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Treponema pallidum invades intercellular junctions of endothelial cell monolayers

(bacterial virulence/syphilis/adherence)

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ABSTRACT **The pathogenesis of syphilis reflects invasive properties of *Treponema pallidum*, but the actual mode of tissue invasion is unknown. We have found two *in vitro* parallels of treponemal invasiveness. We tested whether motile *T. pallidum* could invade host cells by determining the fate of radiolabeled motile organisms added to a HeLa cell monolayer; 26% of treponemes associated with the monolayer in a trypsin-resistant niche, presumably between the monolayer and the surface to which it adhered, but did not attain intracellularity. Attachment of *T. pallidum* to cultured human and rabbit aortic and human umbilical vein endothelial cells was 2-fold greater than to HeLa cells. We added *T. pallidum* to aortic endothelial cells grown on membrane filters under conditions in which tight intercellular junctions had formed. *T. pallidum* was able to pass through the endothelial cell monolayers without altering tight junctions, as measured by electrical resistance. In contrast, heat-killed *T. pallidum* and the nonpathogen *Treponema phagedenis* biotype Reiter failed to penetrate the monolayer. Transmission electron micrographs of sections of the monolayer showed *T. pallidum* in intercellular junctions. Our *in vitro* observations suggest that these highly motile spirochetes may leave the circulation by invading the junctions between endothelial cells.**

The mechanisms by which *Treponema pallidum* subsp. *pallidum* causes the diverse clinical manifestations of syphilis and establishes latent infection are not well understood. There has been considerable interest in the ability of *T. pallidum* to adhere to a variety of cell lines and extracellular matrix components *in vitro* (1–9). Fibronectin-binding proteins of *T. pallidum* have been related to adherence to host cells (10–12).

The clinical and pathological manifestations of syphilis reflect the hematogenous dissemination of *T. pallidum* and its ability to enter certain tissues such as the cardiovascular system and the central nervous system (13, 14). Evidence was presented in the 1930s to suggest that *T. pallidum* enters the bloodstream within 5 min after intratesticular inoculation (15) and that the organisms disseminate into deeper tissues within 3 hr after application directly onto mucous membrane surfaces (16). Infection with *T. pallidum* appears to be principally extracellular, although there have been reports of occasional intracellular organisms in biopsied chancres and infected rabbit testis (17–19) as well as in tissue culture systems (3). Whether intracellularity represents a significant aspect of the pathogenesis of syphilis or its natural history is unknown. A systematic quantitative examination of the ability of *T. pallidum* to attain an intracellular location has not previously been reported.

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We studied the invasiveness of *T. pallidum in vitro* by using a system in which human and rabbit endothelial cells form tight intercellular junctions on the surface of a membrane filter. Virulent *T. pallidum* readily penetrated the monolayer, in contrast to heat-killed *T. pallidum* and the nonpathogen *Treponema phagedenis* biotype Reiter (TpR). Transmission electron microscopy showed treponemes in intercellular junctions, implying that interjunctional invasion was the mode of penetration of the monolayer. We believe that certain aspects of the pathogenesis of syphilis can be explained if *T. pallidum* has specific mechanisms for entering and exiting blood vessels at the endothelial cell level.

MATERIALS AND METHODS

Bacteria. Virulent *T. pallidum* subsp. *pallidum* Nichols strain (*T. pallidum*) was maintained in and harvested from New Zealand White male rabbits as described (3, 10, 20). An extraction medium was used that differed from that of Stamm and Bassford (21) in that it contained 10% heat-inactivated normal rabbit serum, 2 mM amino acids except for 10 mM cysteine and methionine, 0.02% cocarboxylase, 0.04% sodium bisulfite, and 0.03% dithiothreitol. Extracted organisms were separated from host debris by centrifugation twice at 1000 × *g* for 10 min (3). TpR was grown at 34°C in Spirolate broth (Baltimore Biological Laboratory) supplemented with 10% heat-inactivated normal rabbit serum. Three-to 4-day-old (midlogarithmic phase) cultures were used in the experiments.

