formed by centrifugation at 34,000 × g for 20 minutes at 4℃. Grossly visible bands were carefully removed with a Pasteur pipette. The supernatant was then discarded and the sedimented *T. pallidum* was gently aspirated from the top of the Percoll pellet. Percoll was removed by centrifugation at 100,000 × g for 1 hour at 4℃. Treponemes were counted and observed for morphologic integrity and motility by darkfield microscopy. Phagedenis biotype Reiter (CDC), refringens biotype Noguchi (ATCC, Rockville, MD, USA) and denticola biotype MRB (ATCC) were used as representative, nonpathogenic treponemes and were maintained and passaged in thioglycolate medium with 10% heat-inactivated normal rabbit serum at 37℃. For certain experiments, *T. pallidum* was inactivated by heating at 56℃ for 30 minutes.

Radiolabeling of the spirochetes

T. pallidum was intrinsically radio-labeled after being harvested from rabbit testes by a modification of the method described by Stamm and Bassford.[18] Treponemes were initially pelleted by centrifugation (14,000 g, 10 minutes) and then resuspended (10^9 spirochetes/ml) in minimum essential medium (without methionine, reducing agents or cycloheximide) containing 10% normal rabbit serum. *T. phagedenis* was labeled in spirolate broth containing 10% normal rabbit serum. Tran ^{35}S-label was added to a concentration of 100 μCi/ml, and the cultures were incubated for 14 h at 34℃ in an atmosphere of 4.5% O_2 and 5% CO_2. Labeled cultures were harvested by centrifugation (14,000 g, 10 minutes), washed once by centrifugation in RPMI with 15% FCS, and counted by darkfield microscopy. *T. pallidum* was consistently > 75% motile in the different experiments after these labeling and washing procedures. A known number of spirochetes were combined with scintillation fluid and counted to determine the specific activity of intrinsic labeling. Specific activities for labeling *T. pallidum* were found to be comparable with those reported by Stamm and Bassford.[16]

Inhibition assays for HDMEC - *T. pallidum* and fibronectin - *T. pallidum* adherences

Confirmation of an RGD dependent mechanism

A HDMEC monolayer or fibronectin was preincubated with RGD or RGE. HDMECs were placed in a flat, 96-well organ culture kit at a density of 4×10^4 cells per well, and cultivated for 48 hours to form a monolayer. Wells were reacted with RGD peptide (Sigma) at 0.1, 0.5, 1.0, 5.0 or 10.0μg/ml, or RGE peptide at 0.1, 0.5, 1.0, 5.0 or 10.0μg/ml as a positive control, or with culture media on ice for 1 hour.

A crop of 1×10^8 *T. pallidum* in 100μl of normal saline labeled with isotope ^{35}S was added to each well, and incubated, to allow adherence, for 4 hours in 37℃ BBL® Compy Pouch. *T. pallidum* adhering to HDMECs was dissolved in 1% Triton-X, and radioactivity was measured in supernatant using a beta-meter.

In the second experiment, 100μl of solution per well containing 2.5μg/ml fibronectin was added to the cell culture and allowed to spread over the glass surface for 2 hours at room temperature, then RGD, RGE or the control culture media was added to each well and adherence inhibition assays were performed.

Confirmation of the integrin receptor mechanism

In the same way as described above, another adherence inhibition experiment was performed by using mouse monoclonal antibodies against α 2, α3, α4, α5, αv, αIIb, β1, β3, and β6 integrins (Chemicon, Temecula, CA, USA), which are known ligands of fibronectin.

After reacting ^{35}S-labeled *T. pallidum* with monoclonal anti-integrin antibodies for 1 hour, the adherence experiment was carried out on a HDMEC monolayer in a flat 96-well HDMEC culture kit that also contained fibronectin. *T. pallidum* free of anti-integrin antibodies was used as a control to determine whether integrin cell adherence molecules are involved in *T. pallidum* adherence.

Analysis of the T. pallidum surface adherence molecule against HDMECs or fibronectin by ELISA

To investigate the specificity of the surface receptor against fibronectin, quantitative analysis of the total protein content was performed on *T.*

pallidum, T. phagedenis, T. denticola and *T. refringens* using a modification of the Lowry method.[17] Each strain was then reacted with fibronectin that was coated on the glass surface of a flat 96-well organ culture kit in the same procedure as preceding experiments, and then diluted anti-fibronectin antibody (Zymed Laboratories Inc., San Francisco, CA, USA) was added. The whole mixture was incubated for 1 hour at 37℃.

