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# In Vitro Association of Leptospires with Host Cells

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**Interactions of *Leptospira interrogans* with cultured endothelial and kidney epithelial cells were assayed by examining (i) cytoadherence of intrinsically radiolabeled leptospires to eucaryotic cell monolayers and (ii) penetration of leptospires through cell monolayers grown on polycarbonate filters in chemotaxis chambers. *L. interrogans* serovars attached to cultured cells in a dose- and time-dependent manner. Adherence was diminished following pretreatment of organisms with proteases, rabbit immune serum, or heat. When observed by scanning electron microscopy, most leptospires attached by both ends, rather than just one tip like *Treponema pallidum*. In penetration assays, 9.7% of added *L. interrogans* migrated through the monolayer-filter barrier, while only 0.3% of *L. biflexa* penetrated in the same time interval. Transmission electron microscopy revealed that organisms entered the host cell cytoplasm. These in vitro results indicate that leptospires have an invasive capacity that may be related to pathogenicity in vivo and suggest that further investigation of interactions with host cells may enhance knowledge of leptospiral virulence.**

Leptospirosis is a worldwide zoonotic disease that affects primarily cattle and swine herds and occasionally pets, as well as wild animals (5, 11). Cases involving humans result from accidental or occupational contact with infected animals or their urine or contaminated bodies of water. During infection with pathogenic leptospires, a host may experience symptoms that include fever, myalgias, meningitis, petechial skin rashes, and jaundice. The etiologic agent of the infection is a spirochete, *Leptospira interrogans*. More than 150 serovars of *L. interrogans* have been identified (11). Despite the large economic burden posed by leptospiral infection of domestic animal herds, the mechanism of pathogenesis of these organisms is poorly understood.

We are interested in how spirochetes disseminate and target various host systems. Because leptospires are excreted in urine and are often found microscopically to be associated with small blood vessels, we examined the bacterium-host cell interaction in vitro by using kidney cells and endothelial cells in culture. Previous studies by others on the leptospire-host cell association have focused on cytotoxic products and enzymes produced by the spirochetes or on phase-contrast and electron microscopic observation of attachment to host cells (1, 3, 6, 8, 10, 14, 15). We sought to explore the interaction between leptospires and relevant cultured cell types with techniques that might yield better quantitation of the association and lead to molecular characterization of the spirochete and host components involved. The present report describes (i) quantitation of host cell cytoadherence of radiolabeled pathogenic leptospires and (ii) penetration of cell monolayers in vitro.

## MATERIALS AND METHODS

**Bacteria.** Virulent *L. interrogans* serotypes canicola, pomona, and grippotyphosa were obtained from the American Type Culture Collection (Rockville, Md.), as was the noninfectious, saprophytic *L. biflexa* serotype patoc. Organisms were grown at 32 to 34°C in PLM leptospira medium (Intergen, Kankakee, Ill.) and used for experiments when the log phase of growth was reached. All of the serotypes used exhibited characteristic vigorous motility. Leptospires were used at passage 5 or earlier and were hamster infective. For some experiments, leptospires were radiolabeled by a modification of the method of Nunes-Edwards et al. (9). Briefly, 5 to 10 μCi of [$^{35}$S]methionine (specific activity, 1,051 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, Calif.) per ml was added to early-log-phase cultures, and incubation was continued at 32°C until a density of $5 \times 10^7$ to $8 \times 10^7$ organisms per ml was reached. Bacterial cell counts were made by dark-field microscopic examinations based on calibrations incorporating latex particles.