Intrinsic Radiolabeling of *T. pallidum*. Treponemes were incubated at a concentration of 1.5×10^8 to 2×10^9 organisms per ml for 16 hr at 34°C in a sterile screw-cap bottle in extraction medium containing cycloheximide (100 μg/ml), cysteine (1 mg/ml), and [^{35}S]methionine (10 μCi/ml; specific activity, >800 Ci/mmol; 1 Ci = 37 GBq; Amersham). In some experiments, TpR was labeled with [^{35}S]methionine (200 μCi/ml) for 6 hr. Prior to use in experiments, ^{35}S-labeled treponemes were centrifuged at 17,000 × *g* and washed once in extraction medium without radioisotope or cycloheximide. After centrifugation, >90% of the organisms were motile.

Cell Lines. Rabbit aortic endothelial cells (RAECs), human aortic endothelial cells (HAECs), and human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion, identified as endothelial, and propagated as described (22). RAECs were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 15% fetal bovine serum. HAECs and HUVECs were grown in medium 199 (GIBCO)

Abbreviations: TpR, *Treponema phagedenis* biotype Reiter; RAEC, rabbit aortic endothelial cell; HAEC, human aortic endothelial cell; TEER, transendothelial electrical resistance.

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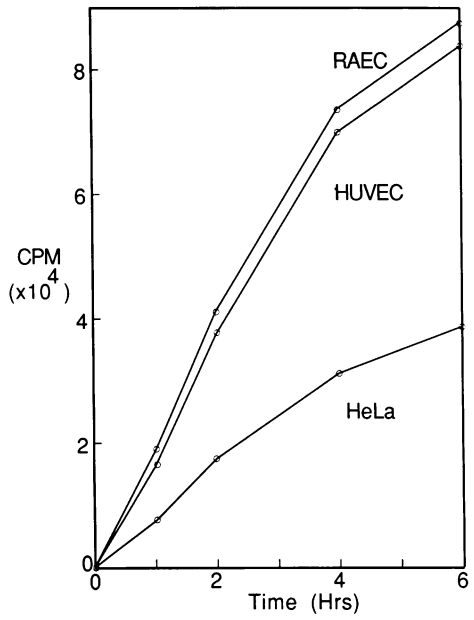

FIG. 1. Time course of ^{35}S-labeled *T. pallidum* association with endothelial and epithelial cell monolayers. A 1.0-ml suspension containing 7×10^7 ^{35}S-labeled treponemes (6.9×10^5 cpm) was added to RAEC, human umbilical vein endothelial cell (HUVEC), and HeLa cell monolayers. After incubation at 37°C, each monolayer was washed three times and disrupted with 0.5% $NaDodSO_4$, and then radioactivity was measured. Data indicated represent mean of three independent determinations performed in triplicate. For each data point, SD was <2.5% of the value.

containing 20% fetal bovine serum. RAECs from passage 7 and HAECs from passage 4 were used in these studies. HeLa cells were grown in minimum essential medium supplemented with 10% fetal bovine serum. All cell lines were maintained at 37°C in a CO_2 incubator.

Attachment Assays. Cells were seeded in 24-well plastic plates (Fisher) at a density of 5×10^5 cells per well and were used at confluency. ^{35}S-labeled motile *T. pallidum* were resuspended in the medium appropriate to the cell type to a concentration of $5–7 \times 10^7$ organisms per ml. Wells were rinsed once with 1 ml of fresh medium per well. One-milliliter suspensions of treponemes were added to each well, and the plates were incubated at 37°C. After incubation, each well was rinsed three times with medium. Monolayers with attached treponemes were then solubilized with 0.5% $NaDodSO_4$. Solubilized monolayers were mixed with Aquasol (New England Nuclear) and assayed by liquid scintillation counting.

