Parasitism by Virulent Treponema pallidum of Host Cell Surfaces

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The interaction between virulent Treponema pallidum extracted from infected rabbit testes and animal cells in culture was examined. The extent of treponemal attachment to monolayers of normal rabbit testicular and HEp-2 cells was dependent upon the incubation temperature and retained motility of the spirochetes. The specific orientation of treponemes to host cell surfaces was demonstrated by dark-field microscopic examination of wet-mount preparations and scanning and transmission electron microscopy. Once attached, T. pallidum organisms remained actively motile yet anchored in place by their terminal tapered structures. After several hours of co-incubation, maximal attachment was attained, and the degree of parasitism seemed regulated not only by available surface sites on individual host cells but also by the proposed membrane response of parasitized cells to continued exposure to treponemes. The avirulent strain, Treponema phagedenis biotype Reiter, did not adhere to monolayer cultures. Characterization of host cell determinants that permitted surface colonization by T. pallidum was attempted. Also, properties of virulent treponemes that enabled surface parasitism were monitored by measuring the effects of enzymes, detergents, and metabolic inhibitors on the host-parasite interaction. Results reinforced the specific nature of the treponemal attachment mechanism. Furthermore, the ability of convalescent rabbit sera to reduce attachment of treponemes to host cells suggested that surface structures on T. pallidum could be masked or inactivated by host components, thus providing a potentially effective research approach for investigating the pathogenesis of syphilis and screening appropriate vaccine candidates.

Syphilis remains a complex and poorly understood disease. The inability to develop satisfactory in vitro methods for cultivation of the causative agent, Treponema pallidum, has hindered attempts to clarify virulence determinants of the parasite and prevent and interrupt the disease. During the past few years, however, several research approaches have focused on defining the metabolic capabilities of virulent treponemes (3, 4, 10, 25) and establishing experimental conditions that prolong their motility and virulence in vitro (11, 13, 30). It appears that co-incubation of treponemes with animal cells in culture under aerobic conditions is beneficial to the survival of these spirochetes. While pursuing this latter approach, we consistently observed, by dark-field microscopy of co-incubated preparations, a specific orientation of treponemes to cultured animal cells. Since the predilection of virulent bacteria to host cells is recognized as an initial and critical stage of infection in several model systems (1, 7, 8, 12, 16, 19), we examined this host-parasite interaction.

In this paper we demonstrate the unique polarity of attachment of virulent treponemes to animal cells in culture and indicate that the terminal structures on virulent T. pallidum represent specialized functional organelles that permit the initial surface colonization of host cells. In addition we examine specific properties of this host-T. pallidum interaction, describe possible mechanisms that explain the surface parasitism, and extend these observations to define the direction of future research.

MATERIALS AND METHODS

Bacteria. Virulent T. pallidum (Nichols strain) was stored in liquid nitrogen before intratesticular inoculation of rabbits at a density of 3 × 10⁸ to 5 × 10⁸ treponemes per testis (4). Infected animals were injected intramuscularly with 7 mg of cortisone acetate (Medwick Laboratories, Inc., Melrose Park, Ill.) per kg of body weight at 3 days postinfection and...
then daily until rabbits were sacrificed 1 to 2 days after the first detection of an orchitis. The avirulent Reiter strain, *Treponema phagedenis* biotype Reiter, was grown in Spirolate broth (BBL, Cockeysville, Md.) containing 10% inactivated normal rabbit serum. Cultures were incubated at 35°C in an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.).

Chemicals. Lipase was purchased from Calbi-ochem, La Jolla, Calif.; collagenase was from Worthington Biochemicals Corp., Freehold, N.J.; sodium deoxycholate and phytohemagglutinin P were from Difco Laboratories, Detroit, Mich.; pokeweed mitogen was from Grand Island Biological Co., Grand Island, N.Y.; concanavalin A was from Miles Laboratories, Inc., Elkhart, Ind.; and digitonin was from Harleco, Gibbstown, N.J. All other chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, Mo., or were of a chemically pure grade.

Animal cell cultures. Primary cultures of rabbit testicular cells were established from a normal rabbit by aseptically removing testes, which were then rinsed with Eagle minimum essential medium (MEM) and minced into 2- to 4-mm³ pieces. Tissue fragments were trypsinized (0.25% trypsin in MEM) for 10 min at room temperature three successive times to release the majority of the testicular cells. After centrifugation of the cell suspensions at 1,000 × g for 10 min, cells were resuspended in MEM supplemented with 10% fetal calf serum (FCS) and then seeded in 32-ounce (ca. 1-liter) glass bottles. Normal rabbit testicular cells (NRT) from passage 1 to 7 were used throughout this study.

