

## Penetration of Endothelial Cell Monolayers by *Borrelia burgdorferi*

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**The ability of *Borrelia burgdorferi*, the agent of Lyme disease, to penetrate cultured human umbilical vein endothelial cell monolayers was investigated. After 4 h of coincubation, approximately 7.7% of added bacteria passed through the host cell monolayers. Electron microscopy revealed that the borreliae entered the endothelial cells and suggested that the organisms penetrated the host monolayers primarily by passing through them.**

Lyme disease, the most prevalent tick-mediated syndrome in the United States (5), is a spirochetosis caused by *Borrelia burgdorferi* (4, 11). We have shown that this organism, like another pathogenic spirochete, *Treponema pallidum*, attaches to human umbilical vein endothelial (HUVE) cells in vitro (12). In earlier studies we also found that *T. pallidum* penetrates cultured endothelial cell monolayers grown in chemotaxis chambers by passing through the intercellular junctions (13). Since *B. burgdorferi*, like *T. pallidum*, disseminates via the bloodstream, the ability of this spirochete to penetrate HUVE cell monolayers in vitro was studied.

*B. burgdorferi* HB19, a human blood isolate (11), was provided by Alan Barbour, University of Texas Health Science Center at San Antonio. This infectious strain was used at passage 12 or lower. Borreliae were maintained in BSK II medium (2) at 34°C and were used at log phase of growth ( $7 \times 10^7$  to  $10 \times 10^7$  organisms per ml). For some experiments, borreliae were intrinsically radiolabeled by addition of 5 to 10  $\mu$ Ci of [ $^{35}$ S]methionine (specific activity, >800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml to early-log-phase cultures and incubated at 34°C until log-phase density was reached. Greater than 98% of the radioactivity in washed  $^{35}$ S-labeled bacterial preparations was precipitable with trichloroacetic acid. *T. pallidum* subsp. *pallidum* and nonpathogenic *Treponema phagedenis* biotype Reiter were cultivated and harvested as described previously (13).

HUVE cells were regularly isolated from freshly delivered human umbilical cords by the method of Jaffe et al. (8). Cells were maintained in 5% CO<sub>2</sub> at 37°C in medium 199 (M199; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% fetal calf serum (GIBCO), 100  $\mu$ g of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml, and 50 ng of endothelial cell growth supplement (GIBCO) per ml. Endothelial character of the cells was assured by demonstrating that cells at various passages stained positively with fluorescein isothiocyanate-labeled rabbit antihuman factor VIII (von Willebrand factor) (12). Experiments were conducted using cells from passage 10 or lower.

Penetration assays were performed essentially as described previously (13). Briefly,  $5 \times 10^4$  HUVE cells were seeded onto sterile polycarbonate filters (5- $\mu$ m pore size, 13-mm diameter; Nuclepore Corp., Pleasanton, Calif.) which were mounted on plastic chemotaxis chambers (PC-2; ADAPS Inc., Dedham, Mass.). Chambers were placed in

24-well plates containing 1.5 ml of medium per well and incubated until the monolayers were confluent and possessed high transendothelial electrical resistance (13). Bacteria were quantitated by dark-field microscopy, centrifuged for 15 min at 17,000  $\times$  g, and suspended in M199 plus 20% fetal calf serum. For each assay, 0.2-ml samples containing  $5 \times 10^8$  bacteria were added to the upper portions of the chambers (above the monolayers). After incubation for various intervals at 37°C in 5% CO<sub>2</sub> in air, samples from beneath the filters were removed and spirochetes were quantitated by dark-field microscopy.

Borreliae were observed below the filter as early as 45 min after addition to the monolayer. At 2 h after addition of the borreliae to the upper chamber, dark-field microscopy revealed that an average of  $3.6 \pm 0.2\%$  (mean  $\pm$  standard deviation) of the bacteria had penetrated through both the monolayer and the filter and were present in the medium below. By 4 h, this had increased to  $9.6 \pm 0.8\%$ . To improve the accuracy of quantitating penetration, the assay was also performed with intrinsically radiolabeled *B. burgdorferi*. A representative radiolabeled borrelia suspension had a specific activity of  $2.22 \times 10^5$  cpm/ $5 \times 10^8$  organisms. By this method, 17,086 cpm or  $7.70 \pm 0.03\%$  of the added organisms were detected in the lower chamber after 4 h of incubation. In all cases, more than 95% of the bacteria below the filters were actively motile. After the 4-h incubation with borreliae, the HUVE cell monolayers appeared normal and intact on visual inspection, and trypan blue exclusion revealed that >98% of the cells were viable. This value was indistinguishable from that obtained by using monolayers not exposed to borreliae. Additionally, when  $5 \times 10^8$  *T. pallidum* cells were added to HUVE cell monolayers,  $5.8 \pm 0.2\%$  were found in the lower chambers at 4 h. However, fewer than  $0.5 \pm 0.1\%$  of *T. phagedenis* biotype Reiter were found in the lower chamber after the same period. These data suggest that invasion may be a unique property of pathogenic organisms. Further, no *B. burgdorferi* which had been heated at 60°C for 30 min were visible by dark-field examination. When radiolabeled heated organisms were used,  $246 \pm 31$  cpm were found below the filter in 4 h. We have previously shown that heat treatment reduces adherence of the bacteria to host cells by more than 90% (12).

