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Interaction of Lyme Disease Spirochetes with Cultured Eucaryotic Cells

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The association of the Lyme disease spirochete, *Borrelia burgdorferi*, with cultured human endothelial cells was investigated. Attachment was time and temperature dependent, with optimal adherence occurring after 4 h of incubation at 37°C. Pretreatment of borreliae with heat, immune human serum, or monoclonal antibodies directed against outer surface protein B (OspB) reduced the attachment of organisms to host cell monolayers. These results suggest that the adherence of *B. burgdorferi* may be mediated, at least in part, by borrelial surface proteins.

Lyme disease, also known as Lyme borreliosis, is caused by the spirochete *Borrelia burgdorferi* (8, 18). This multisystem disorder is notable particularly for its unusual skin lesions (erythema chronicum migrans), arthritis, and neurologic manifestations (8, 9, 16–19). Presently, this disease is the most prevalent tick-borne infection in the United States (9). Relatively little is known about the pathogenesis of the infection.

Bacterial attachment to host tissues is an important first step in the pathogenesis of many infections (6). The capacity of another pathogenic spirochete, *Treponema pallidum*, to attach specifically to a variety of cultured host cells has been described previously (5, 10–12, 20). Only pathogenic treponemes adhered to host cells (10, 12), implying that cytoadherence has a role in pathogenesis. We studied the attachment of the Lyme spirochete to cultured human cells to begin to understand how this organism disseminates and targets various organ systems.

B. burgdorferi B31 (ATCC 35210) was obtained from the American Type Culture Collection, Rockville, Md. Strain HB19 was provided by Alan Barbour, University of Texas Health Science Center at San Antonio. Organisms were maintained in BSK II medium as previously described (1) and used for experiments when the log phase of growth (7×10^7 to 10×10^7 organisms per ml) was reached. For some experiments, borreliae were intrinsically radiolabeled by the addition of 5 to 10 μCi of [^{35}S]methionine (specific activity, 1,045 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, Calif.) per ml to early-log-phase cultures and incubated until the level of organisms reached 7×10^7 to 10×10^7/ml. A full complement of borrelial proteins was labeled by this procedure.

HeLa cells were obtained from the American Type Culture Collection and maintained in minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (GIBCO). Human umbilical vein endothelial (HUVE) cells were isolated from freshly delivered umbilical cords by the method of Jaffe et al. (13) and grown in medium 199 (GIBCO) containing 20% fetal bovine serum, 100 mg of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml, and 50 ng of endothelial cell growth supplement (GIBCO) per ml. The endothelial nature of the cells was confirmed with a fluorescent-double-antibody assay for factor VIII protein. To ensure the endothelial character of HUVE cells, we used only HUVE cells from passages three through eight for experimentation.

The capacity of *B. burgdorferi* to adhere to host cells was assessed in a tissue culture system like that used to study the adherence of *T. pallidum* (12, 20). Eucaryotic cells harvested with trypsin-EDTA were seeded into 24-well tissue culture clusters (Costar, Cambridge, Mass.) at a density of 2×10^5 cells per well and incubated at 37°C for 16 h. It was determined previously that these conditions resulted in the formation of confluent monolayers. ^{35}S-labeled borreliae were washed once and suspended in medium 199 with 20% fetal bovine serum. Suspensions (0.5 ml) containing 5×10^7 organisms were added to the monolayers and incubated for 4 h at 37°C in 5% CO_2 unless otherwise specified. Following incubation, the monolayers with attached organisms were washed three times with warm medium, solubilized with sodium dodecyl sulfate, mixed with scintillation cocktail (Universol ES; ICN), and counted by scintillation.

Although *B. burgdorferi* attached to both HeLa and HUVE cells, the amount which adhered to HUVE cells was approximately double that which adhered to HeLa cells at both 2 and 4 h postinoculation. At 4 h, 3.6 ± 0.13% of HB19 and 1.47 ± 0.09% of B31 attached to HeLa cell monolayers, while 7.34 ± 0.19% of HB19 and 3.12 ± 0.16% of B31 attached to HUVE cell monolayers. Since these organisms disseminate via the bloodstream, endothelial cells would most likely be the first barrier between the bacteria and the target organs. Therefore, HUVE cells were used for subsequent experimentation. Further, the data showed that the extent of adherence also varied between the two borrelia strains tested. Strain B31, the type strain, has been passaged for several years and does not infect hamsters (14), while strain HB19, a human blood isolate (18) used for experimentation at passage 12 or lower, is virulent for hamsters. In subsequent studies, the parameters for optimal binding were the same for both strains. However, strain HB19 consistently gave two to three times greater attachment to host cells. Except where noted, results obtained with HB19 are presented.