Table 1. Resistance of cell-associated *T. pallidum* to trypsin treatment

Sample	Time of incubation, hr	Recovered cpm	
		Before trypsin*	After trypsin†
Motile *T. pallidum*	3	23,217 ± 2503	6277 ± 1090
Heated *T. pallidum*	3	791 ± 64	102 ± 86
Motile *T. pallidum*	6	36,383 ± 1584	9416 ± 235
Heated *T. pallidum*	6	2,780 ± 206	266 ± 102

A 1.0-ml suspension of 7×10^7 ^{35}S-labeled *T. pallidum* (7.0×10^5 cpm) was added to each HeLa cell monolayer and incubated at 37°C for the indicated period. Heated *T. pallidum* was incubated in a 56°C water bath for 30 min prior to addition to monolayers. Data represent mean ± 1 SD of six determinations.

*Treponemes associated with HeLa monolayers.

†Treponemes associated with monolayers after trypsin treatment (0.08% for 15 min at 37°C). See text for details.

Table 2. Penetration of aortic endothelial cell monolayers by *T. pallidum*

Organism	Time, hr	Barrier	Organisms below filter*	% below filter†
T. pallidum	2	F + EC	1.5×10^7	3.0
TpR	2	F + EC	0	0.0
Heated *T. pallidum*	2	F + EC	0	0.0
T. pallidum	2	F	2.8×10^7	5.6
TpR	2	F	2.4×10^7	4.8
T. pallidum	4	F + EC	2.7×10^7	5.4
TpR	4	F + EC	3.0×10^6	0.6
Heated *T. pallidum*	4	F + EC	0	0.0
T. pallidum	4	F	4.0×10^7	8.0
TpR	4	F	3.9×10^7	7.8
T. pallidum	6	F + EC	3.6×10^7	7.2
TpR	6	F + EC	6.0×10^6	1.2
Heated *T. pallidum*	6	F + EC	0	0.0
T. pallidum	6	F	5.3×10^7	10.6
TpR	6	F	4.8×10^7	9.6

A suspension containing 5.0×10^8 motile *T. pallidum* or TpR was placed on RAEC monolayers (EC) grown on 5-μm polycarbonate filters (F). Controls were filter samples without host monolayers.

*Organisms were quantitated by dark-field microscopy (23). Data represent the mean of three separate experiments. Each determination was performed in triplicate. For each data point, the SD was <5% of the value except for the 2-hr time point of *T. pallidum* invading RAEC monolayers, where the SD was 9%.

†Percentage of added organisms that passed to underside of filter.

Trypsin Treatment of Monolayers. Some monolayers with adherent *T. pallidum* were treated with 0.08% trypsin in 0.14 M NaCl at 37°C for 15 min, washed three times with medium, and then treated with 0.25% trypsin for 5 min at 37°C to remove cells from the plastic, or they were solubilized for counting. Trypsin-treated cells were centrifuged 5 min at 500 × *g* to separate host cells from free treponemes. Pellet and supernatant samples were mixed with Aquasol and assayed by liquid scintillation counting.

Endothelial Cell Monolayer Penetration Assay. RAECs or HAECs (5×10^4) were seeded on polycarbonate filters (5 μm; diameter, 13 mm; Nuclepore no. 110413) pretreated with gelatin (22). The filters had been mounted on plastic chemotaxis chambers (PC-2, ADAPS, Dedham, MA), gas sterilized, and placed in 24-well plates or 6-well plates (Fisher) with an adaptor prior to seeding with cells. At confluence, transendothelial electrical resistance (TEER) was measured by a modification of the method of Navab *et al.* (22). Only monolayers with a TEER of ≥13 Ω·cm^2 (indicative of peak formation of tight junctions) were used. In addition, the integrity of the cells was measured before and after experiments by trypan blue exclusion.

Table 3. Specificity of endothelial cell monolayer penetration by *T. pallidum*

Sample	Organisms below filter*	% below filter†	cpm
T. pallidum	3.4×10^7	6.8	NA
TpR	4.2×10^6	0.8	4.1×10^4
T. pallidum + TpR	3.5×10^7	7.0	3.2×10^4

Suspensions containing 5×10^8 motile *T. pallidum* or ^{35}S-labeled motile TpR (4.9×10^6 cpm per 5×10^8 organisms) or both were added to RAEC monolayers on 5-μm polycarbonate filters. Treponemes were incubated on monolayers for 6 hr at 37°C. NA, not applicable.

