A Flagella-less Mutant of *Borrelia burgdorferi*

Structural, Molecular, and In Vitro Functional Characterization

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Abstract

A nonmotile mutant of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, was isolated and characterized. The mutant was compared with the wild-type predecessor as well as with a motile back-revertant of the same genetic background. The mutant lacked, by morphologic, biochemical, and immunologic criteria, the major structural protein of flagella, flagellin. This mutation was not associated with major DNA rearrangements or with failure of transcription. An apparent consequence of a loss of flagella was reduced ability to penetrate human endothelial cell layers in vitro. In another assessment of functional significance, the flagella-less mutant was equal if not superior to flagella-bearing, isogenic isolates when examined in an enzyme-linked immunosorbent assay for anti–*B. burgdorferi* antibodies in the sera of Lyme disease patients. These studies of a mutant, the first among pathogenic *Borrelia* spp. to be characterized, indicate that the flagellum and motility it confers play a role in *B. burgdorferi*'s invasion of human tissues. A flagella-less *B. burgdorferi* may be useful as the basis of a more specific immunoassay and a vaccine for protection against Lyme disease. (*J. Clin. Invest.* 1991. 88:82–92.) Key words: Borrelia • flagella • Lyme disease

Introduction

Lyme disease is a common, disabling tickborne infection in surburban and rural areas of the Northern Hemisphere's temperate latitudes. The clinical features and epidemiology of Lyme disease have been well characterized (1), and the etiologic agent, the spirochete *Borrelia burgdorferi*, has been identified (2). *B. burgdorferi* enters the host's vascular system from the tick bite site and then is distributed via the blood to different organs and tissues, including the brain and joint synovium. In these different tissues the microorganism can persist for months to years. The properties of *B. burgdorferi* that confer invasiveness in human and other mammalian hosts have yet to be identified.

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Ignorance of Lyme disease pathogenesis is in part attributable to the paucity of basic information about all spirochetes. Spirochetes comprise a separate division of bacteria and are only remotely related to the better-known Gram-negative and Gram-positive bacteria (3). The structure of the spirochetal cell is unique in several features (4). Most pathogenic members of the spirochete group are difficult to cultivate, if they can be cultivated at all. Classical genetic experiments with spirochetes would be expected to be time-consuming, and molecular genetic progress is slowed by want of an effective way to transfer DNA into these cells. There are few reports of useful mutant strains among spirochetes; none to date have been described for *B. burgdorferi.* Moreover, discoveries about the virulence factors of other pathogenic bacteria may not be relevant to spirochetes.

One cell structure that spirochetes and other types of bacteria do have in common is the flagellum. The flagellar apparatus is a motility mechanism in spirochetes like other bacteria (5). However, in spirochetes the flagellum is not located at the cell surface, as it is in other bacteria, but instead is entirely periplasmic in location and oriented in the long axis of the cell (4). The axial flagella of spirochetes extends from insertion pores at each cell pole to overlap in the space between the outer sheath and protoplasmic cylinder of the spirochete. In *B. burgdorferi* the flagella appears to be primarily constituted of a single flagellin protein (6). The polypeptide deduced from the nucleotide sequence of the flagellin gene has a molecular mass of 35.8 kD and is similar to the flagellar structural proteins of other bacteria (7, 8).

Surface flagella are virulence factors in some pathogenic bacteria, including *Campylobacter jejuni* (9), *Vibrio cholerae* (10, 11), and *Pseudomonas aeruginosa* (12). In contrast, *Salmonella typhimurium* mutants lacking flagella remained virulent for mice (13, 14). The role of flagella and motility in the pathogenesis of spirochetal diseases is not known. The effects of motility mutations in the spirochete pathogens *Leptospira interrogans* (15) and *Treponema phagedenis* (16) on virulence, attachment, or invasion have not been reported.