Electron microscopy was implemented as another method of examining the borrelia-host cell interaction. Following 4 h of incubation of unlabeled borreliae with HUVE cell monolayers (grown on 12-mm round cover slips), the monolayers were washed as described above and fixed for 15 min at

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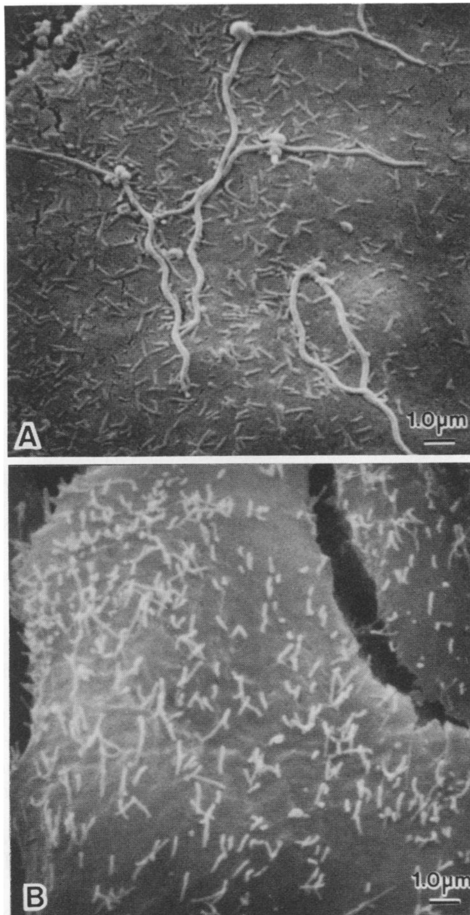

FIG. 1. Scanning electron micrographs of *B. burgdorferi* adhering to cultured HUVE cells. HUVE cell monolayers (2×10^5 cells) were incubated with 5×10^7 borreliae for 4 h at 37°C. (A) HUVE cell monolayer with attached organisms. (B) Control monolayer not exposed to spirochetes. Magnification, ×6,500; tilt, 30°.

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. The cover slips were mounted on specimen stubs and sputter coated with gold-palladium (60:40) prior to observation in a Philips SEM-501 scanning electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

Figure 1A shows an electron micrograph of a section of an HUVE cell monolayer with adhering *B. burgdorferi*. An association between the body of the spirochete and the eucaryotic cell is apparent, suggesting that borreliae can attach along the lengths of their bodies. Some organisms seemed to be contiguous with microvilli on the HUVE cell surface; however, we cannot be certain that this appearance was not an artifact of sample preparation. Figure 1B shows an HUVE cell monolayer not exposed to spirochetes. Microvilli characteristic of this cell type in culture are readily visible. When adherent *B. burgdorferi* were observed by dark-field microscopy, most organisms appeared vigorously motile, suggesting that organisms may attach via their tips as well. Using scanning electron microscopy, Kurtti et al. observed *B. burgdorferi* attached by one or both ends to cultured tick cells in cocultivation experiments (15). Our

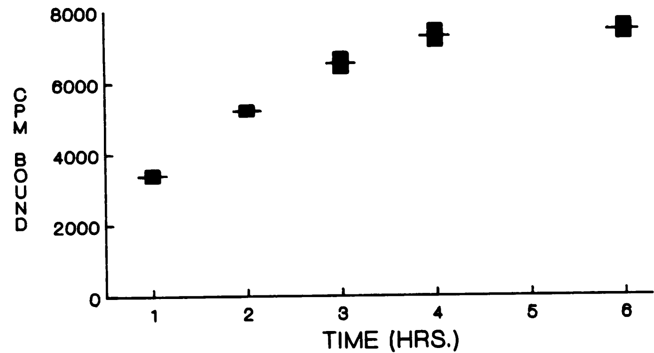

FIG. 2. Time course of attachment of *B. burgdorferi* to cultured HUVE cells. Conditions were as described in the legend to Fig. 1, except that 5×10^7 ^{35}S-labeled *B. burgdorferi* HB19 cells (9.93×10^4 cpm) were added to each monolayer. Bars denote standard deviations, and hash marks represent the average of four determinations for each time point. The data represent results obtained from three experiments.

results suggest that attachment by length and by the tip(s) occurs.