**Host cells.** The eucaryotic cells used included Madin-Darby canine kidney (MDCK) and PK-15 porcine kidney epithelial cells obtained from the American Type Culture Collection, and human umbilical vein endothelial (HUVE) cells isolated from fresh umbilical cords by the method of Jaffe et al. (4). MDCK cells were maintained in Dulbecco minimum essential medium containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), while PK-15 cells were grown in minimum essential medium with 10% fetal bovine serum and HUVE cells were cultured in medium 199 containing 20% fetal bovine serum, 50 ng of endothelial cell growth supplement (Sigma Chemical Co., St. Louis, Mo.) per ml, and 100 mg of heparin (Sigma) per ml. All cells were grown at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

**Attachment assay.** The methods used for the attachment assay were based on those used in our earlier studies (12, 13). Radiolabeled leptospiral suspensions ($5 \times 10^7$ organisms per determination) were added to host cell monolayers prepared in 24-well microtiter plates, and the inoculated monolayers were incubated for various intervals at 37°C in 5% $CO_2$. Following incubation, the suspensions were removed and the monolayers were washed three times with warm medium. Next, the monolayers with attached organisms were solubilized with 300 μl of 1% sodium dodecyl sulfate, mixed with cocktail (Universol ES; ICN), and counted by scintillation. In some experiments, monolayers were prepared on round cover slips. In these experiments, cover slips were removed at the conclusion of the wash steps

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and inverted on a slide for dark-field observation or fixed for electron microscopy.

Various modifying agents were tested for the ability to inhibit adherence of leptospires to host cells. For treatment of host cells, the medium on the monolayers was removed and replaced with the solution to be tested. All test agents were dissolved in phosphate-buffered saline. Monolayers were incubated for 1 h at 37°C before removal of the solution and addition of the spirochete suspension. When effects on the leptospires were tested, samples of organisms were mixed with solutions of the test agents and the mixtures were incubated for 1 h at 34°C. The organisms were pelleted by 5 min of microcentrifugation, followed by resuspension in tissue culture medium and addition to host cell monolayers. Leptospires were also pretreated with a subagglutinating concentration of serum produced by immunization of a rabbit with three intramuscular injections of whole, heat-killed *L. interrogans* serotype canicola cells. Serum was harvested from blood 2 weeks after the last biweekly boost. Additionally, leptospires which had been heat killed by incubation at 60°C for 30 min were tested for extent of adherence.

**Scanning electron microscopy.** At the end of incubation of unlabeled leptospires with host cell monolayers grown on 12-mm-diameter round cover slips, the monolayers were washed three times with warm medium and fixed for 15 min at room temperature with 2.5% glutaraldehyde in phosphate-buffered saline. Cover slips were removed from the plates, dehydrated through a graded series of ethanol to 100%, and dried to the critical point. Cover slips were mounted on specimen stubs and sputter coated with gold-palladium (60:40) before observation in an SEM-501 scanning electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

**Invasion assay.** For experiments designed to assess the ability of leptospires to penetrate host cell monolayers, we used a modification of a system described previously (2). Briefly, cultured eucaryotic cells were seeded at a density of $5 \times 10^4$ per plastic chemotaxis chamber (PC-2; ADAPS, Inc., Greenlodge, Mass.). Each sterile chamber contained a polycarbonate membrane (5-μm pore size; Nuclepore Corp., Pleasanton, Calif.). Chambers were placed in 24-well tissue culture plates, and the cells were incubated at 37°C until confluence (high electrical resistance) was reached. Radiolabeled or unlabeled bacteria were quantitated, and $10^8$ organisms were added to each upper chamber. Following incubation at 37°C, leptospires in samples of medium from below the filters were counted by dark-field microscopy and/or scintillation.

**Transmission electron microscopy.** After 2 h of coincubation of leptospires with cultured cell monolayers, the bacterial suspensions were removed from the host cells and the monolayers were washed three times with phosphate-buffered saline. Samples were fixed for 15 min at room temperature in 2.5% glutaraldehyde–phosphate-buffered saline, and the filters were carefully removed from the chambers. Each sample was postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. Transverse thin sections were cut, mounted on copper grids, and stained with lead citrate and uranyl acetate. Grids were examined on a Philips TEM 400 microscope.