Irrespective of their role in virulence and invasion, the surface flagella of pathogenic bacteria often elicit an immune response in infected animals and humans. In *Campylobacter jejuni* (16) and *Salmonella typhi* (17) infections patients develop antibodies to flagella during the course of disease. Antibodies to the major flagellar proteins are also common in these spirochetal diseases: syphilis (18), relapsing fever (6, 19), and Lyme disease (20–22). Isolated flagella of *B. burgdorferi* have been used as the basis of an enzyme-linked immunoabsorbent assay (ELISA) for Lyme disease (23, 24).

An intriguing aspect of *B. burgdorferi* flagella that also prompts further study of this organelle is molecular mimicry between borrelial flagella and human antigens. Aberer et al. (25) and Sigal and Tatum (26) have found that a monoclonal antibody directed against a *Borrelia*-specific flagellar epitope binds to human nerve cells and other tissues. The clinical features and immunologic epiphenomena of Lyme disease have suggested that autoimmunity plays a role in the pathogenesis of the neurologic and arthritic sequelae of *B. burgdorferi* infection (27).

From the foregoing one appreciates that a mutant lacking axial flagella would be useful for studying the pathogenetic mechanisms and immunology of *B. burgdorferi*. We have isolated a nonmotile variant of the Lyme disease. Structural, molecular, and functional features of this mutant and a motile revertant are described herein.

Methods

Strains and culture conditions. Strain HB19, the first human blood isolate of *B. burgdorferi* (28, 29), had been cloned four times previously by limiting dilution or colony plating (30). A low-passage, uncloned isolate of *B. burgdorferi* HB19 was also available. Borrelia were grown at 34°C in tightly capped polystyrene tubes containing BSK I broth medium, which contains CMRL 1066 tissue culture medium (Gibco-Bethesda Research Laboratories, Gaithersburg, MD), fraction V of bovine serum albumin, Neopeptone (Difco Laboratories, Inc., Detroit, MI), *N*-acetylglucosamine, and rabbit serum (31). In some experiments BSK II medium was used (32); this is BSK I medium supplemented with Yeastolate (Difco Laboratories, Inc.), the dessicated, clarified, soluble portion of autolyzed yeast.

Cells were harvested by centrifugation (10,000 *g* for 20 min) and washed three times with phosphate-buffered saline, pH 7.2 (PBS), with 5 mM Mg (PBS/Mg). For routine passage 0.4% vol/vol of a culture at a cell density of 10^8/ml was inoculated into a fresh tube of medium. Cells were counted with a Petroff-Hauser chamber adapted for phase contrast microscopy. For cloning by limiting dilution in broth medium, 10-fold dilutions were inoculated into each of 10 tubes as described (30). After 3 wk of incubation apparently negative cultures were blindly passed to fresh medium and examined by phase-contrast microscopy for an additional 3 wk. Borreliae were also grown as colonies on BSK I medium with 1.5% agarose in candle jars at 34°C (30). Colonies on the plates were picked as plugs with sterile Pasteur pipettes and inoculated into broth BSK I medium supplemented with rifampin (50 μg/ml) and phosphomycin (100 μg/ml).

Monoclonal antibodies and human sera. Monoclonal antibody H9724 binds to native and denatured flagellins of different *Borrelia* spp. (6), and monoclonal antibody H605 is directed against the flagellin of *B. burgdorferi* (33). Sera from 17 adult patients with Lyme disease of duration 6 wk or more from Connecticut, Wisconsin, and Lithuania, areas with a high incidence of Lyme disease, were supplied by L. Magnarelli, New Haven, CT; K. Case, La Crosse, WI; and J. Bunikis, Vilnius, Lithuania, respectively. Sera from 18 healthy adult residents of Rocky Mountain states, a region with a low incidence of Lyme disease, were provided by W. Letson, Centers for Disease Control, Fort Collins, CO.

