INFECTION AND IMMUNITY, Aug. 1989, p. 2267–2271
0019-9567/89/082267-05$02.00/0

Vol. 57, No. 8

In Vitro Model of *Treponema pallidum* Invasiveness

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Received 13 February 1989/Accepted 26 April 1989

The purpose of this investigation was to develop an in vitro model with which invasion of tissues by pathogenic *Treponema pallidum* could be studied. Double-sided culture chambers were created by mounting abdominal walls excised from mice between two halves of small dialysis cells. The integrity of tissue barriers was confirmed by dye exclusion. *T. pallidum* subsp. *pallidum*, including intrinsically radiolabeled organisms, was introduced into one side of each chamber, and fractions from the other side were evaluated over time by dark-field microscopy and scintillation counting. Tissues were evaluated by scanning electron microscopy and immunologic staining. Motile *T. pallidum*, but not nonpathogenic, host-indigenous *Treponema phagedenis* biotype Reiter, was able to pass from one side of the chamber to the other side within 10 h. Up to 12% of the inoculum crossed the chamber within 24 h. Spirochetes were found within tissue in the greatest numbers between 6 and 8 h postinoculation. The murine abdominal wall has epithelium only on the peritoneum side, and results showed that *T. pallidum* required an epithelial surface on the entry side of the double-chambered cell in order to traverse the tissue barrier. This new in vitro technique may be of value in studying spirochete virulence and host resistance.

Treponema pallidum subsp. *pallidum* (referred to hereafter as *T. pallidum*) survives inflammatory and immune responses in infected hosts (4, 13) and disseminates to remote anatomical sites by mechanisms that have been difficult to elucidate. *T. pallidum* has been found within connective tissue following application to the surface of intact rabbit mucosa (9), and it has been observed to spread from the site of inoculation to distant anatomical locations within infected rabbits (12). *T. pallidum* can penetrate living cells in vitro (5) and in vivo in rabbits (7, 14) and humans (1, 15). Intracellular *T. pallidum* can travel between vascular and extravascular compartments (11) inside mobile host cells. *T. pallidum* can also disseminate by intercellular migration through tissues. Thomas et al. (16) have shown that *T. pallidum* can pass through a monolayer of endothelial cells in vitro by actively moving between cells with tight intercellular junctions. Thus, *T. pallidum* can move through complex tissues of infected hosts by intracellular migration, intercellular penetration, or a combination of both mechanisms. However, it is difficult to track the movement of this organism through tissues and organs of infected hosts.

The work of Thomas et al. (16) raises the possibility that in vitro models can be used to study the ability of pathogenic spirochetes to move within and through intact tissues. However, the internal milieu of infected hosts is much more complicated than endothelial cell monolayers, and an in vitro model more representative of in vivo environments would be of some benefit. We examined the invasive potential of *T. pallidum* in vitro by determining whether it would pass through a relatively complex tissue barrier represented by the murine abdominal wall, which includes epithelium, connective tissue, and muscle layers. These experiments were conducted with dialysis cells, to create two chambers separated by intact tissue. Tissue barriers excluded trypan blue and the nonpathogenic spirochete *Treponema phagedenis* biotype Reiter, but *T. pallidum* (Nichols strain) passed through all layers of the abdominal wall to emerge as motile cells on the other side of the chamber within 10 h.

MATERIALS AND METHODS

Spirochetes. *T. pallidum* subsp. *pallidum* (Nichols strain) was harvested from infected New Zealand White rabbit testes by an established protocol (2, 6). In brief, testes were minced and spirochetes were extracted at room temperature in phosphate-buffered saline (PBS; pH 7.2) containing 10% heat-inactivated normal rabbit serum (NRS). Host debris was removed by two sequential centrifugations at 1,000 × *g* for 10 min each time. For some experiments *T. pallidum* that was freshly extracted from rabbit testes was suspended in PBS supplemented with 10% NRS, frozen, packed in dry ice, and sent to the University of Missouri-Kansas City by overnight mail (generously provided by J. N. Miller, School of Medicine, University of California, Los Angeles). Treponemes were stored at −70°C until they were used for dark-field analysis and immunohistochemical studies. In radiolabeling experiments, treponemes were used directly after isolation from infected rabbits. Cell counts were based on dark-field microscopic examination of motile cells by the method described by Miller (10). Cell counts of thawed samples were based on fully motile, vigorous treponemes which appeared indistinguishable from freshly extracted *T. pallidum*.