*Organisms were quantitated by dark-field microscopy (23). Data represent the mean of three separate experiments. Each determination was performed in triplicate. For each data point, the SD was <5% of the value.

†Percentage of added organisms that passed to underside of filter.

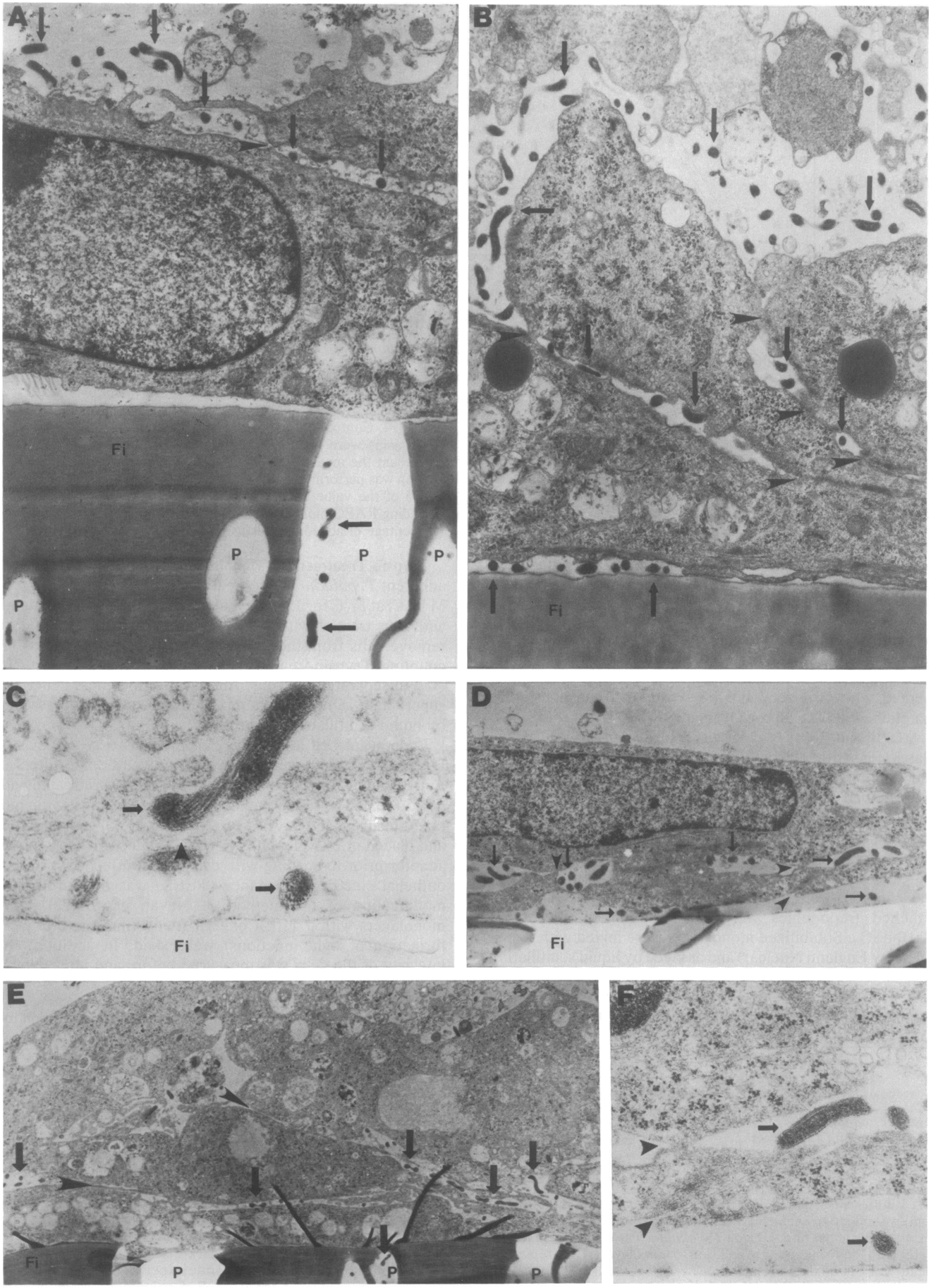

FIG. 2. *(Legend appears at the bottom of the opposite page.)*

Freshly extracted *T. pallidum*, intrinsically radiolabeled motile *T. pallidum*, and motile TpR were centrifuged at 17,000 × *g* for 10 min and resuspended in the appropriate medium for the endothelial cell type to a density of 1×10^9 organisms per ml. Aliquots (500 μl) were added to endothelial cell monolayers on polycarbonate filters in ADAPS chambers. A total volume of 1.5 ml of medium was present in the well below the chamber. Tissue culture plates containing ADAPS chambers were incubated in 5% CO_2/95% air at 37°C. At various time points, aliquots of medium from beneath the filters were observed by dark-field microscopy, at which time organisms were counted and motility was assessed. Enumeration of *T. pallidum* by dark-field microscopy was performed as described by Miller (23). In experiments with radiolabeled TpR, samples of the lower chamber medium were assayed by liquid scintillation counting. Heated organisms (56°C for 30 min) served as a control for each experiment. In some experiments, TEER was measured after incubation of treponemes with cells for 6 hr.

Electron Microscopy. Chambers were incubated in the presence of 5×10^8 *T. pallidum* or TpR as described above. The monolayers were then rinsed three times with medium 199 at 37°C and were fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C, postfixed with 1% osmium tetroxide in PBS, dehydrated in ethanol, and Spurr embedded. Sections were cut on a Sorvall MT 6000 microtome, stained with uranyl acetate and lead acetate, and examined with a JEOL JEM-100CX electron microscope at 80 kV.

RESULTS