After being washed with HBSS three times, the mixture was reacted with peroxidase-conjugated goat anti-mouse IgG, diluted to a titer of 1 : 1,000 with HBSS containing 5% fetal calf serum for 1 hour at 37℃, and then once again re-washed with HBSS three times. The product obtained was allowed to react with stromal media (prepared as described below) in the dark at room temperature. Finally, 8N H_2SO_4 25μl was added to halt the reaction and the amount of surface protein was measured at 450 nm using an ELISA reader, to quantify the protein expression in reaction to fibronectin.

The stromal media was prepared as follows; a stock solution was made by dissolving 100 mg of tetramethylbenzidene in 10 ml of acetone. Distilled water 10 ml and 30% hydrogen peroxide 1 μl were then added to 100μl of the stock solution just before use, and 100 ml of the resultant solution was added to each well.

RESULTS

Survival rate of *T. pallidum* in culture

The number of *T. pallidum* harvested from rabbit testis was 2-8 × 10^8/ml of testis extract 10 days after inoculation. When *T. pallidum*, extracted by Percoll density gradient centrifugation, was cultivated with various growth cofactors in endothelial basal media containing 30% human AB serum in a microaerophilic conditions at 37℃, it showed 70% survival for up to 16 hours. (Data are not shown.)

Treponemal attachment to HDMECs or fibronectin

We analyzed the binding capacities of intrinsi-

cally radiolabeled treponemes, *T. pallidum*, heat-killed *T. pallidum*, or *T. phagedenis* to HDMEC monolayers. This experiment showed 11.23 ± 1.87% of *T. pallidum* binding to HDMECs. However, heat-killed *T. pallidum* and *T. phagedenis* showed negligible adhesion, at 0.53 ± 0.24%, and 0.23 ± 0.10%, respectively.

The adherence of pathogenic *T. pallidum* to fibronectin in the extracellular matrix was also examined. The percentage of *T. pallidum* binding to fibronectin was significantly higher than that of the other treponemes, for 3.70 ± 1.54% of the pathogenic *T. pallidum* attached to fibronectin, whereas the non-pathogenic *T. phagedenis* only showed an attachment rate of 0.72 ± 0.28%, and the heat-killed *T. pallidum* revealed negligible binding, at 0.18 ± 0.20% (Fig. 1).

T. pallidum attachment to fibronectin

After solubilizing the entire pool of treponemal surface proteins, ELISA was performed to detect the fibronectin receptor. Each strain was reacted with fibronectin in a 96-well organ culture kit, and was observed at 450 nm by measuring the optical density by ELISA using monoclonal antibody against fibronectin. Hardly any optical signal was detectable in nonpathogenic treponemes, and the surface protein regarded as a receptor for fibronectin was obtained only in the pathogenic strains of *T. pallidum* at an O.D. of 0.2020, whereas *T. phagedenis*, *T. denticola* and *T. refringens* had O.D. values of 0.0190, 0.0130 and 0.0110, respectively (Fig. 2).

Blockade of *T. pallidum*-HDMEC or *T. pallidum*-fibronectin attachment with RGD

In order to elucidate the adhesion mechanisms operative in *T. pallidum*-HDMEC and *T. pallidum*-fibronectin adhesion, we examined the effect of synthetic peptide RGD on *T. pallidum*-HDMEC and *T. pallidum*-fibronectin binding. The influences of RGD peptide against HDMEC- *T. pallidum* and fibronectin - *T. pallidum* adherence are shown in Figs. 3 and 4. While no significant inhibition by RGE was observed, RGD peptide clearly inhibited the adherence of *T. pallidum* to HDMEC, and adherence inhibition by RGD peptide was significant

at a concentration as low as 0.1 μg/ml of RGD peptide. Moreover, the degree of its inhibition increased in a dose-dependent fashion; the percentage of adherence was 11.24 ± 0.71% at baseline and fell to 1.6 ± 0.44% at 10 μg/ml (Fig. 3).

