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Rapid Translocation of Polarized MDCK Cell Monolayers by *Leptospira interrogans*, an Invasive but Nonintracellular Pathogen

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Pathogenic spirochetes of the genus *Leptospira* are a major cause of human zoonotic infectious disease worldwide. After gaining entry through the skin, the organism causes disease by hematogenously disseminating to multiple organs. The mechanism by which it penetrates the mammalian cell barriers to disseminate is not well understood. In this study, we used a low-passage-number isolate of *Leptospira interrogans* to elucidate the invasive potential of this spirochete. Quantification of bacteria by dark-field microscopy revealed that pathogenic spirochetes were able to translocate through polarized MDCK cell monolayers at a rate significantly greater than that of nonpathogenic *Leptospira* or a recognized invasive bacterial pathogen, *Salmonella*. In contrast to *Salmonella*, *L. interrogans* did not alter transepithelial electrical resistance during cell translocation. Both transmission and scanning electron microscopy revealed tight association of the extracellular spirochetes with the host cell plasma membrane, without membrane perturbations suggestive of cytoskeletal rearrangement. Spirochetes were not observed within intercellular junctions or membrane-bound compartments inside cells. They were found within the cytoplasm of only 8% of the counted cells. These results indicate that *Leptospira* is an invasive but not a facultative intracellular organism. We propose that the rapid translocation of mammalian cells by pathogenic *Leptospira* is a mechanism designed to evade killing by host cells that permits the organism to quickly reach the bloodstream and disseminate to multiple organs.

Leptospirosis is a worldwide zoonosis caused by spirochetes belonging to the genus *Leptospira* (7, 21). The disease has a wide spectrum of clinical manifestations, from mild febrile illness to a severe form known as Weil's syndrome, characterized by jaundice, conjunctival suffusion, and renal failure. The hallmark of infection with *Leptospira* species is its rapid hematogenous dissemination after the organism gains entry into the host through abrasions in the skin. How the spirochete penetrates the mammalian cell barriers to establish disseminated infection is not known.

Experiments performed with other pathogenic spirochetes such as *Treponema pallidum* and *Borrelia burgdorferi* have provided some insight into how these helical pathogens associate with eukaryotic cells (5, 15, 37). Motile *T. palladium* but not nonpathogenic treponemes enter intercellular junctions of human endothelial cells (15, 37) and cross murine abdominal epithelial cell barriers within 10 h (33). *B. burgdorferi* was also shown to penetrate and invade both cultured tick and human umbilical vein endothelial cell (HUVEC) monolayers (5, 22, 25). Indirect immunofluorescence studies with HUVECs showed intracellular localization of *Borrelia* within 24 h of infection (25). These studies were confirmed by transmission electron microscopy (TEM), which provided evidence that the organisms were inside membrane-bound compartments inside cells (22).

Early in vivo experimental studies in guinea pigs support the observation of rapid hematogenous dissemination of *Leptospira* (6, 16). Faine reported the presence of *Leptospira* in the livers and kidneys of intravenously infected guinea pigs after only 30 min (6). Several reports suggest that in vitro, infection of tissue culture cells with *Leptospira interrogans* appears to be primarily extracellular, and adhesion to cell surfaces has been described as an important property of virulent leptospires (2, 7, 29, 38, 39). Studies have also shown that leptospires enter both phagocytic and nonphagocytic cells (26, 30, 36). In vitro, *Leptospira* localized free throughout the cytoplasm or in membrane-bound vesicles inside epithelial cell monolayers (30, 31, 36), while in vivo studies with experimentally infected sheep found leptospires associated with the apical plasma membrane of the kidney (27). In these studies, intact organisms were not seen invading or within cells (28). These apparently conflicting observations may be due to differences in experimental conditions and the *Leptospira* serovar used by various investigators.