## RESULTS

**Visualization of adherence to host cell monolayers.** In the tissue culture system described above, pathogenic leptospires were found to attach to the epithelial and endothelial cells tested. When examined by scanning electron microscopy, most leptospires appeared to be attached by one or both ends. Leptospires were also frequently observed to be attached at various points along their lengths (Fig. 1).

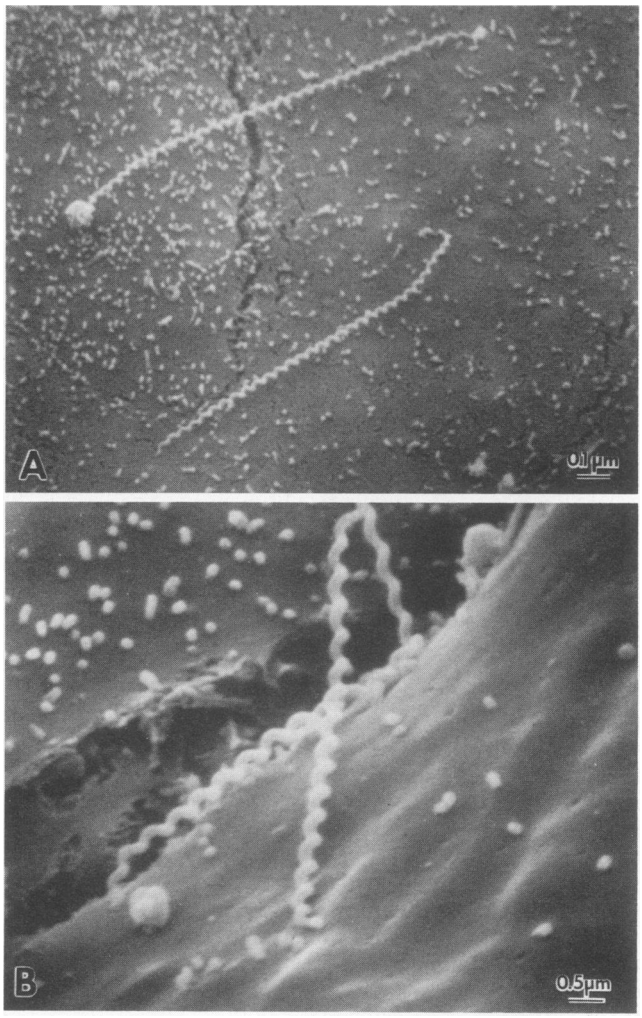


FIG. 1. Scanning electron microscopic visualization of *L. interrogans* serovar canicola adhering to cultured kidney cells. Monolayers of host cells ($5 \times 10^5$) were incubated with $2 \times 10^7$ leptospires for 2 h at 37°C. (A) Low magnification of organisms attached at one or both ends. (B) Higher magnification of attachment mediated by ends and by points along the length of the organism.

**Quantitation of adherence.** Intrinsically radiolabeled organisms were used to quantitate the adherence of leptospires to cultured cells. All serotypes tested adhered to the cell monolayers (Table 1). However, adherence of known pathogenic serotypes was 1.8 to 5 times greater than that for the nonpathogenic serotype patoc. Serotype canicola gave the greatest adherence values, with 11.9% of the inoculum adherent. Attachment was time dependent (Fig. 2). The association curve was approximately linear through 3 to 4 h, when adherence began to plateau. Similar results were obtained with MDCK and HUVE cells. Experiments testing the effect of temperature on the extent of adherence were performed next (Table 2). All serotypes of pathogenic *L. interrogans* showed the greatest attachment to kidney cells at 37°C. The extents of adherence of all serotypes were equal