These results prompted a transmission electron microscopic investigation to determine whether *B. burgdorferi*, like *T. pallidum*, penetrated via the intercellular tight junctions of the endothelial cell monolayer or by a different mechanism. After 2 h of incubation of bacteria with HUVE

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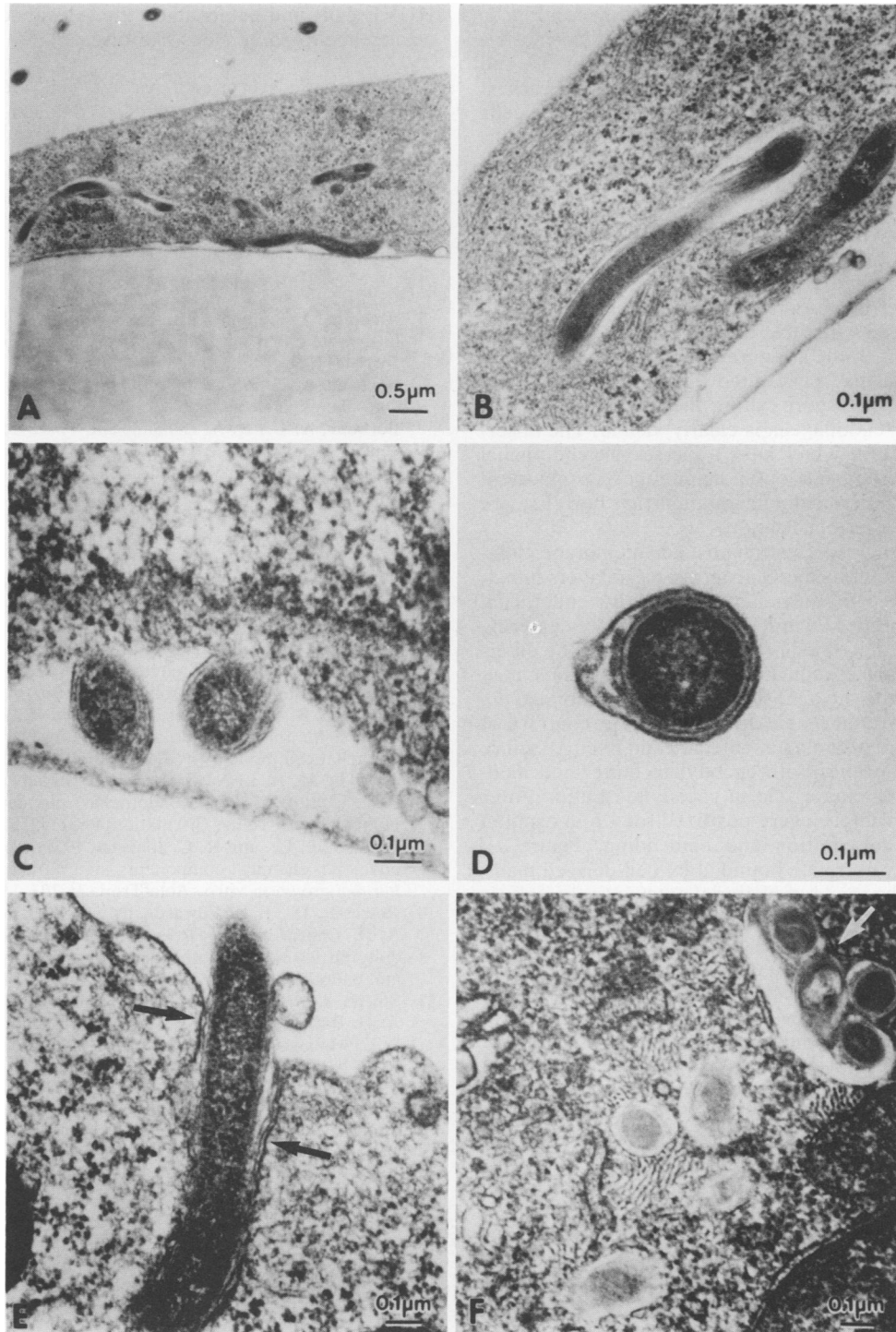