T. phagedenis biotype Reiter (originally obtained from J. N. Miller; referred to hereafter as *T. phagedenis*) was grown in spirolate broth (catalog no. 81-1636-0; BBL Microbiology Systems, Div. Becton Dickinson and Co., Cockeysville, Md.) supplemented with 10% NRS (catalog no. 200-6120AG; GIBCO Laboratories, Grand Island, N.Y.). Cultures were harvested during the early to mid-logarithmic growth phase (3×10^8 to 5×10^8 spirochetes per ml) and washed in PBS as described above.

Radiolabeling of treponemes. *T. pallidum* and *T. phagedenis* were radiolabeled with [^{35}S]methionine as described previously (16). In brief, 1.5×10^8 to 2×10^9 *T. pallidum* isolates per ml were incubated with [^{35}S]methionine (10 μCi/ml; specific activity, >800 Ci/mmol; Amersham Corp.,

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Arlington Heights, Ill.) for 16 h at 34°C. *T. phagedenis* was labeled by the addition of [^{35}S]methionine to the culture medium and incubation for a further 6 h at 34°C. Prior to use in experiments, ^{35}S-labeled treponemes were centrifuged at 17,000 × *g* and washed once in PBS. After centrifugation, >99% of the organisms were motile. Only freshly extracted *T. pallidum* was used for these experiments. Specific activities were 4.8×10^6 cpm per 5×10^8 *T. pallidum* isolates and 2.9×10^7 cpm per 5×10^8 *T. phagedenis* isolates.

Mice. Female BALB/c, CD4, and male C3H/He mice (ages, 3 to 6 months; Sasco, Inc., Omaha, Nebr., and Charles River Breeding Laboratories, Inc., Raleigh, N.C.) were sacrificed by cervical dislocation; and the abdominal wall covering the peritoneal cavity was excised from under the rib cage to just above the pubic area, providing a piece of tissue approximately 2 by 3 cm. The peritoneum side, which is covered with epithelium, was oriented to distinguish it from the ventral side, which is covered with loosely organized connective tissue. Excised abdominal walls were kept for less than an hour in sterile, ice-cold PBS until they were used to construct chambers.

Chambers. Culture chambers were assembled by placing a piece of abdominal wall, trimmed to fit within the posts of a dialysis cell (total volume, 2 ml; catalog no. H40260; Bel-Art Products, Pequannock, N.J.), between the halves (referred to hereafter as the entry side and the exit side) and tightening the assembled chamber to provide a watertight barrier. A ring of sterile filter paper, about 1 cm wide and trimmed to fit around the margins of the chamber in each cell half, facilitated the stretching of the tissue across the cell. In order to determine whether tissue barriers were intact, a sterile 0.4% solution of trypan blue in 0.9% saline (catalog no. 630-5250AG; GIBCO) was added to the entry side and PBS was added to the exit side. Chambers were incubated at room temperature for 15 to 30 min. The exit side of chambers became blue quickly if the seal around the tissue was faulty or if tissue was damaged. Before use, entry-side compartments of intact chambers were rinsed with sterile PBS until the rinse solutions were clear. The contents of both sides were removed, and sterile PBS containing 10% heat-inactivated (56°C for 30 min) NRS was then added to the entry side and neat NRS was added to the exit side. Washed spirochetes were suspended in PBS–10% NRS and added to the entry side of chambers to a final concentration of about 5×10^8 cells per ml. The entry and exit sampling ports were closed and chambers were incubated aerobically at 34°C.