Polyacrylamide gel electrophoresis and Western blot analysis. Whole-cell lysates of the different strains were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (30). The acrylamide concentration was 12.5%. Western blot analysis was carried out as described previously (30). Hybridoma supernatants were used at a dilution of 1:10, and bound antibody was detected with ^{125}I-labeled Protein A (New England Nuclear, Boston, MA).

Nucleic acid analyses. Total borrelia DNA for Southern blot analysis was extracted by a modification of previously described methods (34, 35). A washed pellet of $\sim 10^9$ borreliae were suspended in 1 ml of 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, centrifuged (10,000 *g* for 3 min), resuspended in 250 μl of 25% (wt/vol) sucrose/10 mM Tris, pH 8.0/50 mM EDTA, and placed on ice for 10 min. To this suspension was added 5 μl of Proteinase K (20 mg/ml of water) and 40 μl of 10% SDS. After incubation of this mixture at 56°C for 1 h, the lysate was extracted with phenol/chloroform and precipitated with ethanol (35). The procedure for Southern blot analysis was essentially as described (30). Restriction enzymes were obtained from Boerhinger-Mannheim Biochemicals, Indianapolis, IN, and used according to recommendations. Restriction fragments were transferred to a Nytran membrane with 0.2-μm pores (Schleicher & Schuell, Inc., Keene, NH). The prehybridization and hybridization solutions were 600 mM NaCl/60 mM sodium citrate/0.1% SDS/0.01% salmon sperm DNA/10× Denhardt's (30) at 45°C. The blots were washed with 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS/1 mM EDTA at 45°C.

Total RNA was extracted and subjected to Northern blot analysis as described (30). Markers in the formaldehyde denaturing gel were *Hae* III fragments of bacteriophage M13. The hybridization and washing conditions were those described above.

An oligonucleotide probe was based on nucleotide sequences common to the flagellin genes for the North American strain B31 and the European strain GeHo of *B. burgdorferi* (7). The 32-mer oligonucleotide was synthesized on a DNA synthesizer (Applied Biosystems Inc., Foster City, CA) as the complement to nucleotides 103–135 of the published sequence of Gassmann et al. (7). The oligonucleotide was labeled at the 5′ end with [γ-^{32}P]ATP as described (35).

Enzyme-linked immunoabsorbent assay (ELISA). The ELISA with whole spirochetes was a modification of the method of Magnarelli and Anderson (36). Harvested borreliae were suspended in PBS/Mg; the total cellular protein in the suspension was estimated with the Bradford reagent (Bio-Rad Laboratories, Richmond, CA). The cell suspension was adjusted with carbonate buffer (36) for a protein concentration of 1.4 mg/ml, and 50 μl of the suspension was added to flat-bottomed wells of polystyrene microtiter plates. After incubation of the plates for 18 h at 37°C, 200 μl of 1% dried nonfat milk in PBS was added to each well. Plates were incubated for 1 h at 37°C and washed four times with 200 μl of PBS; 60-μl volumes of serum diluted 1:500 in 1% nonfat milk/PBS were added to each well in triplicate. The plates were incubated for 1 h at 37°C and then washed with PBS. Bound IgG antibody was assayed with horseradish peroxidase-conjugated, anti-human IgG (γ chain) goat antisera (Calbiochem-Behring Corp., San Diego, CA) in 1% nonfat milk/PBS buffer. The substrate for the peroxidase reaction was *O*-phenylenediamine dihydrochloride, and absorbance values at 490 nm to a maximum of 1.500 were recorded on an ELISA reader (model 580, Dynatech Laboratories, Inc., Alexandria, VA). Results were plotted with a Macintosh II computer (Apple Computer) running Cricket Graph (version 1.3.1; Cricket Software); a simple best-fit curve was generated for each pairwise comparison.

Endothelial cell association and penetration assays. Assays for association and penetration of intrinsically labeled spirochetes with and through human umbilical vein endothelial (HUVE)[1] cells were carried out as described by Comstock and Thomas (37). HUVE cells were isolated from freshly delivered human umbilical cords by the method of Jaffe et al. (38). All assays were done in triplicate.