An established cell line of human epithelial cells (HEp-2) was kindly provided by Edward Hayes, Duke University. Cultures were routinely maintained in Dulbecco-modified MEM (D-MEM) supplemented with 0.35% glucose, 10% tryptose phosphate (TP), and 10% FCS. Both cell types were grown at 37°C in an atmosphere of 5% CO₂ in air. Media changes were performed at 3-day intervals, and cells were passed when the monolayers reached confluence.

Extraction of virulent *T. pallidum*. Treponemes were extracted from infected rabbit testicular tissue as previously described (3) with the following modifications. The tissue fragments were shaken (frequency of 74 cycles/min, amplitude of 3 inches [7.62 cm]/stroke) under aerobic conditions in D-MEM supplemented with 0.35% glucose, 10% TP, 10% FCS, 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), cysteine at 0.8 mg per ml, and sodium thioglycolate at 0.7 mg per ml (D-MEM+S). After the initial 5 min the extraction product was discarded, the second and third 10-min extraction products were combined, and the majority of contaminating erythrocytes, spermatozoa, and cellular debris were removed by centrifuging twice at 500 × g for 5 min.

Co-incubation methods. Suspensions of NRT and HEp-2 cells were seeded at a density of 1 × 10⁶ to 2 × 10⁶ NRT or 5 × 10⁴ HEp-2 cells in Leighton tubes containing a single cover slip (9 by 35 mm). Cells were grown for 24 to 48 h in D-MEM supplemented with 0.35% glucose, 10% TP, and 10% FCS in an atmosphere of 5% CO₂ in air at 37°C. On the day of the experiment, cell monolayers had reached partial confluence and were preincubated at 33°C for several hours before use.

Suspensions of treponemes diluted in D-MEM+S to a final concentration of 3.5 × 10⁷ organisms per ml were added to animal cell cultures, and incubation was continued for 4 h at 33°C unless otherwise specified. To establish lower dilutions of these organisms for specific experiments, infected testicular extract was centrifuged at 18,000 × g for 15 min, and the supernatant was used as the diluent. In one series of experiments, treponemes were treated with various compounds at 37°C for 1 h and then centrifuged at 18,000 × g for 15 min. The pellet was then gently resuspended in fresh D-MEM+S before addition to animal cell cultures.

HEp-2 and NRT cell cultures were also exposed in vitro to the avirulent Reiter strain. Before co-incubation, Reiter cultures were centrifuged at 18,000 × g for 15 min, and the pellet was resuspended in D-MEM+S. Cell suspensions containing 7 × 10⁶ treponemes per ml were added to animal cell cultures grown aerobically or preincubated for 5 h under anaerobic conditions.

Bacterial cell counts. At specified times, cover slips were taken from Leighton tubes, rinsed twice in phosphate-buffered saline to remove nonadhering treponemes, drained, and placed on slides with the cell side facing downward. Infected-cell cultures were observed under dark-field microscopy using a 45 × objective. The degree of surface parasitism was measured by counting the number of treponemes attached to 20 individual animal cells of approximately the same size at different locations on duplicate cover slips.

Fluorescence microscopy. The standard fluorescent treponemal antibody absorption (FTA-ABS) test was kindly performed by Charles Pavia on heat-inactivated rabbit serum samples. Serum was obtained from rabbits infected with virulent *T. pallidum* for 14 to 135 days.

Scanning electron microscopy. Cover slip preparations of infected animal cell monolayers were fixed in 2.5% glutaraldehyde-2.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at 4°C. Samples were extensively washed in phosphate buffer at 4°C, dehydrated in ethanol, transferred through a gradient of Freon 113 in ethanol, and then critical-point-dried with Freon 13 in a Bomar SPC 50/EX apparatus. After the cell preparations were coated with gold in a Polaron sputter coater, specimens were viewed with an ETEC Autoscan electron microscope operating at 10 to 20 kV.

Transmission electron microscopy. After co-incubation of virulent *T. pallidum* with rabbit testicular cells for 2 h at 33°C, the culture fluid was removed and the monolayer was washed with phosphate-buffered saline. Sample fixation was accomplished by adding glutaraldehyde-paraformaldehyde as described in the previous section. Cells were then scraped from the cover slip and centrifuged. The cell pellet was resuspended in the same fixative for an additional 30 min at 4°C before postfixation in 1% OsO₄-Veronal acetate buffer (pH 7.3 for 90 min at
4°C, dehydration, and embedding in Epon. Thin sections were cut on a LKB-Huxley microtome, stained with uranyl acetate and lead citrate, and examined in an AEI-6B electron microscope.