Fractions of media from the entry and exit sides of each chamber were examined for spirochetes by dark-field microscopy at ×400, by measuring radioactivity at various times from 1 h through 4 days, or by both methods. At the end of the examination period, the contents of both sides of some chambers were removed and tissue barriers were washed several times with sterile PBS. Chambers were then disassembled, and the tissue was prepared for further examination.

Scanning electron microscopy. Immediately after washing, approximately one-third of each tissue specimen to be examined was placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and held at 4°C for 4 h. During fixation each specimen was subdivided into smaller pieces (approximately 3 by 3 mm) with a scalpel. Following fixation, specimens were rinsed three times in cacodylate buffer and then dehydrated through a graded series of ethanol up to 100%. The specimens were subjected to critical-point drying, coated with 20.0-nm evaporated gold-palladium, and examined in a scanning electron microscope (Philips 515).

Immune rabbit serum. Pooled hyperimmune rabbit serum was generously provided by S. Lukehart (Seattle, Wash.). Three seronegative rabbits were immunized by intramuscular injection with 200 μg of sonicated *T. pallidum* in incomplete Freund adjuvant. Rabbits were boosted with 200 μg of sonicate in the same manner at 8 and 10 weeks and with 100 μg at 12 weeks. Rabbits were boosted intravenously with 200 μg in saline 8 months after the initial immunization and were bled 1 month after the last injection. Serum was separated, pooled, and stored at −70°C.

Immunologic detection of *T. pallidum* in tissue. Chamber tissue was rinsed extensively and repeatedly in sterile PBS before processing. The entry-side surface of tissue was noted and samples were immediately cut into small pieces (about 3 by 3 mm), placed in O.C.T. (optimum cutting temperature) compound (catalog no. 4583; Ames Division, Miles Laboratories, Inc., Elkhart, Ind.), and snap frozen in liquid nitrogen. Cross sections (thickness, 5 μm) were placed on glass slides treated with an adhesive (chrome alum-gelatin in distilled water) and stored at 0°C.

Sections were fixed with 100% acetone for 10 min at 4°C, air dried, and rehydrated in PBS. Slides were blotted dry, and sections were incubated with protein-blocking reagent (catalog no. 7501; Lipshaw, Detroit, Mich.) for 30 min at 34°C in a humidor. Slides were blotted to remove excess blocking solution, and 100 μl of either NRS or immune rabbit serum (IRS) diluted 1:10 in PBS was added to cover the sections. Slides were incubated for 30 min as described above. Slides were washed by immersing them in three changes of PBS for 2 min each time. Sections were covered with 100 μl of a 1:100 dilution in PBS of biotinylated-rat anti-rabbit immunoglobulin G (catalog no. 04-1640; Zymed Laboratories, San Francisco, Calif.) and incubated for another 15 min. Slides were washed as described above, and sections were covered with 100 μl of a 1:100 dilution in PBS of strepavidin–β-galactosidase (catalog no. 9536SA; Bethesda Research Laboratories, Bethesda, Md.). After washing, sections were covered with 100 μl of a working solution of substrate and chromogen. The working solution was composed of 0.05 ml of a 20-mg/ml solution of Bluo-Gal (catalog no. 5519UA; Bethesda Research Laboratories) in dimethylformamide and 2.3 ml of a buffer composed of 100 mM Na_2HPO_4 (pH 7.2), 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, and 50 mM 2-mercaptoethanol. Sections were incubated for 60 min as described above. An equal volume of 1 M Na_2CO_3 (pH 11.0) was then added, and incubation was continued for another 10 min at room temperature. Slides were washed in PBS and mounted with a glycerol-based medium. Rabbit testes infected with *T. pallidum* (the generous gift of S. Lukehart, Seattle, Wash.) provided positive control tissue. Normal mouse abdominal wall was used as negative control tissue. A positive reaction was defined by the presence of a dark blue stain in experimental and positive control tissues treated with IRS, as long as NRS failed to generate stain in any tissue and IRS failed to stain normal or negative control tissue.