For assessing HUVE association, borreliae were intrinsically radiolabeled with [^{35}S]methionine as described (37), washed with PBS, and resuspended to a density of 10^8 bacteria and specific activity of 2.4×10^5 cpm per ml of Medium 199 (M199) with 15% fetal calf serum (FCS). To confluent HUVE cell monolayers grown in 24-well plates were added 0.5-ml aliquots. After a 3-h incubation at 37°C, monolayers with associated organisms were washed, solubilized, mixed with scintillation cocktail, and counted by scintillation (39).

For penetration experiments, 2.5×10^4 HUVE cells were seeded onto sterile polycarbonate membrane culture plate inserts (3-μm pore

1. *Abbreviation used in this paper:* HUVE, human umbilical vein endothelial (cells).

size; 6.5 mm diam; Nuclepore Corp., Pleasanton, CA). Chambers were placed in 24-well plates containing 1 ml of M199/15% FCS per well and incubated for 48 h, at which time the monolayers were confluent and possessed high transendothelial electrical resistance. Bacteria were quantitated by darkfield microscopy, centrifuged for 15 min at 17,000 *g*, and resuspended in M199/FCS. For each assay, 0.2-ml samples containing 3×10^8 bacteria were added to the upper portions of the chambers (above the monolayers). After a 4-h incubation at 37°C in 5% CO_2 in air, aliquots from beneath the filters were removed and spirochetes were counted by darkfield microscopy (37). In this study 3×10^8 spirochetes with a specific activity of 5×10^5 cpm were added to each chamber in a volume of 0.2 ml, and the samples were incubated at 37°C for 4 h. At the end of the incubation the amount of radioactivity in the lower chamber was measured.

Electron microscopy. For studies with negative stains harvested borreliae were resuspended in one-tenth volume of PBS, applied to a carbon-coated 300-mesh grids, and stained for 30 s with 2% (wt/vol) ammonium molybdate, pH 7.2, in distilled water; air-dried grids were immediately examined in a transmission electron microscope (EM 1200, JEOL USA, Inc., Peabody, MA). For thin section studies of interactions of borreliae membrane filters with attached monolayers and associated borreliae were prepared as described previously (37). The membrane filters were cut from the culture plate inserts, rinsed in PBS, fixed in glutaraldehyde, stained, dehydrated, and embedded in epoxy resin. Transverse thin sections were placed on copper mesh grids and stained with lead citrate and uranyl acetate prior to examination in a transmission electron microscope (TEM 400, Philips Electronic Instruments, Inc., Mahway, NJ).

Results

Isolation of a nonmotile mutant of B. burgdorferi. The population of HB19 cells used for cloning was designated "W" for "wild-type"; ~ 300 generations had occurred since the last single cell cloning. Of the 3 out of 10 tubes at the highest dilution that had borrelial growth, one contained cells that differed substantially in appearance from W-type cells in the other two tubes. When examined by phase-contrast microscopy, the variant cells were nonmotile and straighter than W cells. Dark field examination also showed the non-motile isolate to be straighter, but, by this examination, the nonmotile mutant retained its basically helical character, albeit one of lower amplitude and greater pitch. The greater motion of W cells was captured as the slight blurring of the cell images in the photograph for Fig. 1.

The variant was also distinguished from W cells by its tendency to form aggregates visible by microscopy but not by naked eye (Fig. 1) and by its lesser ability to turn the medium's phenol red indicator yellow at equivalent cell densities. The latter characteristic suggested that the nonmotile variant had a lower metabolic activity than its motile parent.

A sample of the variant cell population was plated on BSK agar for a second round of cloning. Four well-isolated colonies were picked and grown in broth medium. These other clonal populations had the same nonmotile phenotype when examined by phase contrast microscopy. One of the second group of clones was arbitrarily selected for use in subsequent experiments and was designated "M" for "mutant."