Time Course of Treponeme–Host-Cell Association. As shown in Fig. 1, *T. pallidum* association with endothelial and epithelial cell monolayers was approximately linear over the 6 hr studied. The number of host-cell-associated treponemes was at least 2 times greater for endothelial cells than for HeLa (epithelial) cells over the time course examined. Based on the specific activity of the labeled treponemes, 9–12 *T. pallidum* were bound to each HeLa cell.

Demonstration of Trypsin-Resistant *T. pallidum*–Host-Cell Association. Intrinsically labeled motile treponemes were incubated with HeLa cell monolayers for 3–6 hr. Treatment with 0.08% trypsin for 15 min at 37°C was sufficient to remove all attached treponemes (>100 per high power field, ×600 magnification), as visualized by dark-field microscopy, but did not disrupt the cell monolayer. As shown in Table 1, we found that ≈26% of the *T. pallidum* counts that had become cell-associated by 3–6 hr remained with HeLa monolayers after the mild trypsin treatment. Heated *T. pallidum* associated only minimally with host cells. Endothelial cells were more sensitive to trypsin treatment than the HeLa cells, and conditions could not be found that released attached treponemes without release of the monolayer (data not shown).

Nature of Trypsin-Resistant *T. pallidum*–Host-Cell Association. The fate of treponemes associated with HeLa cell monolayers was determined by adding a 1.0-ml suspension of 7×10^7 ^{35}S-labeled *T. pallidum* (6.8×10^5 cpm) to each HeLa cell monolayer. The following data represent the mean ± 1 SD of six determinations. After incubation for 3 hr at 37°C, 22,857 ± 1991 cpm had become cell-associated without trypsin treatment. After treatment with 0.08% trypsin, 6160 ± 815 cpm, or ≈26%, of treponemes remained cell-associated. Next, HeLa cells with remaining treponemes were released from the plastic by using 0.25% trypsin, centrifuged to pellet the HeLa cells, yielding 1134 ± 184 cpm (18%) in the pellet and 5181 ± 317 cpm (82%) in the supernatant. These data are consistent with the idea that these organisms had not been trypsin resistant on the basis of an intracellular location but had instead found a trypsin-resistant niche between the HeLa cells and their underlying plastic support.