TABLE 1. Adherence of leptospires to cultured epithelial and endothelial cells[a]

| Leptospiral serotype | Avg ± SD % of bacteria that adhered to[b]: | | |
|---|---|---|---|
| | MDCK cells | PK-15 cells | HUVE cells |
| Pathogenic | | | |
| Canicola | 8.5 ± 0.6 | 11.9 ± 0.7 | 7.1 ± 0.4 |
| Pomona | 4.3 ± 0.2 | 4.8 ± 0.3 | 4.4 ± 0.1 |
| Grippotyphosa | 5.6 ± 0.4 | 5.5 ± 0.4 | 8.3 ± 0.5 |
| Nonpathogenic Patoc | 1.7 ± 0.1 | 2.6 ± 0.1 | 2.4 ± 0.2 |

[a] Suspensions containing leptospires were added to host cell monolayers and incubated at 37°C for 3 h. Following incubation, the monolayers were washed and solubilized with sodium dodecyl sulfate and counted by scintillation.

[b] Percentage of the added organisms which became cell associated. The data represent the results of three separate experiments.

at 4°C; however, at 23 and 37°C, more than twice as many serotype canicola organisms were attached than organisms of either serotype pomona or grippotyphosa. Incubation at temperatures greater than 37°C was detrimental to adherence.

**Effects of modifying agents.** To further characterize the attachment of *L. interrogans* to host cells, the susceptibility of the adherence to inhibition with various modifying agents was investigated. Pilot studies examining the monolayers by Giemsa staining and trypan blue exclusion following exposure to the modifying agents indicated that these treatments were not sufficient to cause release of the cells from the plastic. Typical results are shown in Table 3. When cell monolayers were pretreated with trypsin or other proteases before addition of leptospires, a 60% decrease in the extent of attachment was noted. Neuraminidase, lipase, and periodate treatments had little or no effect. When leptospires were pretreated with various agents before incubation with cultured cells, proteases and formaldehyde inhibited adherence. Additionally, heat-killed leptospires did not adhere significantly. After preincubation with a subagglutinating concentration of rabbit immune serum, attachment of pathogenic leptospires to each cell type was diminished by approximately 65%.

**Penetration of host cell monolayers.** Plastic chemotaxis

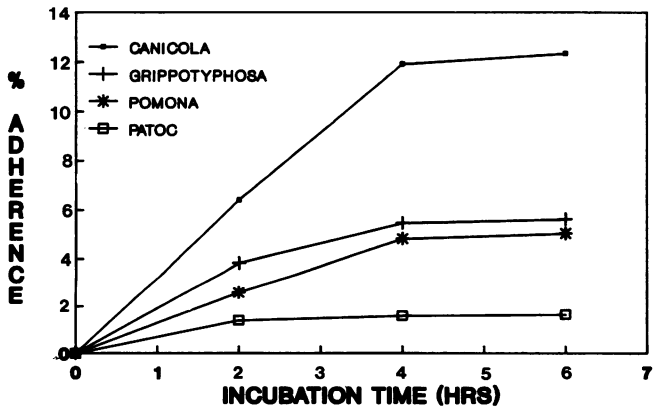


FIG. 2. Time course of attachment of *Leptospira* species to cultured PK-15 cells. [$^{35}$S]methionine-labeled leptospires ($5 \times 10^7$ cells containing $3.8 \times 10^4$ cpm) were added to each monolayer. The data represent averages of four determinations performed in triplicate. In each case, the standard error was ≤5%.

TABLE 2. Effect of temperature on adherence of leptospires to cultured cells

| Serotype | Avg ± SD % adherence to PK-15 cells[a] at: | | |
|---|---|---|---|
| | 37°C | 23°C | 4°C |
| Canicola | 11.6 ± 1.1 | 8.3 ± 0.6 | 1.5 ± 0.2 |
| Pomona | 5.1 ± 0.4 | 3.8 ± 0.2 | 1.4 ± 0.1 |
| Grippotyphosa | 5.3 ± 0.3 | 2.7 ± 0.3 | 1.3 ± 0.2 |