When M cells were passed in medium without Yeastolate, at least 99% cells in each tube's population remained nonmotile after 10–15 passages, or 80–120 generations. However, when the M mutant was passed in complete BSK II, which contains Yeastolate, motile bacteria with a morphology like W cells constituted at least 0.1% of the cell population in the culture tubes by three to six passages, or 24–48 generations. When these mixed cultures were subsequently continuously passed in BSK II medium the motile cells consistently came to predomi-

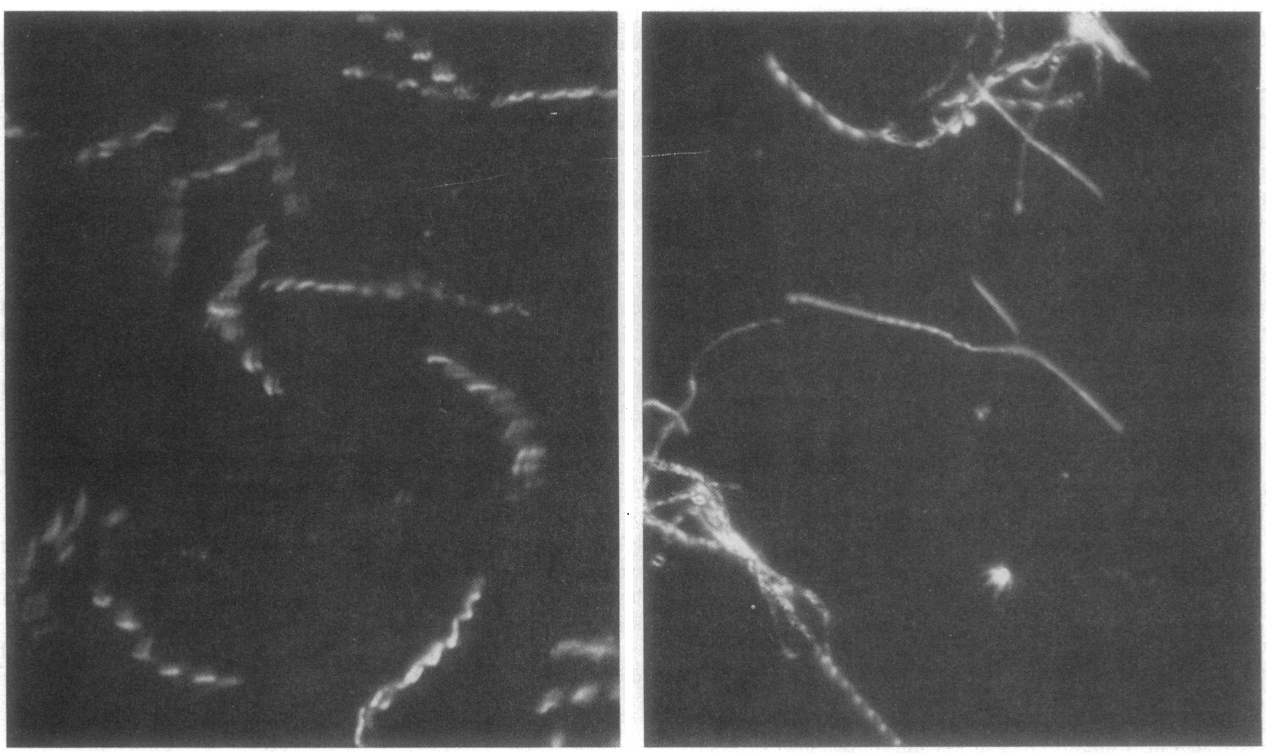

Figure 1. Dark field photomicrographs of motile wild-type cells (*left*) and a nonmotile mutant (*right*) of *B. burgdorferi* strain HB19. The magnification is 1,260.

nate. Thereafter, the population retained the wild-type phenotype even when recultivated in BSK I medium. Cultures of these revertant motile cells, unlike M cells, changed the color of the phenol red in the medium at the same cell concentration as W cells. This revertant motile population, designated "R," was cloned by limiting dilution for subsequent experiments.