RESULTS

Movement of *T. pallidum* across the mouse abdominal wall. The mouse abdominal wall consists of a single-cell layer of epithelium on the peritoneum side, vascular connective tissue with a rich capillary bed, muscle layers, adipose tissue, and loosely arranged connective tissue on the ventral side (Fig. 1). The epithelial surface consists of a sheet of

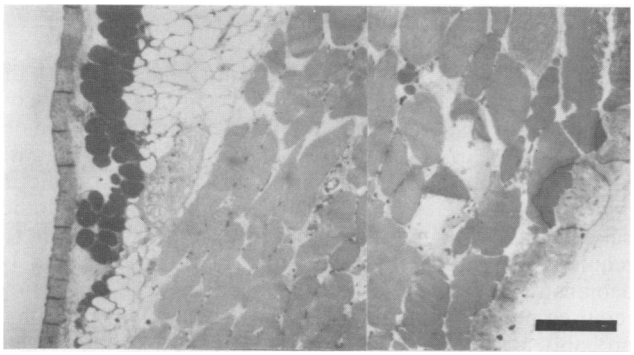

FIG. 1. Full-thickness cross-sectional view of normal mouse abdominal wall with epithelium on the left. Hematoxylin and eosin stain was used. Bar, 0.1 mm.

intact squamous epithelial cells displaying tight intercellular junctions (Fig. 2a).

In order to determine whether the barrier would exclude nonpathogenic and, presumably, noninvasive treponemes, four experiments were conducted in duplicate by using *T. phagedenis*. No spirochetes were seen by dark-field microscopy on the exit side of any of the four chambers through 48 h of observation. Two similar experiments were conducted in duplicate with *T. pallidum* by using spirochetes which were frozen at −70°C and freshly extracted spirochetes. In both experiments, no penetration of *T. pallidum* could be detected up to 4 h; however, following overnight incubation, motile spirochetes were observed in the exit side of each chamber. The number of spirochetes on the exit side did not increase during 4 days of observation.

Penetration of tissue by *T. pallidum* was assessed by both dark-field microscopy and by determining the radioactivity in the exit sides of two additional chambers. *T. pallidum* crossed tissue barriers, reaching peak numbers on the exit sides at about 20 to 22 h (Table 1). Approximately 12% of the spirochetes placed in the entry side passed through the tissue to the exit side of the chambers.

A mixing experiment was conducted in which freshly extracted, radiolabeled *T. phagedenis* and unlabeled *T. pallidum* were added together to the entry sides of two chambers, and the exit sides were evaluated by both dark-field microscopy and scintillation counting. *T. pallidum* but not *T. phagedenis* was able to traverse tissue barriers (Table 2). The morphology of spirochetes in the exit sides of chambers was consistent with that of *T. pallidum*, which is much smaller than *T. phagedenis*, and was easily distinguished from *T. phagedenis* by dark-field microscopy. Scintillation counting documented the presence of 4×10^3 to 5×10^3 *T. phagedenis* isolates in the exit side after nearly 4 days of culture; this is 4 orders of magnitude less than the direct counts of *T. pallidum*.

TABLE 1. Passage of ^{35}S-labeled *T. pallidum* through murine abdominal wall[a]

Time (h)	No. of spirochetes (mean ± 1 SD)[b] on exit side determined by:	
	Dark-field microscopy (10^7)	Scintillation counting (10^5 cpm)
16	3.8 ± 0.4	3.4 ± 0.3
20	5.7 ± 0.3	5.6 ± 0.3
22	6.3 ± 0.4	6.1 ± 0.3
24	6.3 ± 0.3	6.0 ± 0.3

[a] A total of 5×10^8 *T. pallidum* isolates (4.8×10^6 cpm) were introduced into the entry sides of two chambers.
[b] Data for isolates derived from the exit sides of both chambers.