Regarding adherence to fibronectin, significant adherence inhibition was observed only after incubation with RGD peptide, and this occurred in a dose-dependent manner, starting at 1.0 μg/ml. The percentage of bound organisms was 3.06 ± 0.37% at baseline and declined to 1.99 ± 0.14% at 1.0 μg/ml, and 1.26 ± 0.37% at 10.0 μg/ml (Fig. 4). No such pattern was observed for RGE (Arg-Gly-Glu) peptide.
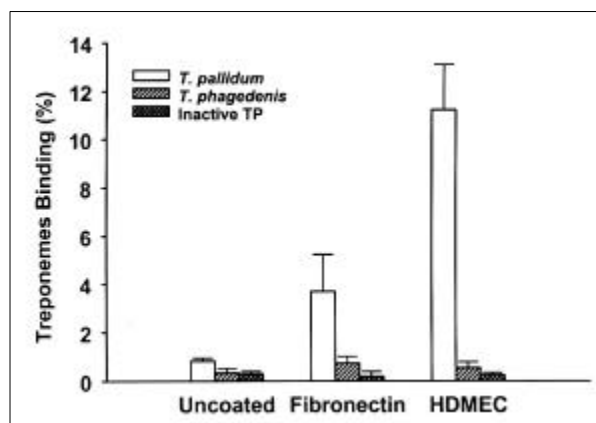

Fig. 1. The binding of intrinsically radiolabeled treponemes, to a HDMEC monolayer.
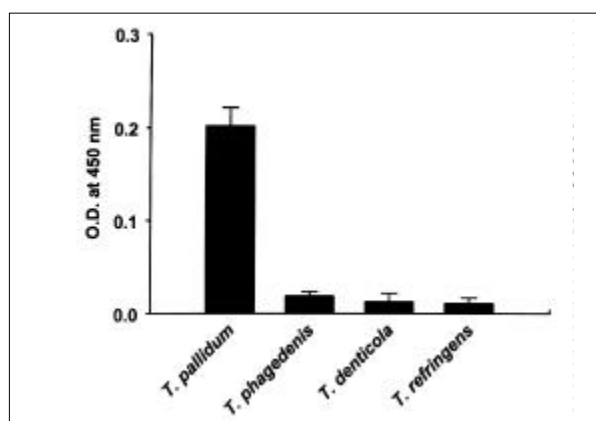

Fig. 2. Fibronectin affinity of the surface proteins of each treponemal strain Each treponemal strain that reacted with fibronectin was evaluated at 450nm by measuring optical density by ELISA using monoclonal antibody against fibronectin.

Influence of anti-integrin antibody against HDMEC - *T. pallidum* and fibronectin - *T. pallidum* adherence

While adherence inhibition was not shown by anti- $\alpha2$, $\alpha3$, $\alpha4$, αv, αIIb or $\beta3$, significant inhibition was shown by anti- $\alpha5$ integrin antibody with respect to the adherence of *T. pallidum* to HDMECs;

the lowest percentage of bound organisms was observed with $\alpha5$ at 4.70 ± 0.38%, as compared with a baseline value of 8.00 ± 0.57% (Fig. 5). And the same held true in the case of the adherence of *T. pallidum* to fibronectin; the lowest percentage of bound organisms was observed with $\alpha5$ at 0.98 ± 0.21%, as compared with a baseline value of 1.95 ± 0.64% (Fig. 6).
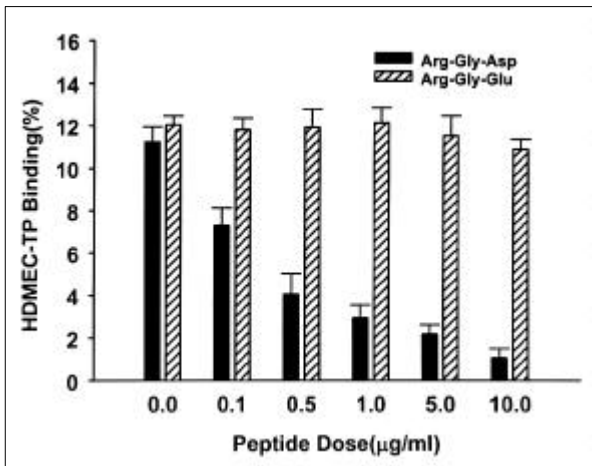

Fig. 3. The inhibition of the adherence of *T. pallidum* to HDMEC by RGD peptide After RGD peptide or RGE peptide had been added to a cultivated HDMEC monolayer for 1 hour, *T. pallidum* labeled with 35S was added, and an adherence assay was carried out over 4 hours at 37°C. TP: *T. pallidum*.
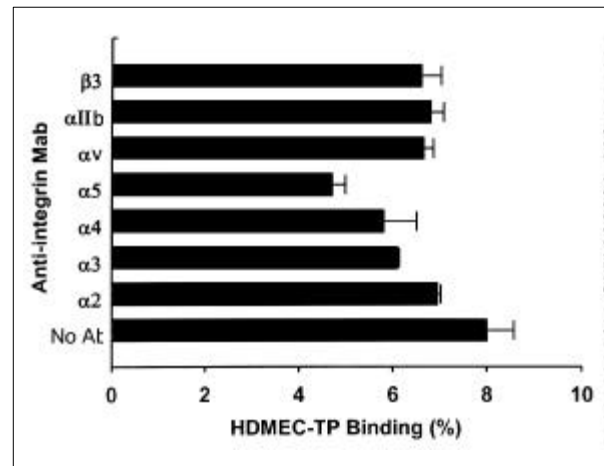