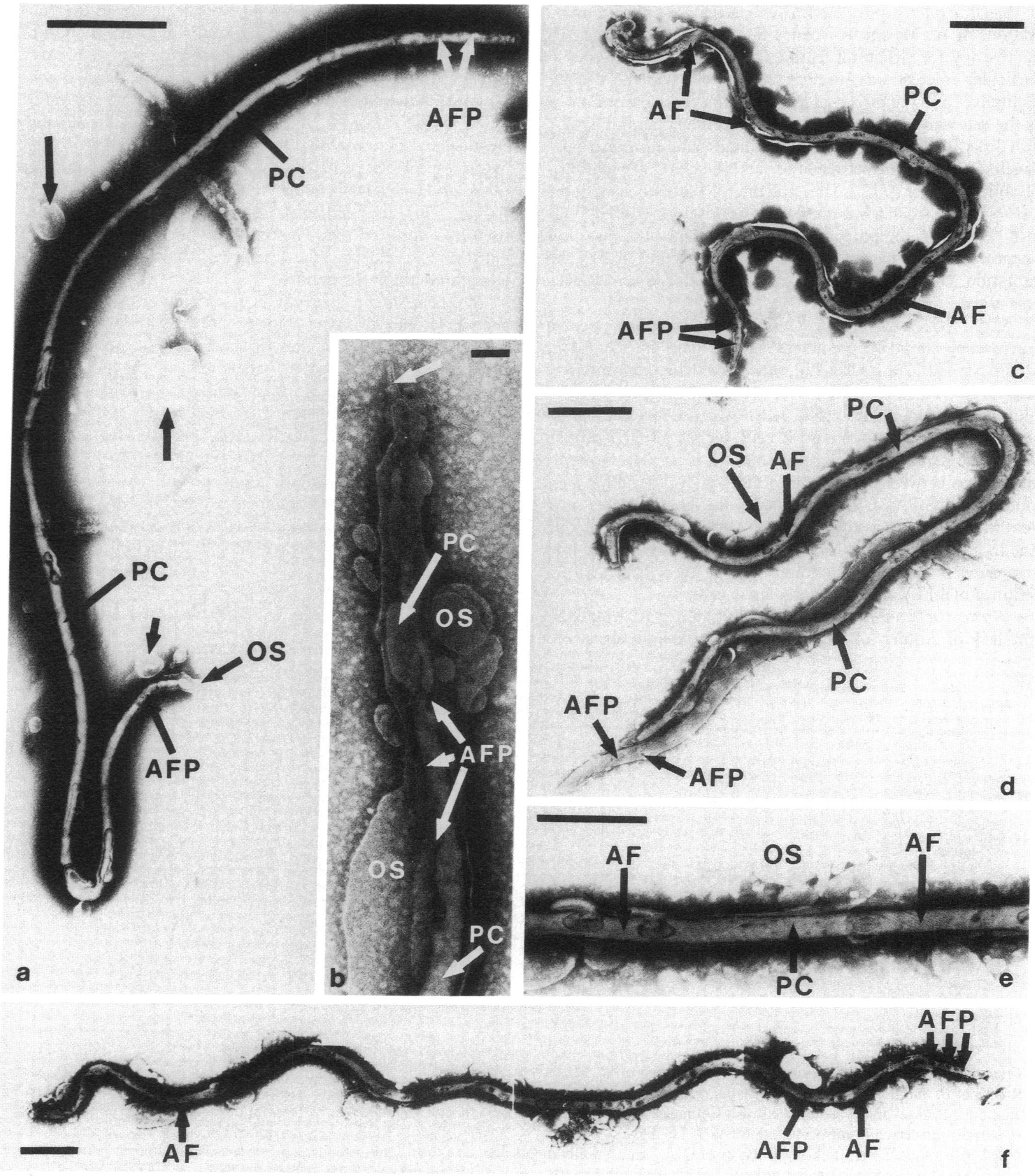

Figure 2. Electron photomicrographs of negatively-stained W, M, and R cells of *B. burgdorferi* strain HB19. W cells (plates *c, d,* and *f*) possess numerous axial flagella (*AF*) which overlay the helical protoplasmic cylinder (*PC*). The loose-fitting outer sheath (*OS*) encloses the cell. Axial flagella pores (*AFP*) are also apparent. M cells (*a* and *b*) possess a loosely outer sheath and axial flagella pores with emerging hooks but not axial flagella themselves. R cells (*e*) have axial flagella. Bar, 1.0 μm (*a, c, d, f*), 0.5 μm (*e*), or 0.1 μm (*b*).

From these studies we concluded that a mutation had occurred in a population of W cells and that this mutation conferred a nonmotile phenotype to the cells bearing it. We next compared the structure and cellular proteins of the M cells with W and R cells.

Electron microscopy of spirochetes. Negatively stained preparations of W, M, and R isolates were examined by electron microscopy for structural features of spirochetes (Fig. 2). Of particular interest was whether the M cells had the flagella themselves and the proximal hooks, which emerge from pores in the cell wall to connect the flagella to the apparatus' insertion disks (4). The motile W and R isolates had full-length axial flagella (*AF*) running between the outer sheath (*OS*) and protoplasmic cylinder (*PC*). The straighter M isolate had pores (*AFP*) for the flagella but not flagella themselves. Hooks could also be seen in the pores of the M cells. Thus, the insertion apparatus for the flagella did not appear to be affected by the mutation. No difference in the morphology of W and R cells was noted.

SDS-PAGE and Western blot analysis. From the electron microscopy study we predicted that the flagella-less mutant would have little or no flagellin, the major structural protein of flagella (6). This proposition was examined using SDS-PAGE and Western blot analysis (Fig. 3). SDS-PAGE revealed that M cells, in distinction to W and R cells, lacked a major protein with an apparent molecular weight of 37×10^3 (K). No