RESULTS

Effect of temperature on T. pallidum adherence to animal cells. Several investigations have suggested that virulent T. pallidum organisms readily adhere to animal cells in culture (11, 30). Similar observations in our laboratory stimulated this investigation of the interaction of T. pallidum with animal cells in vitro under aerobic conditions. By examining cover-slip preparations stained with Richardson blue, we observed adherence of treponemes to numerous animal cell types. Since virulent T. pallidum multiplies in vivo in rabbit testes, cultures of normal rabbit testicular cells were routinely used for this study. HEp-2 cells were also included, since they are of human origin and represent an established epithelial cell line with differing morphology from rabbit testicular cells.

A simple, reliable, and reproducible method was employed to quantitate the degree of adherence of T. pallidum to animal cells and to simultaneously observe motility. Nonconfluent monolayer cultures of NRT and HEp-2 cells were established on cover slips in Leighton tubes before exposure to treponemes for 4 h. Cover slips were then processed for observation and counting by dark-field microscopy as described in Materials and Methods. As shown in Fig. 1, T. pallidum adheres to both cell types at temperatures ranging from 25 to 37°C, and attachment increases gradually as the temperature of incubation is raised. However, the extent of adherence was at least fourfold higher with NRT cells when compared with HEp-2 cells. Microscopic observations showed that treponemes adhered by one or both ends to greater than 95% of the NRT and HEp-2 cell populations and remained anchored, but actively motile, on the surfaces of the cells as well as in the culture fluid during the 4-h incubation. At 4°C, movement of treponemes decreased slightly and adherence to either cell type was impaired by more than 90%. When treponemes were heat-killed at 80°C or treated with 1% formaldehyde at 4°C for 15 min before co-incubation at 33°C (data not shown), a similar loss of adherence resulted, suggesting that metabolically active treponemes were required for the surface parasitism.

T. pallidum-host cell interactions as recorded by electron microscopy. A high-resolution view of the parasitized NRT cell surface was provided by scanning electron microscopy.

![Fig. 1. Effect of temperature on adherence of virulent T. pallidum to monolayers of rabbit testicular and HEp-2 cells. Animal cell cultures and extracted treponemes were preincubated separately for 2 h and 30 min, respectively, at the indicated temperatures before co-incubation. After 4 h of co-incubation, the degree of adherence of treponemes to animal cells was measured as described in Materials and Methods. Each point represents the mean of 40 determinations (± standard deviation). Symbols: □, HEp-2 cells; ■, rabbit testicular cells.](http://iai.asm.org/)

Uninfected NRT cells grown as monolayers appeared flat and elongated with a relatively smooth surface and few microvilli (Fig. 2A). Because of this unstructured host cell surface, low magnifications of infected cultures revealed numerous treponemes apparently attached to host cells by one or both terminal ends or by body spirals or waves (Fig. 2B). These various host-parasite associations are depicted in Fig. 2C, with treponemes appearing as spirals or fully extended structures. A higher magnification of a selected area demonstrates the tapered ends and spiral or extended shapes of T. pallidum (Fig. 2D). Close examination of wet-mount preparations before fixation indicated that all attached treponemes showed vigorous motility and that the majority of treponemes were anchored to host cell surfaces by their tapered ends. This observation is dramatically recorded in Fig. 3A, where treponemes are joined by one or both terminal ends to individual cells and can be seen bridging two adjacent host cell processes. This frequent occurrence in wet-mount samples permits close study of the
FIG. 2. Scanning electron micrographs. (A) Low magnification of a monolayer of uninfected rabbit testicular cells. Cells appear as smooth-surfaced, highly flattened structures (1 μm = 3.5 mm). (B) Low magnification of rabbit testicular cells exposed to 3.5 × 10^7 virulent T. pallidum per ml of medium for 4 h at 33°C. Preparations were then washed and fixed as described in Materials and Methods. Note the spiral or wave-shaped treponemes associated with host cell surfaces (1 μm = 1.6 mm). (C) Portion of a testicular cell monolayer 4 h postinfection. Treponemes appear as both wavy and fully extended structures (1 μm = 4 mm). (D) Higher magnification of a section of (C). Tapered ends of the treponemes are in close contact with the host cell surface (1 μm = 8 mm).
Fig. 3. Scanning electron micrographs. (A) Two parallel rabbit testicular cell processes with attached T. pallidum as described in the legend of Fig. 2B. Note the ability of single treponemes to associate by their terminal ends to host cell surfaces and to bridge the two adjacent testicular cells (1 μm = 3.5 mm). (B) Extended configuration of T. pallidum is observed while part of the organism is firmly attached to the host cell surface after 4 h of co-incubation (1 μm = 11.2 mm). (C) Rabbit testicular cell infected for 22 h with T. pallidum. Note the wavy shape of the treponemes and their attachment by tapered ends to the host cell surface. Treponemes remain actively motile while anchored to the testicular cell. Several microvilli appear as short distinct bodies (1 μm = 18 mm). (D) Attachment of several treponemes to testicular cell membranes 22 h post-infection. Note the orientation of the treponemes mediated by their tapered ends and apparent disk-like organelle (1 μm = 28 mm).
extraordinary movements of the extended *T. pallidum* organisms. A higher magnification of the animal cell surface discloses the unusual elasticity of the treponemes (Fig. 3B). Careful examination of Fig. 3C indicates that the tapered ends of *T. pallidum* appear to anchor the spirochete to the testicular cell membrane, whereas host cell contact by treponemal spirals or waves seems less critical for successful surface-surface interaction. This observation was reinforced by viewing many preparations under dark-field microscopy and documenting on videotape and 16-mm film the specific orientation and attachment of the majority of virulent treponemes by their tapered end structure, as seen in Fig. 3D.