In this study, we analyzed the interaction of a well-characterized low-passage-number patient isolate of *L. interrogans* with polarized cell monolayers. In an attempt to better characterize the invasive potential of this spirochete, we examined the host cell-pathogen interaction by (i) quantifying the per-

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centage of migrating leptospires through an intact polarized cell monolayer, (ii) assessing intercellular junction integrity of the infected monolayer, (iii) characterizing cytoskeleton involvement, and (iv) visualizing *L. interrogans* associating with cell monolayers by both transmission and scanning electron microscopy.

MATERIALS AND METHODS

Bacterial isolates. *Leptospira* organisms were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris medium (Difco Laboratories, Detroit, Mich.) at 29°C and counted in a Petroff-Hausser counting chamber (Fisher Scientific, Pittsburgh, Pa). A low-passage-number clinical isolate from Brazil (21), *L. interrogans* serovar copenhageni strain L1-130 was used in all assays. It has a 50% lethal dose of 10^4 in hamsters. This strain was passaged and reisolated from hamsters twice after isolation from a blood culture of a patient with leptospirosis and stored at −70°C. Frozen aliquots were thawed and passaged in liquid medium less than two times prior to use as a low-passage-number isolate in the infection experiments. Saprophytic *Leptospira biflexa* Patoc 1 strain (World Health Organization Collaborative Laboratory for Leptospirosis, Royal Tropical Institute, Amsterdam, The Netherlands), *Salmonella enteritidis* strain 4386, and *Escherichia coli* DH5α were used as control bacteria in the cell monolayer assay.

Cell culture. Madin-Darby canine kidney cells (MDCK-II) were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in minimal essential medium (MEM) with sodium bicarbonate (Cellgrow; Mediatech, Herndon, Va.), supplemented with 10% fetal calf serum (Sigma, St. Louis, Mo), 5 mM L-glutamine, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml. Cells were cultured in a 37°C incubator containing 5% CO_2, grown to confluence in T75 flasks (Falcon, Oxnard, Calif.), and transferred to Transwell polycarbonate filters for infection assays.

Electrical measurements. In this study, 4- and 6-day-old confluent monolayers were used. MDCK-II cells form tight junctions and polarize within a few hours under optimal conditions (13). The formation of tight junctions can be evaluated by measuring the transepithelial electrical resistance (TER) across the cell monolayer (12, 15). High electrical resistance correlates with well-developed tight junctions, while a decrease in TER indicates a disruption in the cell integrity (12). The TER of cells grown on polycarbonate filters was monitored daily with an EVOMmeter (World Precision Instruments Inc., Sarasota, Fla.) and recorded for each well before and after the infection of each cell monolayer. An average TER measurement (with standard deviation) was calculated for each time point, and each measurement was repeated in triplicate. The average TER measurement of polycarbonate filters in the absence of a cell monolayer was 90 Ω/cm^2 (baseline).

Infection of polarized cell monolayers. Epithelial cells were seeded at a density of 4×10^5 cells/cm^2 in the apical side of the Transwell chamber lined with a 12-mm-diameter polycarbonate filter with a pore size of 3.0 μm (Costar Corp., Cambridge, Mass.). The chamber was placed inside another well (lower chamber) in a 12-well tissue culture plate. MDCK-II cells were washed daily with phosphate-buffered saline (PBS), pH 7.4, and replenished with fresh MEM (containing antibiotics). Spirochetes were quantified by dark-field microscopy and resuspended in l00 μl of MEM (with no antibiotics). Prior to infection, MDCK cell monolayers were washed seven times in 1× PBS (to remove any residual antibiotics) and were then infected at a multiplicity of infection (MOI) of 100 from the apical chamber. At 15, 30, 60, 120, and 240 min, 100-μl aliquots of MEM from the lower chamber were collected, and the spirochetes were quantified by dark-field microscopy.