[a] Suspensions containing leptospires were added to host cell monolayers and incubated for 3 h at the indicated temperatures. Following incubation, the monolayers were washed and solubilized with sodium dodecyl sulfate and counted by scintillation. The data represent percentages of the inocula bound to host monolayers.

chambers were used in studies on the invasive potential of pathogenic leptospires. Results from a typical experiment are shown in Table 4. Each of the pathogenic serovars tested was able to penetrate the monolayers in a time-dependent fashion. Serovar canicola was most able to penetrate, with 9.7% of the organisms in the lower chamber after 3 h of incubation. In contrast, the nonpathogenic serovar patoc did not penetrate significantly. All organisms could pass through the pores of filters with no cell monolayers to the same extent.

When monolayers fixed for transmission electron microscopy following incubation with leptospires were viewed, bacteria were frequently found in the cytoplasm of both kidney epithelial and endothelial cells (Fig. 3). The bacteria were found singly and in groups. However, not all intracellular organisms had the same appearance. Some leptospires were seen in cytoplasmic vesicles, while others appeared to be free in the cytoplasm. In some instances, intracellular

TABLE 3. Effects of modifying agents on leptospiral adherence to host cells

| Pretreatment | Avg ± SD cpm attached[a] | % Inhibition[b] |
|---|---|---|
| Host cells[c] | | |
| None | 4,497 ± 269 | 0 |
| Trypsin (125 μg/ml) | 1,827 ± 174 | 59.4 |
| Neuraminidase (2.5 mg/ml) | 4,201 ± 298 | 6.6 |
| Lipase (2.5 mg/ml) | 4,971 ± 317 | 0 |
| Sodium metaperiodate (2.5 mg/ml) | 5,228 ± 294 | 0 |
| Leptospires[d] | | |
| None | 4,355 ± 277 | 0 |
| Heat (60°C, 30 min) | 995 ± 87 | 77.2 |
| Immune serum (1:250) | 1,529 ± 121 | 64.9 |
| Sodium metaperiodate (2.5 mg/ml) | 4,435 ± 301 | 0 |
| Trypsin (125 μg/ml) | 1,912 ± 165 | 56.1 |
| Proteinase K (125 μg/ml) | 2,185 ± 173 | 49.8 |
| Formaldehyde (0.5%) | 2,808 ± 196 | 35.5 |
| β-Mercaptoethanol (0.5%) | 3,425 ± 215 | 21.4 |

[a] Radiolabeled leptospires (serovar canicola) were added to MDCK cell monolayers and incubated at 37°C. Counts per minute attached were determined when the monolayers were washed, solubilized, mixed with cocktail, and counted by scintillation.

[b] Percent decrease in host cell association measured against an untreated control.

[c] Host cell monolayers were pretreated by incubation with the agents listed for 30 min at 37°C. Treatment solutions were aspirated before addition of leptospires. Attachment was assessed as previously described.

[d] Leptospires were preincubated in solution with the indicated concentrations of modifying agents for 30 min at 37°C. Following incubation, the spirochetes were pelleted by centrifugation, suspended in tissue culture medium, and added to host cell monolayers. Host cell association was assessed as described in footnote *a*.

TABLE 4. Penetration of MDCK monolayers by leptospires[a]

| Time and serotype | Avg ± SD no. ($10^7$) in lower chamber[b] | % of added organisms[c] |
|---|---|---|
| 1 h | | |
| Canicola | 1.32 ± 0.08 | 5.3 |
| Pomona | 0.87 ± 0.05 | 3.5 |
| Grippotyphosa | 0.79 ± 0.06 | 3.1 |
| Patoc | 0.05 ± 0.01 | 0.2 |
| 3 h | | |
| Canicola | 2.42 ± 0.13 | 9.7 |
| Pomona | 1.61 ± 0.07 | 6.4 |
| Grippotyphosa | 1.33 ± 0.06 | 5.3 |
| Patoc | 0.07 ± 0.01 | 0.3 |

[a] Leptospires ($2.5 \times 10^8$ per sample determination) were added to MDCK monolayers grown on filter membranes in plastic chemotaxis chambers and incubated at 37°C. Samples from below the membranes were examined at 1 and 3 h postinoculation by dark-field microscopy.