A two-dimensional view of the association between *T. pallidum* and rabbit testicular cells was provided by transmission electron microscopy. In Fig. 4, the terminal end or nosepiece (36) of the darkly staining treponeme is observed in close proximity with the surface of the testicular cell. There is a right-angle orientation of the nosepiece to the host cell membrane, with the contact point of the treponeme being the terminal tip. The axial fibrils of *T. pallidum* are visible and terminate just posterior to the mesosome and nosepiece as described by Wiegand et al. (36).

Rate and extent of adherence of *T. pallidum* to rabbit cell monolayers. In an attempt to define the optimal conditions for attachment of *T. pallidum* to rabbit testicular cells, the rate and extent of this association were examined using different treponeme cell densities. As shown in Fig. 5, the number of treponemes attached to testicular cells increased with the size of the inoculum during a 2-h incubation. From 2 to 4 h, the level of adherence remained unchanged, and a slight decline was observed at 21 h. Maximal adherence occurred when testicular cells were initially exposed to $70 \times 10^6$ treponemes per ml, but treponemes on individual host cells were too numerous to count (greater than 35 treponemes per cell).

In general, adhering and nonadhering treponemes remained actively motile during the first 4 h of co-incubation. However, it appeared that treponemes adhering to NRT or to HEp-2 cells during prolonged co-incubation retained vigorous motility longer than organisms remaining in the culture fluid. Experiments were then designed to explain the absence of any additional attachment by actively motile treponemes that remained in the culture fluid after 2 h of co-incubation (Fig. 5). Treponemes at a concentration of $14 \times 10^6$ organisms per ml were initially added to monolayer cultures of testicular cells. After a 2-h attachment period, the culture fluid containing nonadhering treponemes was discarded and replaced with varying dilutions of fresh suspensions of *T. pallidum* organisms for 2 h more. As shown in Fig. 6, the extent of adherence remained unchanged and

**Fig. 4.** Transmission electron photomicrograph. Specific attachment of *T. pallidum* to rabbit testicular cell membrane via terminal organelle. Characteristic axial fibrils are seen (1 μm = 64 mm).
independent of the second inoculum size. An additive effect was not observed, as might be anticipated (expected values are represented by the solid bars). If these secondary suspensions of treponemes were added to uninfected cultures for the same incubation period, the degree of adherence was dose dependent, as previously recorded in Fig. 5. Furthermore, it was shown that 25 to 30% of the nonadhering motile treponemes remaining in the supernatant after a 2-h co-incubation still maintained the capacity to readily adhere to uninfected testicular cells. This observation was determined by transferring 2-h culture fluid containing nonadhering motile treponemes to uninfected monolayers and monitoring treponeme attachment, which paralleled data presented in Fig. 5 and 6. These results suggest that the membrane of the host cell is modified as a result of surface parasitism by virulent T. pallidum and at certain times postinfection, putative sites on the host cell membrane are no longer accessible to virulent treponemes.

Since virulent T. pallidum adhered to both NRT and HEp-2 cells, it was important to examine whether the avirulent strains possessed a similar capability. Reiter treponemes grown under anaerobic conditions were centrifuged and resuspended in D-MEM+S to a final density of 70 x 10^6 organisms per ml. Organisms were then added to NRT and HEp-2 cell cultures for co-incubation at 33°C for 2 and 18 h under aerobic conditions. No adherence of motile Reiter treponemes was observed (data not shown). Similar results were obtained when
Reiter treponemes were added to cell cultures preincubated for 5 h in an anaerobic hood and then co-incubated for 2 h under anaerobic conditions. Our findings are consistent with those of Fitzgerald and co-workers (11), who reported that *Treponema denticola* and *T. phagedenis* did not adhere to animal cells cultured in vitro.