The viability of MDCK-infected monolayers was assessed by trypan blue dye exclusion and examined by bright-field microscopy at intervals during the 15 min to 4 h of infection. Since this pathogenic strain of *L. interrogans* does not form distinct colonies that permit quantification by enumeration of CFU, both strains of leptospires were counted in a Petroff-Hausser chamber (25 squares counted; experiment done in triplicate) immediately following each time point. All spirochetes were visibly motile after recovery from the lower chamber. Serial dilutions of *S. enteritidis* and *E. coli* were plated at each time point (in triplicate) and quantified by enumeration of CFU. To maintain consistency of the results, we counted each well in triplicate and calculated the mean and standard deviation of the percentage of initial inoculum that had penetrated the monolayer. Means were compared with the Student's *t* test.

F-actin staining. F-actin staining was performed with rhodamine-labeled phalloidin (Molecular Probes, Eugene, Oreg.). Briefly, 2×10^5 MDCK cells/cm^2 were layered over round glass coverslips in 24-well tissue culture plates (Falcon). Following incubation with bacteria (15, 30, 60, and 120 min) at an MOI of 100, cell monolayers were washed three times in PBS, fixed in 2% glutaraldehyde for 10 min at room temperature, washed three times in PBS, and permeabilized with 0.1% Triton X-100 for 5 min. To minimize nonspecific binding, we incubated the cells with 1% bovine serum albumin in PBS for 30 min at room temperature and washed them three times with PBS before incubating them with rhodamine-labeled phalloidin. Slides and coverslips were mounted with Cytoseal and examined with a Zeiss fluorescence microscope.

EM. Monolayers of *Leptospira*-infected MDCK-II cells were washed seven times in PBS, fixed in 2% glutaraldehyde overnight at 4°C, postfixed with 1% osmium tetraoxide and uranyl acetate, and treated with a graded series of ethanol solutions. Polycarbonate filters were cut from the Transwell apparatus and embedded in Epon 812 for TEM. The same samples were cut and prepared for critical point drying and sputter coating (12 nm) for scanning EM (SEM). The specimens for TEM were examined with the FEI Tecnai 12 electron microscope, while those for SEM were examined with the Hitachi S-5000 cold field emission SEM.

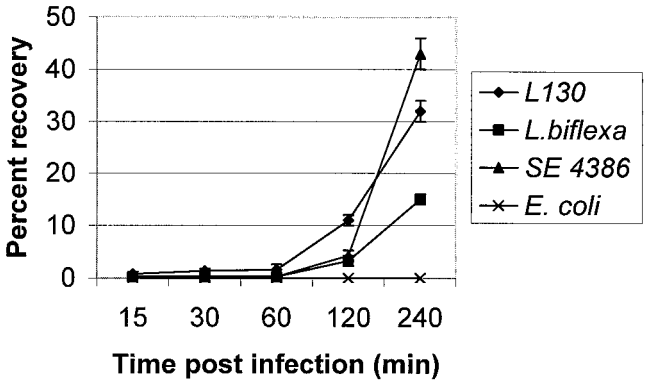

FIG. 1. Percent recovery of *L. interrogans* serovar copenhageni L1-130 (*L130*), *L. biflexa* Patoc 1, *S. enteritidis* strain 4386 (*SE 4386*), and *E. coli* strain DH5α after penetration of polarized MDCK cell monolayers between 15 and 240 min postinfection. Each point is the mean percent recovery ± standard deviation; each assay was done in triplicate for each organism. We counted 25 squares to enumerate the organisms in the lower chamber (experiment done in triplicate).