Fig. 5. The effects of anti-integrin antibodies on the adherence of *T. pallidum* to HDMECs Anti-integrin antibodies were incubated with the cultivated HDMEC monolayer for 1 hour and the *T. pallidum* labeled with 35S was added. The adherence assay was carried out for 4 hours at 37°C. TP : *T. pallidum*
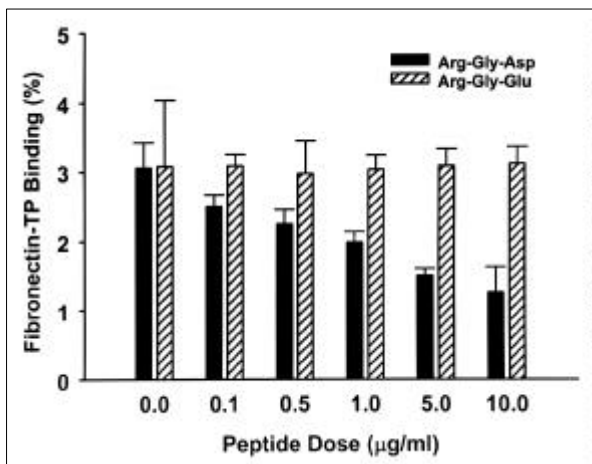

Fig. 4. The inhibition of the adherence of *T. pallidum* to fibronectin by RGD peptide After RGD peptide or RGE peptide had been added to a well coated with fibronectin for 1 hour, *T. pallidum* labeled with 35S was added and an adherence assay was conducted over 4 hours at 37°C. TP: *T. pallidum*.
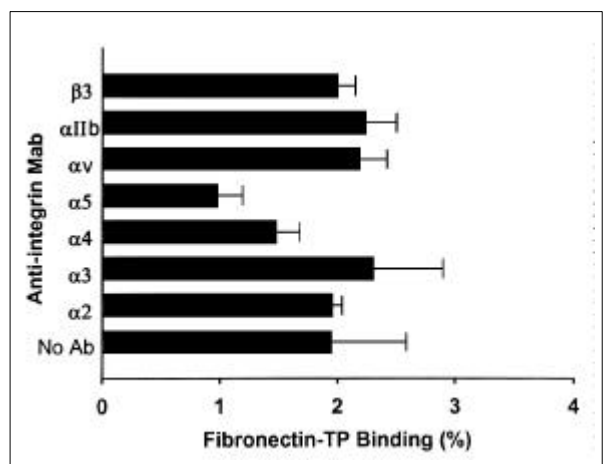

Fig. 6. The effects of anti-integrin antibodies on the adherence of *T. pallidum* to a fibronectin layer Anti-integrin antibodies were incubated with fibronectin coated on glass for 1 hour and then *T. pallidum* with 35S was added. The adherence assay was carried out for 4 hours at 37°C. TP : *T. pallidum*.

DISCUSSION

T. pallidum is known to attach to monolayers of HUVECs and to traverse them by penetrating intercellular junctions.[18] Moreover, our previous experiences with HDMECs[19] are consistent with the present results. While adherence and penetration on the surface of HDMECs and adherence to fibronectin molecules were not observed either by nonpathogenic spirochetes or by heat-inactivated *T. pallidum*, adherences to both were demonstrated by the pathogenic *T. pallidum*. Pathogenicity and viability seem to play an important role in the pathogenesis of syphilis. The requirement for viability and pathogenicity has been noted especially with respect to attachment and penetration.