Penetration of Endothelial Cell Monolayers. As shown in Table 2, both motile *T. pallidum* and motile TpR crossed the 5-μm pores of the polycarbonate filters in the absence of endothelial cell monolayers. However, in the presence of rabbit endothelial cell monolayers with tight junctions (as determined by electrical resistance), motile *T. pallidum* crossed the filters much more efficiently than TpR. By 6 hr, 7.2% of *T. pallidum* had traversed the monolayers, compared to only 1.2% for TpR. No organisms were observed below the filters in the heated *T. pallidum* samples. All treponemes observed below the filters were motile. Similar results were obtained in identical experiments with human aortic endothelial cell monolayers (data not shown). More than 99% of the endothelial cells were viable after the 6-hr exposure to treponemes, as judged by trypan blue exclusion. When intrinsically labeled TpR and unlabeled *T. pallidum* were mixed and added to an endothelial monolayer, >99% of the radioactive counts were retained above the filter (Table 3), indicating that the much greater ability of *T. pallidum* to penetrate the monolayer was a specific property of the motile organism and did not reflect a toxic effect on the monolayer that could have led to the penetration of TpR. In addition, the endothelial cell monolayers were not disrupted because TEER had not diminished in the presence of *T. pallidum*, TpR, or medium alone (data not shown).

Demonstration of *T. pallidum* in Intercellular Junctions. Fig. 2 shows representative electron micrographs of sections of the endothelial monolayer taken 2–6 hr after addition of *T. pallidum*. We systematically studied the first 15 intercellular junctions that were free of wrinkles (Fig. 2*E*) or other artifacts that complicated visualization and found treponemes in 7 of 15 (47%). Intercellular junctions appeared to retain integrity on both sides of the invading organisms (Fig. 2 *A*, *B*, and *D–F*). Presumably, adherent treponemes were found on the apical cell surface (Fig. 2*B*). In general, *T. pallidum* were more numerous between the endothelial cells and the underlying filter (Fig. 2 *B–D*) than in intercellular junctions.

DISCUSSION

The purpose of this study was to look for *in vitro* parallels of *T. pallidum* dissemination, a central feature of the pathogenesis of syphilis. The means by which the organism proceeds from skin or mucous membrane to the circulation and from the circulation to tissue parenchyma are unknown. We first determined that *T. pallidum* binding to endothelial cells of rabbit and human origin was 2.0- to 2.2-fold greater than its binding to the epithelial cells studied in parallel. Although the number of cells used for the binding assays was carefully controlled, the data do not rule out the possibility that the

FIG. 2 *(on opposite page).* *T. pallidum* invading endothelial cell monolayers. Electron micrographs of transverse sections of rabbit (*A–C* and *E*) and human (*D* and *F*) aortic endothelial cell monolayers grown on polycarbonate filters (Fi) with *T. pallidum* (arrows) in intercellular junctions (arrowheads) and filter pores (P). (*A*) *T. pallidum* invading intercellular junction and passing through pore in filter below. (×11,950.) (*B*) *T. pallidum* between and below endothelial cells. (×11,950.) (*C*) High magnification view of *T. pallidum* invading intercellular junction. (×48,400.) (*D*) *T. pallidum* between and below endothelial cells. (×10,600.) Filter separated during sectioning. (*E*) Low magnification view of numerous *T. pallidum* passing between endothelial cells and through pores in filter below. (×4830.) Linear black marks represent wrinkles occasionally seen as a result of embedding process. (*F*) High power view of *D* showing an area of intercellular junction. (×28,800.)

differences in binding observed reflect differences in cell volume. However, the dimensions of HeLa and aortic endothelial cells in culture are very similar (24–26).