RESULTS

Rapid translocation of polarized MDCK cell monolayers. After apical infection of MDCK cells, pathogenic leptospires were observed in the lower chamber of the Transwell apparatus as early as 15 min postinfection. Of the initial inoculum, 1.6% ± 0.2% of leptospires were found in the lower chamber between 15 to 60 min (Fig. 1), while only 0.3% ± 0.04% of the invasive *Salmonella* strain was recovered from the lower chamber during the same time interval ($P < 0.005$). Moreover, both pathogenic and nonpathogenic leptospires were able to penetrate epithelial cell monolayers, but a greater percentage of the inoculum of the pathogenic *L. interrogans* serovar copenhageni crossed the epithelial cell barrier compared to the saprophytic *L. biflexa* Patoc 1. Both pathogenic and nonpathogenic leptospires crossed the polycarbonate filters at the same rate in the absence of cell monolayers (data not shown). By 4 h postinfection, 32% ± 3% of the pathogenic strain had crossed the cell membrane, while only 15% ± 2% of the saprophytic strain penetrated the monolayer ($P < 0.01$). Similarly, by 4 h, 43% of the inoculum of the invasive strain of *S. enteritidis* were recovered from the lower chamber. As expected, no organisms were recovered from the lower chamber in MDCK cell monolayers infected with a laboratory strain of *E. coli*.

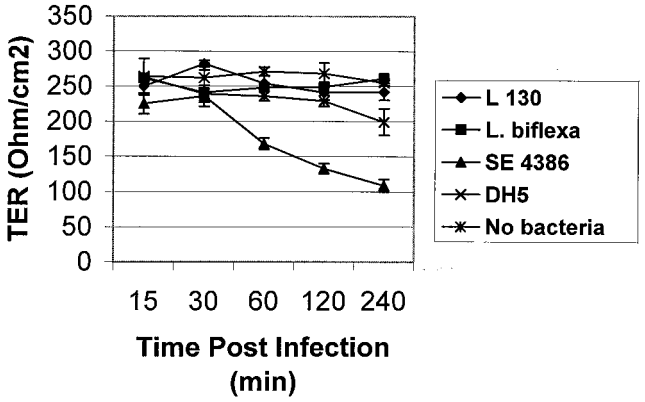

FIG. 2. Change in TER of polarized MDCK cells during 15 to 240 min of apical infection with *L. interrogans* serovar copenhageni L1-130 (L 130), *L. biflexa* Patoc 1, *S. enteritidis* strain 4386 (SE 4386), and *E. coli* strain DH5α (DH5). Each point is the mean TER decrease ± standard deviation. Each assay was done in triplicate. The baseline value for a filter with no bacteria was 98 Ω/cm^2.

***Leptospira* spp. do not affect TER of MDCK cell monolayers.** The integrity of tight junctions of the infected MDCK cell monolayers was assessed by TER measurements (Fig. 2). TER measurements of MDCK cell monolayers were taken before and at each postinfection time period of 15, 30, 60, 120, and 240 min. After infection with *Leptospira*, only a minor decrease in the TER was observed after 4 h. During the first 60 min, TER decreased by approximately 10%. At 120 and 240 min, TER measurements were seen to decrease by 12 and 16%, respectively. Similarly, the TER measurements of uninfected cells changed minimally between 120 and 240 min. However, TER measurements of cells infected with *S. enteritidis* decreased by 19% at 15 min and by 63% at 240 min. These data suggest that *Leptospira* organisms penetrate and translocate polarized cell monolayers without greatly disrupting cell junction integrity.

***Leptospira* penetration of MDCK cells does not rely on actin cytoskeletal rearrangements.** The distribution of rhodamine-labeled phalloidin revealed no evidence of actin cytoskeleton condensation during 4 h of coincubation with pathogenic leptospires (data not shown).

***L. interrogans* was found closely associated with microvilli and inside MDCK cells.** Examination of transmission electron micrographs of leptospire-infected MDCK cell monolayers demonstrated organisms both closely associated with and inside the cell as early as 30 min postinfection. Of the 50 polarized cells examined by TEM, many of the spirochetes were seen in association with and entering the cell membrane (Fig. 3A and D) and in tight association with microvilli of the apical cell surface (Fig. 3B and E), but none were seen in the intercellular spaces. There appears to be no clear staining of the cell plasma membrane at the site of entry of the organism (Fig. 3A). There was no evidence of pedestal or filopodium projections at the site of attachment of the spirochetes. Leptospires were also seen inside the cell cytoplasm (Fig. 3C and F). However, of the 50 cells examined, only 4 (8%) contained any intracellular organisms, and each infected cell appeared to contain only one organism. Moreover, there was no evidence that these organisms were inside a membrane-bound vacuole (Fig. 3F). Intercellular junctions appeared to retain integrity throughout the time course of infection.