Previous studies on the attachment of *T. pallidum* to host cells have shown that only a relatively small percentage show cytoadherence.[18,20-25] Most studies have examined HUVECs in this context. The percentage of *T. pallidum* that eventually succeeded in attachment to HUVECs was low at only 3.3%; once attached, 70-80% of them traversed the endothelial monolayer 6 h after incubation. Microvascular endothelial cells are phenotypically and functionally distinct from large vessel endothelial cells.[26,27] In the present study, we used cultured HDMECs obtained from human neonatal foreskins to simulate the living conditions of *T. pallidum*, i.e. to provide mucocutaneous surfaces. In our experiments using a HDMEC model, 10-13% of *T. pallidum* bound to the HDMEC monolayer after 4 hours of incubation and 9.7% of attached *T. pallidum* were found to traverse the HDMEC monolayer. As previously mentioned, our results also show nonpathogenic *T. phagedenis* and 56℃ heat-inactivated *T. pallidum* neither bind to HDMECs nor penetrate the HDMEC monolayer.

For *T. pallidum* to disseminate and cause systemic infection, it should first contact endothelial cells lining the vasculature. This direct interaction between *T. pallidum* and endothelial cells may be an important early event in the initiation of the host immune response to syphilitic infection. The direct interaction of *T. pallidum* with endothelial cells may activate endothelial cells to express a broad repertoire of molecules relevant to inflammation and immunity, including the cell adhesion molecules. Adhesion molecules allow cells to adhere to other cells or to extracellular matrix. This adhesion process is a prerequisite for cellular interaction and migration, and for adhesion to the endothelium and the stromal infiltration of *T. pallidum*. Moreover, inflammatory cells may also be key initiators of vascular damage in syphilis.[28]

Fibronectin, a major substance of the extracellular matrix, plays a variety of important roles, which include an involvement in cellular adherence. Fibronectin is found both in many biological fluids and on surface of eukaryotic cells, including fibroblasts and hepatocytes.[29,30] It has a domain that accommodates fibrin, heparin and Staphylococci, and another that accommodates collagen (gelatin), surfaces receptors on different kinds of cells, heparin and fibrin. It also contains domains for IgG, C3, thrombospondin, microbe, plasminogen and plasminogen activator, and a domain that allows connection with other fibronectin molecules.[31] *T. pallidum* adheres to fibronectin, and therefore can be blocked from adhering to the host cell and the surface of a fibronectin-coated glass with anti-fibronectin antibody pretreatment.[11,32]

Fibronectin may well be regarded as a determinant for pathogenicity as nonpathogenic strains do not bind fibronectin.[33] Antibodies to fibronectin were found to block treponemal attachment to cultured cells, thus suggesting the probable involvement of fibronectin in treponemal attachment.[8] In addition, antibodies to fibronectin blocked the attachment of treponemes to Hep2 cells, and fibronectin was found to react with three treponemal surface proteins previously implicated by Baseman and Hayes in the attachment process.[2]

In the present study, 3.3% of *T. pallidum* were observed to adhere to a fibronectin coated glass plate, and no conspicuous adherence was noted when the glass was coated with vitronectin or collagen. Thus, this adherence seems to be fibronectin-specific. In contrast, avirulent Reiter treponemes fail to bind fibronectin and other plasma proteins, reinforcing the idea that host protein binding may be an exclusive trait of the virulent strain of *T. pallidum*. Neither nonpathogenic *T. phagedenis* nor heat-inactivated *T. pallidum* bound to fibronectin-coated glass surfaces. These

findings suggest that the receptor enabling *T. pallidum*-fibronectin attachment may contribute to the pathogenicity of syphilis.

Fibronectin has at least two independent cell adhesion regions with different receptor specificities. The cell adhesive region in the central portion of the fibronectin molecule is comprised of at least two amino acid sequences - the RGD sequence and a Pro-Ser-Arg-Asm(PHSRN) sequence. The β1 integrins that can bind fibronectin include $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha v\beta1$ and fibronectin-specific integrin, which consists of an $\alpha5$ subunit and a $\beta1$ subunit, is the major fibronectin receptor on most cells.

In the present study, of all the antibodies directed against members of the integrin family that recognize the RGD sequence on fibronectin, only the anti-$\alpha5$ integrin antibody interfered with *T. pallidum* adherence when added to the media. Moreover, immunoblotting of treponemal surface proteins produced a band only after treatment with anti-$\alpha5$ integrin antibody. No fibronectin receptor was detected on the surface of nonpathogenic *T. pallidum*, *T. phagedenis*, *T. denticola* or *T. refringens*, but a receptor was found on the virulent strain of *T. pallidum*. Immunoblotting of *T. pallidum* confirmed the presence of surface proteins that interact with fibronectin. Given these results, we believe that the *T. pallidum* surface receptor requires the presence of RGD peptide for adherence to HDMEC or fibronectin and that the receptor is inhibited by anti-$\alpha5$ integrin antibody.

ACKNOWLEDGEMENTS

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