other differences between the protein profiles of W, M, and R were noted by SDS-PAGE. The identity of the 37,000-mol wt protein with flagellin was confirmed by Western blot analysis with the monoclonal antibodies H9724 and H604. Even with long exposures of the radioautographs full-length or truncated flagellin protein was not detectable in M cells.

Nucleic acid analyses. The preceding studies indicated that the lack of motility of M cells was attributable to absence of cell-associated flagellin protein. To begin the determination of the genetic basis for this mutation we carried out two studies of the nucleic acids of W, M, and R cells. In the first study we used an oligonucleotide probe for the flagellin gene to assess if there had been deletion of all or a major part of the gene. DNA was digested with *Rsa* I, which would be expected from the published nucleotide sequence of two strains of *B. burgdorferi* to produce a 0.6-kb fragment that contained the complementary sequence to the probe and more than half of the flagellin gene (7). The Southern blot analysis is shown in the left panel of Fig. 4. The expected *Rsa* I fragment was hybridized by the oligonucleotide and there was no difference between the three isolates in this respect. The probe did not bind to lambda bacteriophage DNA or *E. coli* DNA under the same conditions (data not shown). This study indicated that a large deletion in the gene itself had not occurred. The Southern blot analysis also provided genetic evidence that M cells were *B. burgdorferi* and not a contaminant in the culture.

We next sought evidence of transcription of the flagellin gene in M cells. Using the same probe, we examined extracted mRNA of the three isolates by Northern blot analysis. The results are shown on the right panel of Fig. 4. The three isolates produced a mRNA species that hybridized with the flagellin probe. There was no difference between the size of hybridizing RNA bands of W, M, and R cells. The probe did not bind under these conditions to mRNA extracted from *E. coli* and used as a negative control (data not shown).