Scanning electron micrographs revealed *L. interrogans* entering (Fig. 4A) or in close association with microvilli on the apical surface of the monolayer with little disruption or perturbation of the epithelial cell surface (Fig. 4B to D). Moreover, there was no evidence of surface epithelial cell damage attributable to the presence of the spirochetes.

DISCUSSION

The above observations reveal a pattern of interaction with mammalian cells of pathogenic *Leptospira* that is quite distinct from other well-recognized invasive bacterial pathogens, such as *Salmonella*, *Yersinia*, or *Shigella* spp. These latter groups of organisms usually establish invasive intestinal infections after oral inoculation. *Salmonella* and *Yersinia* organisms may disseminate via the bloodstream. Patients who develop severe leptospirosis acquire the infection through exposure to an environment contaminated with animal urine containing *Leptospira* (23). In places where leptospirosis is endemic like Brazil, the sewer rat, *Rattus norvegicus*, is the major reservoir for *L. interrogans* serovar copenhageni (21). The organism is thought to penetrate the skin or breaks in the skin to initiate infection and then rapidly disseminates via the bloodstream to cause multisystem infection, targeting the liver and kidney. In rats, infection results in chronic colonization of the proximal renal tubules, from which the organism is shed in the urine, thus facilitating transmission to other hosts (7).

The ability of *L. interrogans* to cause such a rapid systemic infection after penetration of the skin suggests that it is a highly invasive pathogen. Use of the Transwell chemotaxis chambers allowed us to demonstrate that 1.6% of the initial inoculum of pathogenic leptospires crossed polarized monolayers within 15 min of infection and that after 4 h, 32% of the inoculum of *L. interrogans* was found to have translocated the cellular barrier. Similar results were described in other reports (30, 36), although in our experiments, both pathogenic and nonpathogenic strains were recovered after 4 h. Hence, the ability to cross the monolayer per se does not appear to be a phenotype associated with pathogenicity. The pathogenic strain exhibited significantly faster translocation ability than the nonpathogenic strain (Fig. 1), which suggests that this ability of *Leptospira* to rapidly translocate through the cells may be the distinguishing feature of its pathogenicity.

It has been well established that spirochetes such as *T. pallidum* and *B. burgdorferi* can penetrate and cross cell monolayers. The current paradigm is that these pathogenic organisms disseminate through the interjunctional spaces or through the cell membrane (5, 15, 37). However, studies with leptospires have provided conflicting reports made by both in vitro and in vivo observations regarding the cellular localization (extracellular, phagosomal, cytoplasmic, or interjunctional) of *Leptospira* in nonprofessional phagocytic cells (2, 5, 26–28, 30, 31, 34, 36, 38). These conflicting observations may relate to differences in experimental conditions used, such as differences in the *Leptospira* serotypes, tissue culture cell types, and animal models.