In our experiments, the percentage of bound *T. pallidum* was similar to that reported in other adherence studies using the same basic methodology (5, 8, 11, 12) and ranged from ≈1% to 12% over the 2- to 6-hr incubation periods. We found that counts representing ≈26% of the bound treponemes remained associated with the cell monolayer after mild trypsin treatment sufficient to remove all organisms visible by dark-field microscopy (Table 1). Further trypsin treatment released 82% of the cell-associated treponemes along with the HeLa cells from which they were separated by differential centrifugation. We postulate that these organisms had become resistant to the mild trypsin treatment by migrating between the HeLa cells and the underlying plastic dish. Presumably, this is the same process described by Cox and coworkers, who found that *T. pallidum* cultured *in vitro* for 12 days with Sf1Ep cells could be demonstrated between the cells and their underlying coverslips by electron microscopy (27). We observed *T. pallidum* beneath the endothelial cells and the underlying filter by electron microscopy (Fig. 2) and, therefore, did not conduct separate electron microscopic studies of *T. pallidum* exposed to HeLa cells grown on plastic.

The endothelial cell monolayers used in these studies to investigate treponemal penetration have been previously well characterized. Cultures of aortic endothelial cells on filters produce a confluent monolayer that retains many of the characteristics of *in vivo* endothelium. Cultured endothelial monolayers form gap and tight junctions, desmosomes, and pinocytic vesicles (22, 24). The magnitude of TEER of the monolayer used in the present study was similar to that seen across intact rabbit aortic endothelium (28). Cultured aortic endothelial cells exhibit apical–basal polarity (29, 30). Cultured endothelial monolayers have been used to study granulocyte diapedesis *in vitro* (31).

T. pallidum and the nonpathogenic treponeme TpR pass equally well through filters not containing cells (Table 2). Our conclusion that *T. pallidum* has the ability to pass through the monolayer is therefore dependent on retention of the integrity of the monolayer and its tight intercellular junctions, because damage to the monolayer could permit the passage of motile treponemes. We believe that the passage of *T. pallidum* through the endothelial cell monolayer was a specific and biologically relevant event for the following reasons: The extent of migration of *T. pallidum* through the monolayer was approximately an order of magnitude greater than that by TpR, and it was almost as great as its passage through the membrane in the absence of endothelial cells. When *T. pallidum* and TpR were mixed, the ability of TpR to traverse the monolayer was not enhanced, indicating that the mechanism of *T. pallidum* penetration did not involve nonspecific destruction of the monolayer. Trypan blue exclusion was also used to rule out damage to the monolayer and the TEER of monolayers was unaltered throughout the duration of the experiments.

Our observation by electron microscopy of numerous *T. pallidum* in the intercellular junctions of the endothelial monolayer strongly suggests that interjunctional invasion is the mode by which penetration of the monolayer occurred. After emerging from the intercellular junctions, *T. pallidum* must pass beneath the cells and then through the pores of the filter. Given that *T. pallidum* were more numerous beneath endothelial cells than in their intercellular junctions on electron microscopy, we hypothesize that the rate-limiting step in traversing the monolayer–filter barrier may be in the movement of treponemes between the endothelial cells and the underlying filter prior to entering pores in the filter. We cannot rule out the possibility that some treponemes crossed the monolayer by entering and exiting the endothelial cells. We have noted very rare intracellular treponemes when either *T. pallidum* or TpR were applied to the monolayer (data not shown). The finding of rare intracellular TpR as well as *T. pallidum* suggests that the endothelial cells may be able to ingest both organisms with a very low frequency in a way unrelated to pathogenicity.

Our finding that *T. pallidum* is able to invade through intercellular junctions of endothelial cells *in vitro* is consistent with the hypothesis that it enters tissue parenchyma during natural infection by a similar mechanism. While intracellular invasion is accomplished by pathogenic bacteria of a variety of shapes, the corkscrew shape and vigorous motility of spirochetes may facilitate invasion of intercellular junctions. The goal of studies in our laboratory is understanding the molecular basis of this unique aspect of spirochete–host interaction.

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