Treatment of animal cells and its effect on adherence. Possible cell determinants required for the successful attachment of *T. pallidum* were investigated. Preliminary experiments were performed in an attempt to understand the biological function of host cells in this surface parasitism. Monolayer cultures of NRT and HEp-2 cells were irradiated with 7,000 to 8,000 rads of cobalt, which decreased deoxyribonucleic acid synthesis in the irradiated cells by 95% when compared with control cells, whereas synthesis of ribonucleic acid remained relatively unchanged. When *T. pallidum* was exposed to irradiated and nonirradiated NRT and HEp-2 cells for 4 h, no difference was noted in the extent of adherence. Similar results were obtained when NRT cells were exposed to 10 μg of concanavalin A per ml, phytohemagglutinin P, and pokeweed mitogen. After 2 h, treponemes and the same concentration of lectins were added to these cell cultures for an additional 4-h incubation before microscopic examination. Pretreatment of NRT monolayers with lectins or co-incubation in the presence of lectins did not reduce *T. pallidum* attachment. An average of 25 to 30 treponemes per animal cell was detected in all cases. No difference in adherence of treponemes was observed when HEp-2 cells were pretreated with the enzymes listed in Table 1 (excluding chymotrypsin and lysozyme) at similar or lower levels and shorter incubation times before co-incubation. Attempts to pretreat NRT cells with the same group of enzymes were unsuccessful due to the high sensitivity of these animal cells to the enzymes, which resulted in the sloughing of cells from the monolayer.

*T. pallidum* attachment to host cells after specific treatments. Virulent *T. pallidum* was exposed to a variety of enzymes and detergents in an attempt to identify bacterial surface components responsible for the surface parasitism. As shown in Table 1, treatment of treponemes with a range of enzymes at 37°C for 1 h did not alter the outer surface of *T. pallidum* sufficiently to prevent or decrease adherence to rabbit testicular cells. Similar results were obtained with HEp-2 cells.

| TABLE 1. Adherence of *T. pallidum* after treatment with detergents, enzymes, and metabolic inhibitors |
|----------------------------------------------------------|---------------------------------|
| Test substance*  | Concentration (μg/ml) | Treponemes per testicular cell (±SD)* |
| None            | 20 ± 2.3             |
| Sodium deoxycholate | 500 0                  |
|                | 200 15 ± 4.4         |
|                | 200 11 ± 2.4         |
| Digitonin      | 10 8 ± 2.2           |
|                | 1 18 ± 2.4           |
| Collagenase    | 1,000 22 ± 2.5       |
| Hyaluronidase  | 1,000 26 ± 2.9       |
| Lipase         | 50 22 ± 3.5          |
| Neuraminidase  | 530 21 ± 3.1         |
| Trypsin        | 100 20 ± 2.1         |
| Chymotrypsin   | 330 24 ± 3.5         |
| Lysozyme       | 500 19 ± 2.1         |
| Potassium cyanide | 650 3 ± 1.9        |
|                | 65 21 ± 3.3          |
| Sodium azide   | 650 21 ± 2.4         |
|                | 65 23 ± 2.4          |
| 2,4-Dinitrophenol | 20 20 ± 1.6      |
| p-Hydroxymercuribenzoate | 350 20 ± 2.3 |
| Disodium ethylenediaminetetraacetate | 370 22 ± 2.7 |

* Treponemes were treated for 1 h at 37°C before centrifugation except as indicated by footnote c.

b All treponemes that attached to animal cells demonstrated vigorous motility except in the case of potassium cyanide (650 μg/ml), where the few host cell-associated treponemes were nonmotile. SD, Standard deviation.

c Treponemes were treated for 2 h at 37°C.

d Treponemes were both extracted and treated with enzyme in serum-free culture medium. After treatment, trypsin activity was neutralized with trypsin inhibitor. Chymotrypsin-treated treponemes were centrifuged to remove the enzyme before co-incubation with testicular cells.

e Treponemes were extracted in calcium- and magnesium-free medium, treated with disodium ethylenediaminetetraacetate, and centrifuged before resuspension in the same medium and co-incubation with host cells. The number of treponemes per host cell in control calcium- and magnesium-free cultures was 24 ± 2.9.