FIG. 1. Transmission electron micrographs of *B. burgdorferi* penetrating HUVE cell monolayers. Bacteria were incubated with HUVE cell monolayers on filters in chemotaxis chambers for 2 h at 37°C before washing, fixation, embedding, and sectioning for electron microscopy, as described in the text. Transverse thin sections were observed by using a Philips TEM 400 microscope. (A and B) HUVE cell monolayers (on polycarbonate filters) with intracellular borreliae; (C) spirochete emerging from HUVE cell; (D) cross section of *B. burgdorferi* cell which had traversed the monolayer and was in a filter pore; (E) single borrelia entering cell, with HUVE cell membrane indicated by arrows; and (F) sample prepared as before and stained with tannic acid to enhance membranes (arrows).

cell monolayers, the filters were removed from the chambers and rinsed in phosphate-buffered saline. Samples were fixed in 2.5% glutaraldehyde-phosphate-buffered saline for 15 min at room temperature, postfixed in 1% osmium tetroxide,

dehydrated with ethanol, and embedded in epoxy resin. Transverse thin sections were cut, transferred to copper mesh grids, and stained with lead citrate and uranyl acetate before being viewed.

Representative electron micrographs are shown in Fig. 1. In contrast to *T. pallidum* (13 and this study), *B. burgdorferi* appeared to be within the host cell cytoplasm (Fig. 1A and B). Some organisms were visible inside cells at the earliest time tested, 30 min postinoculation. When 50 HUVE cells and 50 intercellular junctions were examined, 466 organisms were found in the cells while only 33 organisms associated with junctions were observed. Figure 1C shows organisms emerging from a HUVE cell. This occurrence was observed with virtually every HUVE cell examined and suggests that passage through the HUVE cells was a mechanism for penetration of the monolayer by *B. burgdorferi*.

Spirochetal outer membranes have been demonstrated to be fragile compared with the outer membranes of gram-negative bacteria (1, 3, 6, 9). Figure 1D shows a cross section of an organism that had penetrated through the monolayer and was contained in a pore within the filter. Outer membrane surrounding endoflagella is clearly visible. The observation that the bacteria which have traversed the endothelial cell monolayer retain their outer membranes is important and is consistent with our documentation that borreliae are vigorously motile after penetration.

Since bacterial enclosure within host cell membrane might explain how the bacteria emerge from the monolayers intact, we sought evidence of borreliae enclosed by endothelial cell-derived membrane. Figure 1E shows a borrelia entering a HUVE cell. The host membrane visible around the entering organism (arrows) indicates that the organisms may attain intracellularity in a host vesicle. Also, monolayers were treated with tannic acid to enhance the contrast of membrane material. Monolayers infected and fixed as before were washed three times with cacodylate buffer and incubated for 1 h in 1% tannic acid in cacodylate buffer. After three washes, the samples were postfixed for 1 h in osmium tetroxide before dehydration and embedding. Figure 1F shows intracellular borreliae bounded by cell-derived membrane. Although the mode of bacterial entry into the cells is uncertain, these observations imply that the borreliae may be endocytosed by the HUVE cells.

Investigating the mechanism of penetration of *B. burgdorferi* into cultured endothelial cells should increase our knowledge of the pathogenesis of Lyme disease in vivo. Observations made with electron microscopy indicate that borreliae may enter HUVE cells surrounded by a host-derived membrane (Fig. 1) and penetrate by passing through the cells. The entry may be similar to *Salmonella choleraesuis* entry into canine kidney epithelial cells, which has been shown to occur via directional transcytosis (7). It is also possible that entry of borreliae might result from parasite-directed phagocytosis, such as that described for a *Candida* sp. and *Rickettsia prowazekii* penetration of HUVE cells (10, 14). Further studies will be aimed at defining the mechanism of *B. burgdorferi* penetration into and through

HUVE cells and determining the relevance of this in vitro occurrence to pathogenesis of disease.

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