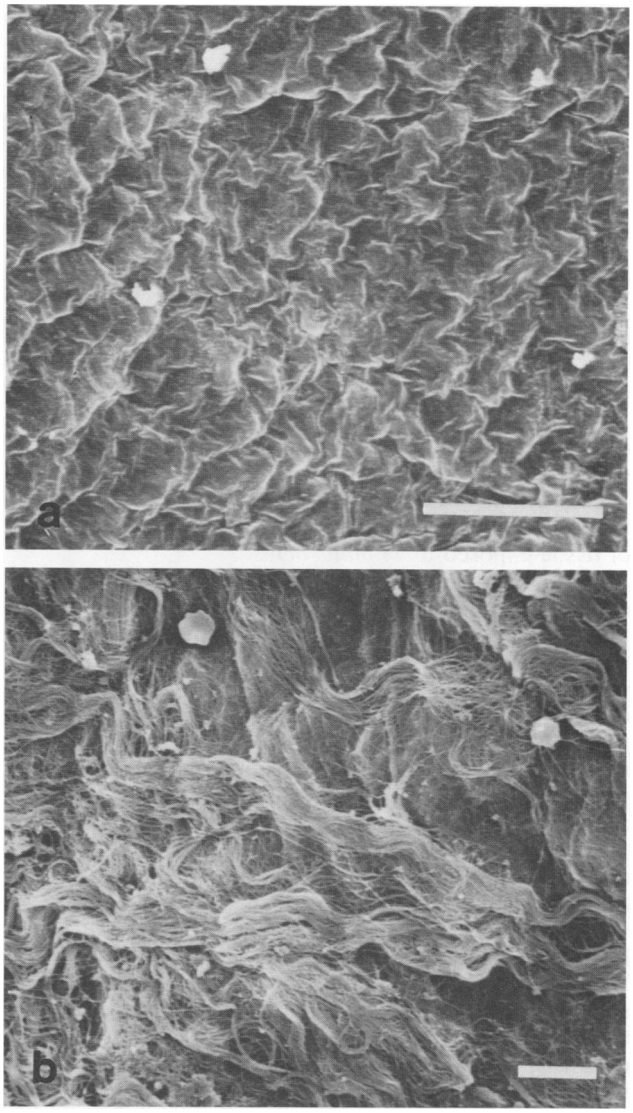

FIG. 2. (a) Scanning electron micrograph of the epithelial side of normal mouse abdominal wall without spirochetes. Bar, 100 μm. (b) Scanning electron micrograph of the connective tissue side of normal mouse abdominal wall without spirochetes. Bar, 10 μm.

Effect of epithelium on the ability of *T. pallidum* to enter tissue. To determine whether the orientation of the abdominal wall would affect the ability of *T. pallidum* to traverse the tissue barrier, the abdominal wall was oriented in one chamber with epithelium on the entry side (chamber 1). The tissue was reversed in the second chamber (chamber 2) so that unorganized connective tissue faced the entry side. After the introduction of thawed *T. pallidum*, chambers were incubated overnight (approximately 14 h) and examined by dark-field microscopy. Motile spirochetes were observed on the entry side of both chambers and on the exit side of chamber 1. The epithelial surface of chamber 1 was covered with cells which appeared to be leukocytes that must have migrated from within the tissue barrier in response to spiro-

TABLE 2. Penetration of murine abdominal wall by mixed *T. pallidum* and *T. phagedenis*[a]

Time (h)	No. of spirochetes (mean ± 1 SD)[b] on exit side determined by:	
	Dark-field microscopy (10^7)	Scintillation counting (10^2 cpm)
18	3.6 ± 0.3	0
22	6.3 ± 0.3	1.6 ± 0.4
42	6.2 ± 0.4	3.2 ± 0.4

[a] A total of 5×10^8 unlabeled *T. pallidum* and 5×10^8 [^{35}S]methionine-labeled *T. phagedenis* isolates (2.9×10^7 cpm) were introduced into the entry sides of two chambers. The small *T. pallidum* was easily distinguished from the large *T. phagedenis* by dark-field microscopy.

[b] Data for isolates derived from the exit sides of both chambers.

chetes on the entry side (Fig. 3). Note that such cells were not found on the epithelial surface of normal tissue (Fig. 2b). No spirochetes were observed on the exit side of chamber 2, nor was a cellular exudate observed on the epithelial surface of tissue exposed to *T. phagedenis*.