Notice and adherence of *T. pallidum* to rabbit testicular cells, although intact treponemes could be observed. Adherence of actively motile treponemes was reduced by 50% after a 2-h treatment with 200 μg of sodium deoxycholate per ml, whereas treatment of treponemes with this concentration of sodium deoxycholate for a shorter period decreased attachment by 25%. Digitonin, at 10 μg per ml, reduced surface parasitism by 60%. In no case did these...
latter treatments result in lysis of the treponemes or loss of motility.

Incubation of treponemes with sodium azide, 2,4-dinitrophenol, p-hydroxymercuribenzoate, and low levels of potassium cyanide did not affect attachment. Adherence was inhibited by 85% after pretreatment of treponemes with 650 μg of potassium cyanide per ml. However, this higher concentration of cyanide was apparently toxic since decreased adherence was accompanied by loss of T. pallidum motility. No effect on T. pallidum adherence was noted when treponemes were treated with disodium ethylenediaminetetraacetate or maintained in a calcium- and magnesium-free medium, suggesting that adherence by T. pallidum was not dependent on divalent cations.

To examine the effect of in vitro incubation on T. pallidum attachment, suspensions of treponemes at a density of 35 × 10^6 organisms per ml were incubated aerobically at 33°C for 21 h in cell-free D-MEM + S medium. Treponemes were then added to monolayer cultures of NRT cells for a 2-h incubation. Adherence of motile treponemes was reduced by 75% as compared with treponemes added directly to animal cell monolayers shortly after extraction from testicular tissue.

Adherence of T. pallidum to the surfaces of animal cells appears to be a specific interaction between host cell membranes and the outer terminal surface of treponemes. To investigate treponeme affinity for additional substrates, extracted treponemes were mixed with previously washed and coated (35) diethylaminoethyl-Sephadex A-50 beads (Pharmacia, Upsala, Sweden), and with a series of diversely charged and chemically modified glass particles, a gift from Corning Glassworks. The diameter of the Sephadex beads after swelling ranged from 40 to 120 μm, whereas the size of the glass particles ranged from 5 to 10 μm and 74 to 125 μm. Treponemes remained actively motile in the presence of the beads or particles, but no attachment was observed by dark-field microscopy. Attempts to coat the Sephadex beads with 1% rabbit serum albumin and 10% calf serum to enhance adherence of T. pallidum were unsuccessful.

**Effect of convalescent rabbit serum on T. pallidum attachment.** To study the possible influence of antibody raised during virulent T. pallidum infection on the host-parasite surface interaction, serum samples were obtained from infected rabbits at different times of infection. Suspensions of treponemes exposed for 1 h to heat-inactivated sera were added to normal rabbit testicular cells for 4 h of co-incubation. Treponemes remained actively motile during the entire experiment. As shown in Table 2, reduced adherence to T. pallidum correlated directly with the level of antibody in the serum. Convalescent sera possessing FTA-ABS titers of 3,125 inhibited attachment by approximately 50% as compared with normal serum, whereas serum with lower FTA-ABS titers had little effect. The two serum samples with high FTA-ABS titers were obtained from rabbits infected with T. pallidum for 30 and 135 days.

**DISCUSSION**

The use of animal cells for prolonged survival, motility, and virulence of T. pallidum in vitro has been described (11, 13, 30). Fitzgerald and co-workers (11) and Sandok et al. (30) reported that T. pallidum adheres to several animal cell lines during in vitro incubation. In our laboratory we have attempted to define the optimal requirements for the interaction between virulent treponemes and cultured host cells. A simple assay using nonconfluent infected animal cell monolayers allowed quantitation, by dark-field microscopy, of T. pallidum attachment. We found that treponemes adhered to more than 95% of the NRT and HEp-2 cells and demonstrated increased affinity for cells of rabbit rather than human origin in contrast to the findings of Fitzgerald et al. (11). It does not seem unusual that treponemes extracted from infected rabbit testes might exhibit a greater predilection for cells derived from the same animal species and organ where they have been maintained. Furthermore, we observed a greater number of treponemes associated with individual NRT cells in our system using a comparable inoculum of treponemes. Possible differences might be explained by the method of counting or degree of confluence of monolayer cultures. Another consideration, however, is that T. pallidum may prefer fibroblasts (NRT) rather than epithelial (HEp-2) cells and fur-

<table>
<thead>
<tr>
<th>Test serum* (titer)</th>
<th>Treponemes per testicular cell (±SD)*</th>
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<tbody>
<tr>
<td>Normal FTA (&lt;5)</td>
<td>26 ± 3.8</td>
</tr>
<tr>
<td>Infected FTA (125)</td>
<td>24 ± 3.4</td>
</tr>
<tr>
<td>Infected FTA (625)</td>
<td>23 ± 1.8</td>
</tr>
<tr>
<td>Infected FTA (3,125)</td>
<td>14 ± 3.0</td>
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<tr>
<td>Infected FTA (3,125)</td>
<td>12 ± 3.0</td>
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* Treponemes were exposed to 25% heat-inactivated rabbit serum for 1 h at 33°C before co-incubation with rabbit testicular cells for 4 h at 33°C. Antibody levels in test sera were determined by the FTA-ABS test.