[b] Organisms in 25 fields were counted for each determination. The experiment was performed three times in triplicate.

[c] Percentages of the added organisms found in the lower chamber under the indicated conditions.

organisms in vacuoles seemed to be in various states of degradation (Fig. 3). The host cell monolayers maintained integrity throughout the experiment as judged by trypan blue exclusion tests.

## DISCUSSION

In this study, we examined the interaction of pathogenic leptospires with cultured host cells in a quantitative manner. Other investigators have counted adherent organisms observed by fluorescence microscopy or scanning electron microscopy (1, 3, 14). We believe that the methods used by us allow for a more meaningful assessment of leptospire-host cell interactions. In addition, the cultured cell types used reflect two sites (endothelium and kidney) targeted by pathogenic leptospires in vivo.

Use of radioactively labeled organisms enabled us to demonstrate that up to about 12% of the added pathogenic leptospires adhered to cultured endothelial cells during the experiments, while only 1 to 2% of nonpathogenic leptospires adhered. These results support the reports of Tsuchimoto et al. (14) and Vinh et al. (15) that virulent organisms attached to a greater degree than avirulent bacteria. Further, the interaction was time and temperature dependent and, we believe, mediated by protein-containing moieties on both the leptospires and the host cells.

Our results indicate that adherence of leptospires to kidney cells was reduced by 50% or more by preincubation of the leptospires with heat, proteases, or rabbit immune serum (Table 3). However, Vinh et al. (15) reported enhanced adherence of leptospires to L cells when the organisms were incubated in immune serum. We can only postulate that the