Merien et al. (30) compared the abilities of pathogenic (*L. interrogans* serovar icterohemorrhagiae) and nonpathogenic

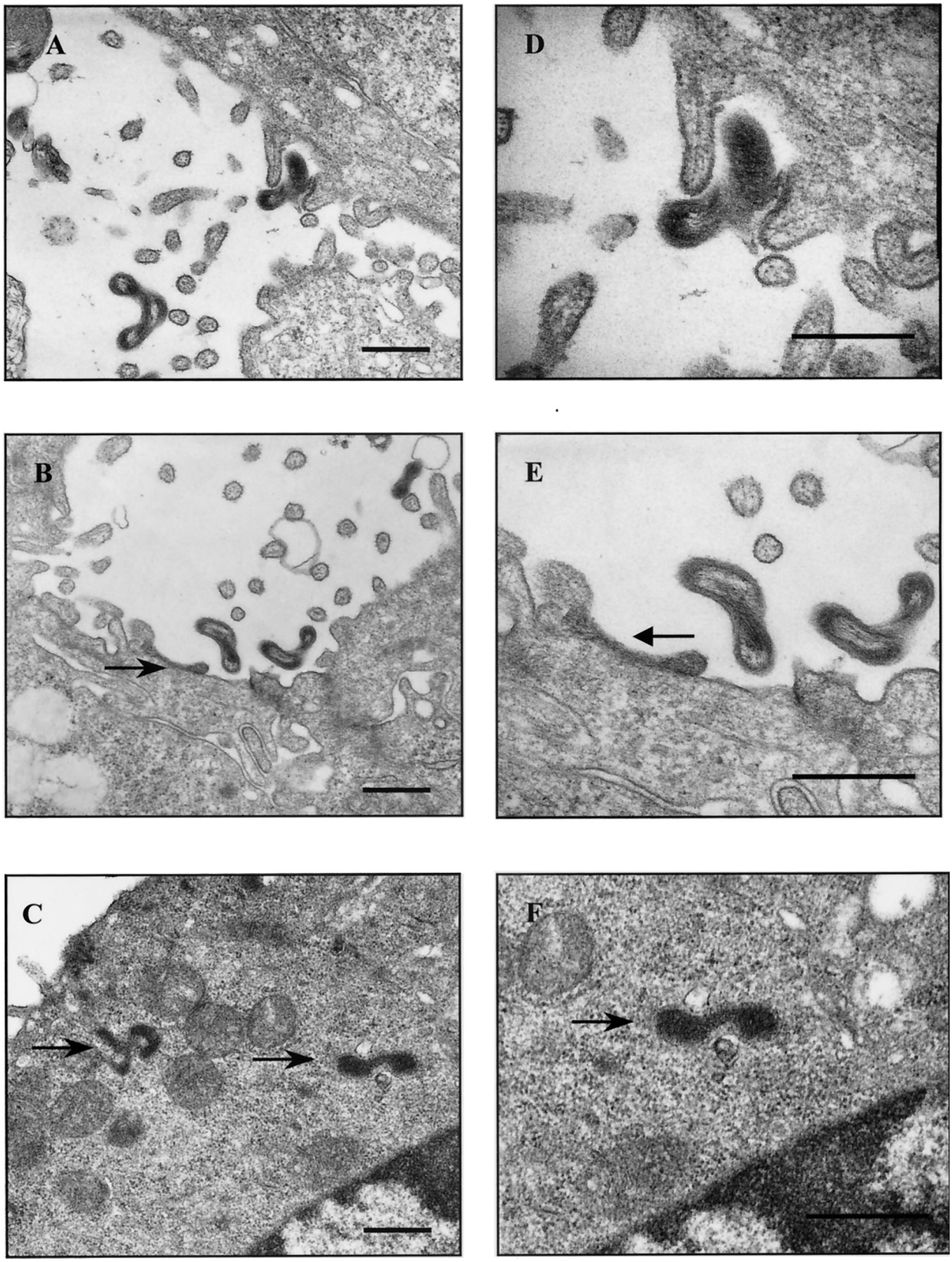

FIG. 3. High- and low-magnification transmission electron micrographs of *L. interrogans*-infected MDCK cells. (A and D) Spirochete *L. interrogans*, 30 min postinfection, entering the cell membrane; (B and E) *L. interrogans* in tight association with the plasma membrane 30 min postinfection; (C and F) *L. interrogans*, free inside the cell cytoplasm 60 min postinfection. Arrows point to *L. interrogans*. Bars, 200 μm