* SD, Standard deviation.
thermore, the former generally afford a larger surface area because of elongated cytoplasmic processes common to this cell type. It was not clear whether *T. pallidum* preferred certain cell types in the NRT monolayer. No obvious decrease in surface parasitism was evident when treponemes were co-incubated with late-passaged NRT cultures in which increased fibroblast growth was apparent. Whatever the case, using partially confluent monolayers in our study, most membrane surfaces of the animal cells were accessible and the orientation and extent of *T. pallidum* attachment could be readily viewed.

The degree of *T. pallidum* parasitism of host cells varied with the temperature of incubation. This observation indicated that the surface interaction was temperature dependent and influenced by the physiological state of host and parasite. Maximal attachment was reached at 37°C. Higher temperatures were detrimental to *T. pallidum* as evidenced by loss of motility, decreased metabolic function (4), and lack of attachment. At a temperature of 4°C, treponemes remained motile but exhibited reduced attachment capabilities. The shifting of parasitized cells from 33 to 4°C did not release treponemes from the host cell surface.

High-resolution electron microscopy, along with detailed examination of wet-mount preparations plus videotape and 16-mm film recordings, demonstrated the specific orientation of virulent *T. pallidum* via their terminal end structures to membrane surfaces of host cells. Once attached either by one or both tapered ends, treponemes remained actively motile but anchored to the parasitized cell. It was impressive to watch *T. pallidum* flex and undulate under these conditions. This unusual motility, along with the full-length extensions of the spirochete across the surface of individual NRT cells or between host cells, reinforces the previous reports by Sequiera (31) and Cox (9) describing *T. pallidum* as a flat structure capable of numerous wave planes of varying amplitude rather than a more stable spiraled or coiled body shape. It was also possible to observe certain treponemes, with one end firmly anchored, stretch across the host cell surface. In addition, some spirochetes appeared associated with the host cell membrane by their wavelike body rather than by the terminal structures, although the majority of treponemes were clearly secured by the latter. These tapered ends have been examined by several investigators and their distinct morphology is documented. Ovchinnikov and Deklertskij (27) reported that the two ends of the treponemes differed anatomically and might be involved in cell division.

Wiegand et al. (36) hypothesized that the nose-piece or terminal structure might be required for penetration by *T. pallidum* of tissue cells. Based upon our data, we submit that the terminal tapered structures of *T. pallidum* represent specialized organelles whose function is the attachment and subsequent surface parasitism and colonization of sensitive host cells. It is difficult to assess this activity in relation to the putative intracellular residence of *T. pallidum* after penetration or engulfment as proposed by others (2, 21, 33). Although frequent reports describe intracellular sightings of *T. pallidum*, there is no evidence to indicate that these treponemes are viable or metabolically active. We observed no substantial decrease in the number of treponemes that were intimately associated with host cell membrane surfaces during 21 h of incubation.

The interaction between virulent *T. pallidum* and cultured animal cells seems a complex process. Shortly after the initial surface parasitism, host cells appear refractory to specific challenge doses of *T. pallidum* (Fig. 6). Although no clear explanation for this host response is available, the fluidity of the host membrane and the functional relationship between surface receptors and microtubules and microfibrils (5, 26) might be considered. Once critical numbers of host cell surface sites are occupied, membrane movements might provoke clustering (Fig. 7a), masking (Fig. 7b), or modifying (Fig. 7c) of available attachment points for *T. pallidum*, as schematically represented in Fig. 7. Large initial doses of treponemes circumvent this "host response" by the early occupation of numerous specific membrane sites. It is also possible that these speculative events might explain the reduced attachment of treponemes to HEp-2 cells. These cells may possess either decreased numbers of receptor sites or an accelerated mechanism for modifying or masking potential targets necessary for *T. pallidum* attachment. This membrane surface response, like a type of negative cooperativity or surface refractoriness, could be considered a possible host defense mechanism mounted against specific parasitic attack. Taken together, these data suggest the existence of host cell surface determinants that regulate the degree of parasitism and may explain the ability of *T. pallidum* to associate with privileged tissue sites in the host for extended periods of time.