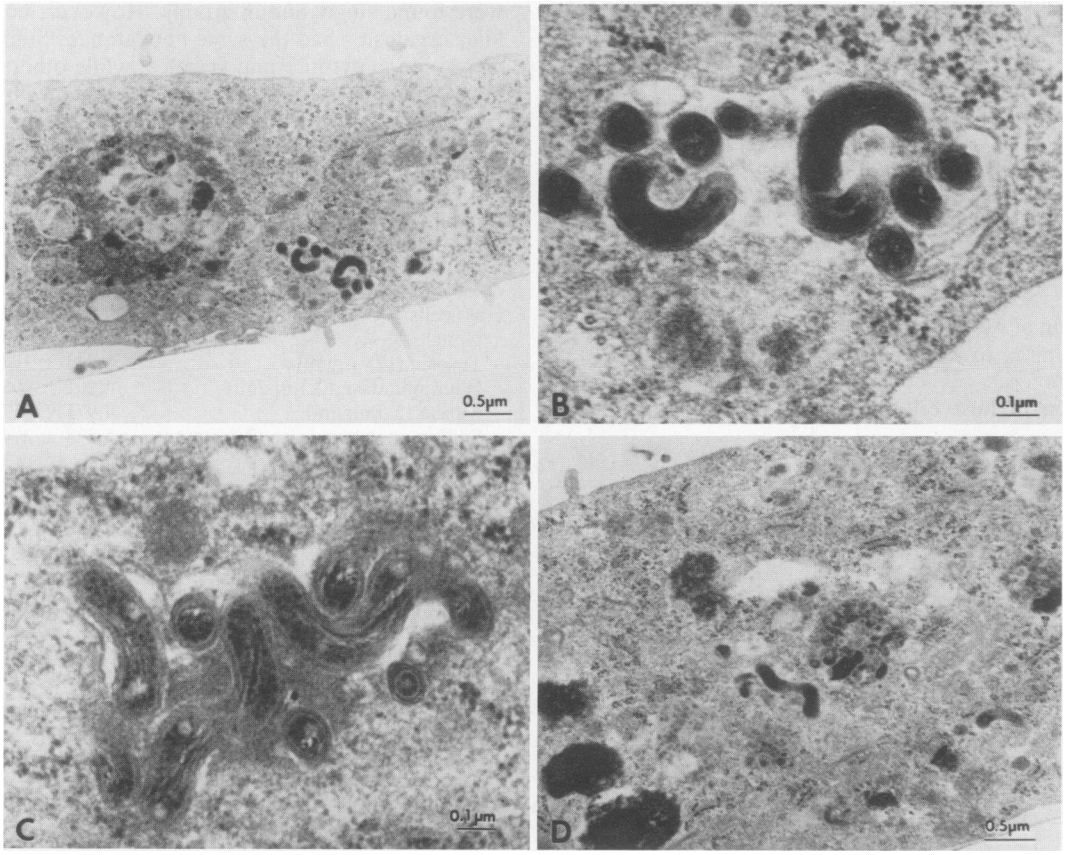


FIG. 3. Transmission electron microscopy of a representative invasion experiment. Leptospiral suspensions were added to confluent kidney epithelial or endothelial cell monolayers grown on porous filters and incubated at 37°C for various intervals. Following coincubation, the filters were rinsed, fixed, embedded, and sectioned. (A) Low-magnification view of an MDCK cell with intracellular *Leptospira* growing on a porous filter membrane. (B and C) Higher magnification of intracellular leptospires in membrane-bound vesicles in MDCK and HUVE cells, respectively. (D) Intracellular leptospires in an MDCK cell. Leptospires free in the cytoplasm and degrading in the vesicle are apparent.

disparity in the results relates either to the differences in the cultured cell types used or possibly to differences in the number of organisms per cultured cell used in the assays. Vinh et al. used approximately a five-times-greater leptospire/cultured cell ratio in their adherence assay on L cells (15). Perhaps surface charge changes related to the density of the organisms present, as well as those due to bound antibodies account for the differences in our results on this point.

Other studies have demonstrated leptospires inside cells in various organs from experimentally infected animals (6, 8), and Rose et al. (10) showed by fluorescent-antibody techniques that leptospires could attain intracellularity in monkey kidney cells. In this study, we showed that pathogenic leptospires can penetrate tight-junctioned epithelial and endothelial cell monolayers in a chemotaxis chamber-filter membrane assembly, while the nonpathogenic leptospires tested could not invade to significant extent. Infected monolayers studied by transmission electron microscopy had virulent leptospires within the individual cells. Most of the spirochetes inside either MDCK or HUVE cells appeared intact, and organisms emerging into the bottom chamber were cultivable. However, some organisms were obviously degraded inside the host cells. Further studies will be required to determine the significance of various fates once the bacteria become intracellular and also to assess the mechanism(s) by which the spirochetes achieve intracellularity.

The results of this study indicate that specific attachment to and invasion of host cells, at least in the in vitro model, are properties of virulent leptospires. These results are consistent with findings for other spirochete genera, including *Treponema* (13) and *Borrelia* (2). *Treponema pallidum* penetration of endothelial cells has been shown to be primarily by passage of the organisms through the junctions between the cells (13). By contrast, *Borrelia burgdorferi* cells pass through the endothelial cell cytoplasm and emerge from the basolateral side of the cells intact and motile (2). In this investigation, leptospires were seen in intercellular junctions as well as within host cells. The significance of these findings is unclear; however, studies of spirochetal penetration of host cell monolayers in vitro may yield important information regarding the dissemination and tissue tropisms of these organisms. Use of a chemotaxis chamber–porous-membrane system could facilitate the study of the bacterium-host cell interaction. Future experiments will be aimed at characterization of leptospiral surface proteins that mediate attachment and entry into cultured cells and at elucidation of the mechanism of host cell entry used by leptospires.

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