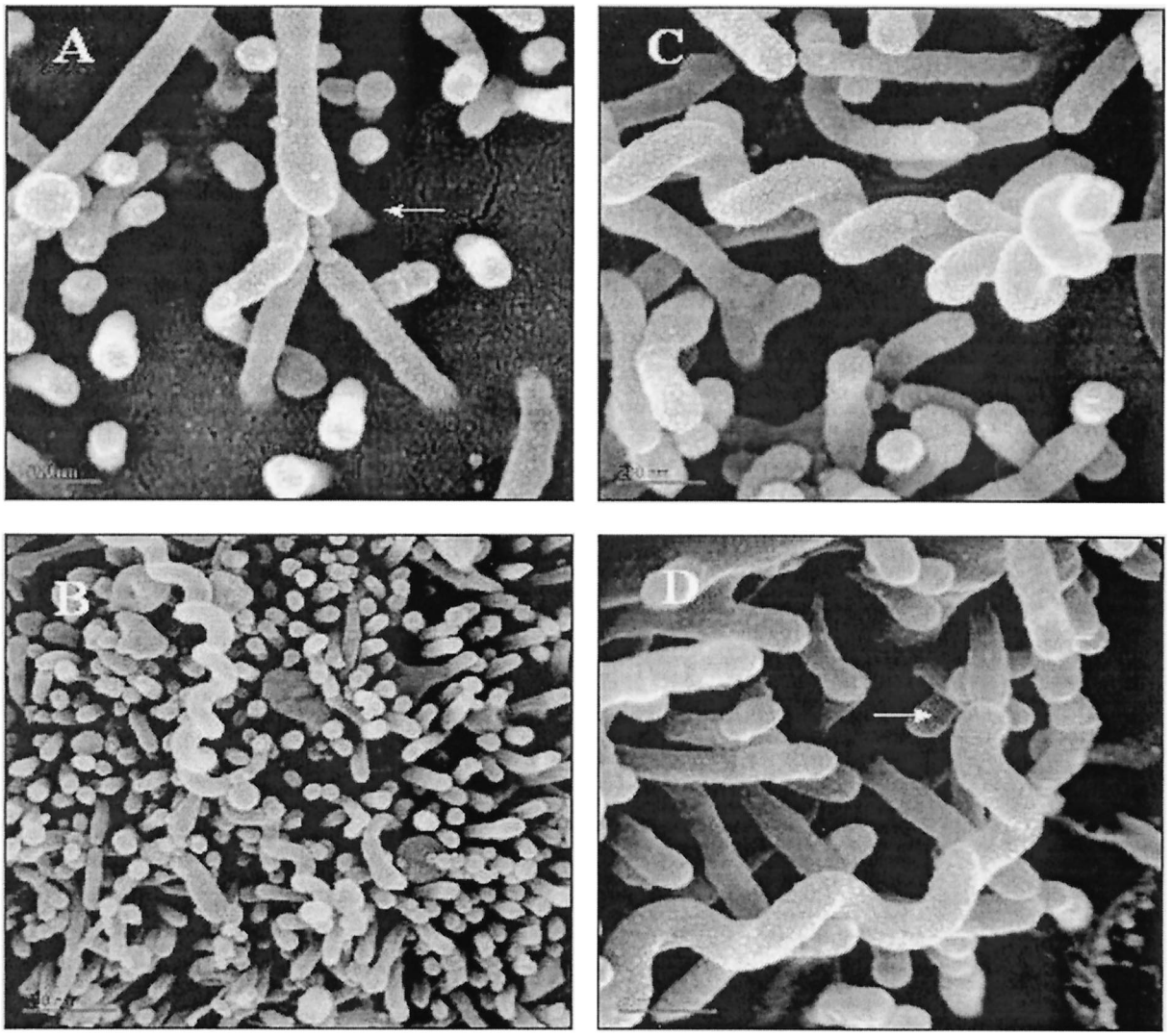

FIG. 4. Scanning electron micrographs of *L. interrogans* (arrow) penetrating the MDCK cell monolayer 30 min postinfection (A) and in tight association with microvilli and the cell membrane (B to D). Bars, 200 μm.