Kinetics of migration of *T. pallidum* through tissue. To explore the kinetics of *T. pallidum* migration through tissue, frozen *T. pallidum* was thawed and introduced into three chambers, one of which was collected at 6, 8, and 10 h after initiation. At each collection period, the exit side was evaluated for spirochetes by dark-field microscopy, and then both sides were washed with PBS and the tissue was snap frozen in liquid nitrogen. Cross sections were incubated with IRS or NRS, and spirochetes within tissue were stained with a biotin-strepavidin system.

T. pallidum was not observed within the exit sides of chambers collected at 6 or 8 h, but motile *T. pallidum* was seen in exit sides of chambers at the 10-h collection period.

IRS, but not NRS, stained positive control infected rabbit testes, but neither serum stained normal mouse abdominal wall. IRS produced a dense band of stain on the epithelium side of chamber tissue collected 6 h after inoculation. Stain was observed scattered throughout tissue at 8 h, often occurring as dense patches. At 10 h little stain was seen in tissue, and no stain was observed in or around blood vessels.

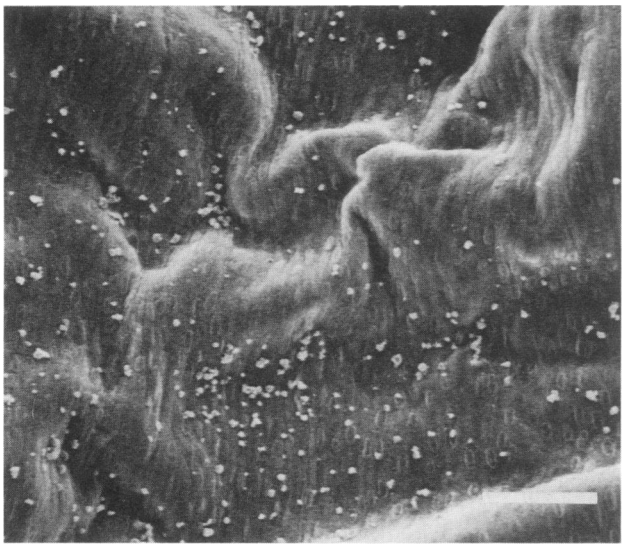

FIG. 3. Scanning electron micrograph of the epithelial surface of mouse abdominal wall 14 h after application of *T. pallidum*. The presence of migrating cells can be seen on the surface. Bar, 100 μm.

DISCUSSION

The observations presented above are consistent with the original work of Thomas et al. (16) regarding the invasive potential of *T. pallidum* and extend their findings to a more complicated barrier consisting of epithelium, connective tissues, and muscle. In the prior investigation, viable *T. pallidum* was able to move through monolayers of endothelial cells in less than 2 h (16), but in this study, spirochetes required more than 8 h of culture before they could be demonstrated on the exit sides of chambers. The longer time required for movement across mouse abdominal wall is probably a reflection of the greater thickness of the barrier, the greater complexity of the involved tissue, or both.

It is unlikely that *T. pallidum* moved through the tissue barrier by association with blood vessels, because antiserum to *T. pallidum* did not produce stain around blood vessels and because movement of spirochetes was at right angles to the vascular bed. It also seems unlikely that *T. pallidum* traversed murine abdominal walls because of an organ culture-generated increase in tissue permeability. Trypan blue in physiologic saline was excluded by the tissue barrier, and *T. phagedenis* was unable to move from one side to the other during the first 24-h period, either alone or when mixed with *T. pallidum*. The small numbers of *T. phagedenis* observed in exit sides of chambers after 2 or more days of culture may be evidence of physical deterioration of the barrier, but these later observation periods proved to be unnecessary. Furthermore, [^{35}S]methionine was not detected in meaningful levels on the exit sides of chambers unless ^{35}S-labeled *T. pallidum* was also found on the exit side. Low-level counts derived from labeled *T. phagedenis* placed on the entry side did not reach the exit side until after the migration of unlabeled *T. pallidum* peaked during the initial 24-h period.