To further clarify the surface determinants on host cells, a variety of experimental conditions were employed either to modify the availability of membrane sites or alter host cell metabolism. No decrease in attachment of *T. pal-
lidum to host cell surfaces was apparent when animal cells were lethally irradiated or killed by heat or formaldehyde. Exposure of host cells to specific lectins was without effect, indicating that lectin-compatible membrane receptors on host surfaces, as well as on the terminal ends of T. pallidum, may not be involved in the parasitism. Furthermore, pretreatment of HEp-2 cells with selected enzymes did not impair T. pallidum attachment. Since NRT monolayers were highly sensitive to these enzymes, as judged by animal cell release from the cover slip, useful data were not obtained.

Results of studies concerning the treatment of T. pallidum with enzymes before co-incubation are difficult to interpret. Treponemes are known to be highly resistant to trypsin (32) and lysozyme (22) (found in testicular extracts). In addition, hyaluronic acid is present in high quantities in the testes of cortisone-treated rabbits infected with T. pallidum and apparently coats the spirochetes (34). Since the infected testicular extract also contains specific substrates for the enzymes tested in this study, characterization of the treponemal surface component(s) responsible for attachment is not readily possible under these experimental conditions. Nonetheless, several aspects of T. pallidum attachment could be evaluated. Pretreatment of treponemes with low concentrations of digitonin and deoxycholate had little effect on motility but significantly diminished the capacity of T. pallidum to parasitize host membranes. This selective action suggested that certain surface components might be more susceptible to detergent than other constituents of the outer layer or protoplasmic cylinder of T. pallidum. Johnson et al. (18) have shown that the outer coat of avirulent treponemes is dissociated by higher levels of surface-active agents. Exposure of treponemes to in vitro conditions such as excessive heat, formaldehyde, and cyanide, which produced loss of motility and metabolic activity, abolished attachment capabilities. On the other hand, retention of motility at 4°C was accompanied by a significant reduction in attachment, implicating the metabolic state of treponemes as an essential factor. Also, the extent of parasitism was dependent on the numbers of treponemes added initially to monolayer cultures.

The fact that motile T. pallidum could not adhere to a series of variably charged and chemically modified glass particles and Sephadex beads, some of which permitted attachment and growth of animal cells (unpublished data), signified that the interaction was not merely an electrostatic phenomenon but required the involvement of host cell determinants, as previously mentioned. Preincubation of treponemes with certain metabolic inhibitors did not influence attachment. However, incubation of T. pallidum in cell-free medium under aerobicosis for 24 h resulted in a decreased capacity to parasitize, with no obvious decline in motility. This latter point disclosed potential deficiencies in T. pallidum cultivation under these environmental conditions and reinforced previous reports that the virulence of T. pallidum decreases without loss of motility after in vitro incubation (11, 13). Also, it suggested that a labile component(s) might be associated with the attachment mechanism at the terminal end of virulent treponemes, and this factor was not regenerated under these in vitro conditions.

The observation that heat-inactivated convalescent rabbit serum can reduce surface parasitism by T. pallidum freshly extracted from infected testicular tissue is significant. It has been established that virulent T. pallidum organisms possess an outer protective layer and that this material allows treponemes to resist or avoid the immediate consequence of treponemal immobilizing and agglutinating antibodies. In detailed studies, Hardy and Nell (14, 15), Metzger et al. (22), and Kent and De Weerdt (20) documented the alterations necessary in T. pallidum before the effectiveness of certain antibodies was manifested. Other investigations including our own indicate that virulent treponemes are unusually resistant to specific enzymes and detergents, further supporting the concept of protective outer layers, as previously reported (6, 37).
A relevant interpretation of data from Table 2 is that specific molecules, presumably immunoglobulins, in convalescent sera can prevent surface parasitism without causing lysis, immobilization, or agglutination of treponemes. This "antibody" apparently reacts quickly and avidly with freshly harvested and virulent treponemes. Whether this antibody is similar to globulins involved in the FTA-ABS, agglutination, and immobilization tests is unknown. However, the fact that this particular serum component prevents the host-parasite interaction at the earliest possible stage of parasitism is important conceptually in the development of a protective and rational injectable or topical vaccine. Conceivably, specific surface antigenic moities responsible for attachment could be isolated from the terminal tip organelle. Antibody raised against these components could then bind to T. pallidum and sterically prevent the host-parasite surface interaction. This approach would also avoid the potential difficulties and serious side-effects of administering complex vaccines, as employed in past studies (17, 23, 24). Furthermore, this research direction might circumvent possible detrimental effects on cell-mediated immunity which we have observed during experimental T. pallidum infection of rabbits (28, 29) and which might arise during large-scale trials in man with chemically and antigenically complex T. pallidum vaccines.

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