leptospires (*L. biflexa*) to be internalized into both Vero and macrophage J774 cells. Unlike our results, they observed that only virulent leptospires were internalized, whereas saprophytic and avirulent strains remained extracellular (30). They also postulated that invasion of epithelial cells may be a way for the organism to escape the host immune response, although our results indicate that very few leptospires reside inside the cell at any time point. The intracellular organisms we saw may simply represent spirochetes in transit at the time of cell fixation for the TEM analysis. There was no evidence for intracellular multiplication, escape from membrane-bound compartments, or cell-to-cell spread of the organism, characteristics of some facultative intracellular bacterial pathogens.

When facultative intracellular bacterial organisms such as *Salmonella* and *Shigella* enter nonphagocytic mammalian cells, they characteristically induce cytoskeletal rearrangement associated with plasma membrane perturbations that lead to phagocytosis of the organism (3, 10, 32, 40). Organisms such as *Yersinia* spp. enter nonphagocytic cells by a receptor-mediated process, sometimes referred to as the "zipper" mechanism, in which the bacteria express a surface ligand that binds to a specific mammalian receptor in a process that allows the cell's plasma membrane to tightly surround the surface of the entire organism (8, 17, 18). We did observe tight association of the plasma membrane with the bacterial surface, resembling the "intimate attachment" seen with organisms such as enteropathogenic *E. coli* (11, 20) (Fig. 3B and E). Interestingly, however, despite the above observation, there was no evidence by transmission electron or fluorescence microscopy of any cell membrane perturbations or evidence of cytoskeleton rearrangement elicited by *Leptospira*. These results are in accordance with reported observations that cytochalasin D, an organic fungal compound that irreversibly binds actin and blocks actin polymerization, had no inhibitory effect on the internalization of leptospires (30). Moreover, TEM revealed no evidence of plasma membrane perturbations suggestive of macropinocytosis or "ruffling" reported with *Salmonella* epithelial cell invasion (3, 9, 19, 40). Clearly, the organism could be seen

inside cells (Fig. 3C), suggesting that it entered the lower chamber of the polarized monolayer by translocating through the cells. It is also possible that the organism crossed the monolayer through interjunctional spaces, but we did not observe any organism in such spaces by TEM. Furthermore, infection by *Leptospira* of MDCK cells had little effect on the monolayer integrity, as previously reported with *T. pallidum* (37). TER decreased only 16% over the course of 4 h in contrast to 63% with *Salmonella* infection.

The ultimate productive outcome of pathogenic leptospires may be the complete cell translocation. In vivo, this would facilitate rapid entry into and out of the bloodstream to infect target organs, such as the kidneys (1). Thus, *Leptospira* may be an invasive but not a facultative intracellular pathogen. We therefore propose that *Leptospira* organisms invade cells but escape them rapidly to avoid intracellular killing. How *Leptospira* organisms achieve this high-speed cell translocation is not known. The characteristic helical morphology may play an important role in their movement through the environment (14, 24), as seen by their ability to bore through highly viscous gel-like media, such as connective tissues, which inhibit the motility of most other bacteria (4). Motility itself as a virulence factor was examined in *B. burgdorferi*, where a nonmotile mutant with a markedly hindered ability to penetrate HUVEC monolayers was found (35). With *Leptospira*, the ability to pass through cells may not be as important as the rate at which they penetrate them. This may facilitate rapid dissemination, before cell barriers or circulating immune cells can inhibit them. This rapid cell translocation phenotype of *Leptospira* may be a characteristic feature of pathogenic members of this spirochete.

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