Photomicrographs from scanning electron microscopic analysis of epithelial surfaces facing the entry sides of chambers showed that *T. pallidum*, but not *T. phagedenis*, elicited the migration of host cells out of abdominal walls onto the surface-facing spirochetes. Since *T. phagedenis* does not attach to host cells (5, 6), it is not surprising that *T. phagedenis* was unable to elicit the same cellular response as *T. pallidum*.

T. pallidum is subject to phagocytosis (8) and could have been transported within phagocytic vacuoles from one site to another (10), especially since *T. pallidum* appears to be more resistant to the degradative effects of some phagocytic cells than *T. phagedenis* is (3). Immunohistochemical analysis in this study suggested that initial penetration of tissue by *T. pallidum* occurred along a wide front after 6 h of culture but that some spirochetes may have been trapped by host cells after about 8 h because of the aggregated stain that was observed at that time. There was much less stain deposited after 10 h, suggesting either that all the spirochetes had migrated through or that host cells had transported them to one side or the other. Further experimentation will be necessary to confirm the role of phagocytic cells in this process and to determine whether phagocytic cells could have transported spirochetes to the exit sides of the chambers.

We used concentrated rabbit serum on the exit side to create a serum gradient to facilitate migration through tissue (D. Thomas, unpublished data). However, we have no reason to believe that tissue penetration would occur only under these conditions. Suspensions of *T. pallidum* contained rabbit serum and tissue components because no

attempt was made to remove anything but easily sedimentable material. This rabbit material did not affect the integrity of tissue barriers because, in coincubation experiments, *T. phagedenis* was excluded while *T. pallidum* crossed through the barrier.

The directional movement of *T. pallidum* from the epithelium into deeper associated connective tissues is consistent with the early observations by Mahoney and Bryant (9). These investigators applied viable *T. pallidum* to the surface of intact rabbit mucosa and found that the spirochetes moved through the epithelium into connective tissues below the site of application. *T. pallidum* attaches to cells by means of receptor-ligand interactions (2) that *T. phagedenis* cannot execute. Hence, in contrast to *T. phagedenis*, *T. pallidum* can attach to a large variety of cell types (5). Results of this study support the key role played by attachment as the initial step in pathogenicity since *T. pallidum* required an epithelial surface in order to cross the tissue barrier in these experiments. When *T. pallidum* confronted a connective tissue surface on the entry sides of chambers, it was unable to traverse the barrier.

There are well-documented differences between some animal species and humans regarding their susceptibilities to infection with *T. pallidum* subsp. *pallidum* (for a review, see reference 13), but differences in propensity for disease does not mean that *T. pallidum* cannot interact with murine tissues as it would with rabbit or human tissues. Indeed, the observation that *T. pallidum* passes through a murine tissue barrier while *T. phagedenis* does not indicates that this model can be used to study spirochetes, whether or not the mouse is accepted as an appropriate animal for syphilis research. In fact, resistant species may offer advantages for investigation with this in vitro model that are not found with susceptible species.

In summary, construction of simple chambers with dialysis cells and abdominal walls of mice permitted in vitro analysis of migration of *T. pallidum* through a complex tissue. It is likely that other murine tissues and tissues from other animals, including humans, could be used in this assay. Other spirochete pathogens and potential pathogens could also be studied. Furthermore, by introducing substances into one chamber or the other, this model permits investigators to evaluate the effects of a variety of factors, such as drugs and immunologic mediators of resistance, on the virulence potential of pathogenic spirochetes.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous gifts provided by Sheila A. Lukehart and James N. Miller. We also thank Sheila A. Lukehart for critical evaluation of this manuscript. We express appreciation to Laurie Comstock and Linda Higbie for assistance with extraction of treponemes from rabbits and to Om Singla and Scott Robinson for preparation of samples for microscopy.

This study was supported, in part, by an award from the Weldon Spring Endowment Fund (to G.R.R.) and by a Burroughs Wellcome Young Investigator Matching Grant award from the National Foundation for Infectious Diseases (to D.D.T.).

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