The time course of attachment of *B. burgdorferi* to HUVE cells is shown in Fig. 2. Attachment increased with time of incubation up to 4 h, when the extent of attachment began to plateau. The number of attached borreliae following 4 h of incubation was equivalent to 7.0 to 7.5% of the added organisms.

The adherence of *B. burgdorferi* to HUVE cells was also temperature dependent. Although borreliae bound at each temperature tested (4, 23, 34, 37, and 42°C), organisms bound best at 37°C (7.8 ± 1.1% of added organisms), while only 4.2 ± 0.6% of added organisms attached at 4°C. Incubation at temperatures greater than 37°C was detrimental to motility and attachment.

The attachment of radiolabeled *B. burgdorferi* was diminished by greater than 95% when a 100-fold excess of unlabeled organisms was mixed with the ^{35}S-labeled organisms prior to the addition to cultured cell monolayers. However, the extent of attachment was not affected by the addition of a 100-fold excess of unlabeled *T. pallidum* or unlabeled *T. phagedenis* biotype Reiter, a commensal nonpathogenic spirochete. Further, unlike *T. pallidum*, borreliae did not adhere to fibronectin-coated plastic (personal observation). Exposure of borreliae to 60°C for 60 min prior to addition to cell monolayers reduced adherence by greater than 90%.

To assess the possible involvement of borrelial surface proteins as mediators of adherence, we tested polyclonal and monoclonal antibodies for their ability to inhibit attachment. Radiolabeled *B. burgdorferi* was preincubated with dilutions of immune human sera (provided by Allen Steere, Tufts University) or hybridoma supernatants of monoclonal immunoglobulin G antibodies (provided by Alan Barbour) (2–4) prior to adherence assays. When organisms were preincubated with heat-inactivated immune human serum, attachment to HUVE cells was reduced by 65% (Table 1). Incubation of organisms with normal or immune serum did not affect bacterial motility under the assay conditions used. Monoclonal antibodies to the OspB protein (FR14, FR8, and H5TS) brought about inhibition of attachment of the organisms to HUVE cells (39, 36, and 12%, respectively), indicating that OspB may be involved in mediating adherence. A monoclonal antibody against OspA did not affect attachment significantly. However, since only one OspA monoclonal

TABLE 1. Screening of monoclonal antibodies for inhibition of attachment of *B. burgdorferi* to human endothelial cell monolayers[a]

Treatment[b]	Recovered cpm[c]	% Inhibition
None	24,332 ± 484	0
Normal mouse serum[d]	24,172 ± 578	1
Immune human serum	8,516 ± 401	65
MAb H9724 (flagellin)	20,196 ± 523	17
MAb H5332 (OspA)	22,629 ± 611	7
MAb FR14 (OspB)	14,843 ± 495	39
MAb FR8 (OspB)	15,572 ± 658	36
MAb H5TS (OspB)	21,412 ± 594	12

[a] Monoclonal antibodies (MAbs) were characterized and provided by Alan Barbour.

[b] Suspensions containing 5×10^7 organisms (3.37×10^5 cpm) were preincubated for 1 h at 37°C with 1 μl of heat-inactivated whole sera or 50 μl of hybridoma supernatants before being added to HUVE cell monolayers in 24-well microdilution plates. By using indirect immunofluorescence assays (4), the immune serum titer was determined to be 1:350 and all hybridoma supernatant titers were determined to be 1:200.

[c] Average of three determinations plus or minus the standard deviation. Data represent results obtained from three separate experiments.

[d] Heat-inactivated normal human serum was also tested and had no inhibitory effect on adherence to cultured cells.

antibody was available, the possible involvement of this protein as a binding molecule cannot be ruled out. A monoclonal antibody against the flagellin molecule inhibited attachment 17%, a result which was surprising since spirochetes possess flagella which are contained within the periplasm. The *T. pallidum* literature, however, suggests that the flagella may be transiently exposed to the outside (7).

This study demonstrated that the attachment of *B. burgdorferi* to HUVE cells was time and temperature dependent and specific in the sense that (i) cytoadherence was inhibited by treatment with immune human serum but not normal serum and (ii) attachment of radiolabeled organisms was abolished by the presence of a 100-fold excess of *B. burgdorferi* but was not affected by a similar excess of *T. pallidum* or the nonpathogenic spirochete *T. phagedenis* biotype Reiter.

Further investigation of the attachment of *B. burgdorferi* to cultured host cells could lead to an increased understanding of the pathogenesis of Lyme disease. Therefore, future studies will be aimed at identifying *B. burgdorferi* surface molecules implicated in mediating adherence and determining what role attached organisms may play in vivo.

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