The finding that the flagellin gene was transcribed to its full or near-full length suggested that either the mutation involved only a small number of nucleotides in the flagellin gene itself or

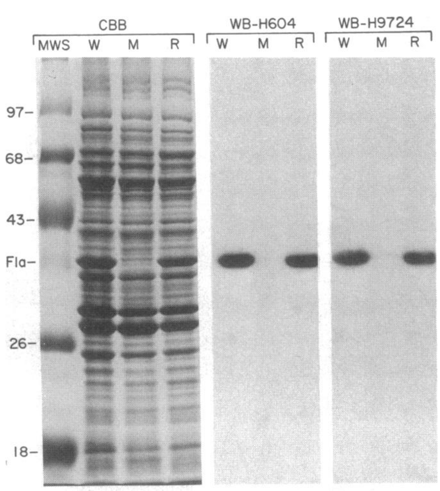

Figure 3. SDS-PAGE and Western blot (*WB*) analyses of W, M, and R cells of *B. burgdorferi* strain HB19. Total cell lysates were fractionated on a 15% gel and either stained with Coomasie Brilliant Blue (*CBB*) or transferred to nitrocellulose for WB. The blots were incubated with monoclonal antibody H604 or H9724. Bound antibody was detected with radioiodinated protein A. The position of the flagellin (*Fla*) protein in the gel is indicated. The molecular weight standards (*MWS*), given in thousands as indicated on the left, were phosphorylase B (97), bovine serum albumin (68), ovalbumin (43), α-chymotrypsinogen (26), and β-lactoglobulin (18).

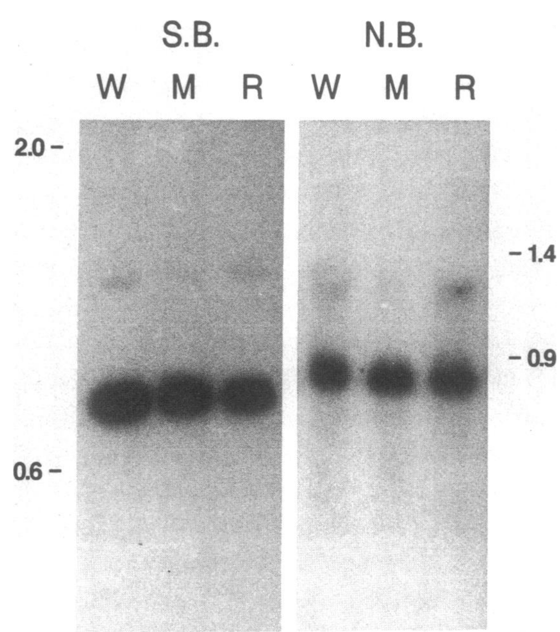

Figure 4. Southern blot (*S.B.*) and Northern blot (*N.B.*) analyses of W, M, and R cells of *B. burgdorferi* strain HB19. In the Southern blot *Rsa* I digests of DNA were separated on a 1.0% agarose gel; the position of the double-stranded size standards (in kilobase pairs) in the gel are indicated are on the left. In the Northern blot total RNA was separated in a 1.5% agarose denaturing gel; the positions of the single-stranded size standards (in kilobases) in the gel are shown on the right. For both blots the probe was an oligonucleotide specific for the flagellin gene of *B. burgdorferi* (see text).

was located elsewhere in the genome. Mutants of the latter type would be expected to have a comparatively higher number of revertants to wild-type phenotype among its progeny than of those cells bearing mutations caused by losses or rearrangements of larger amounts of DNA (40).

ELISA of patient and control sera. Antibodies to flagellin have been reported to be a prominent part of the immune response in early and late Lyme disease (20–22, 41, 42). To assess the contribution of antibodies to flagellin in a standard immunologic assay for anti–*B. burgdorferi* antibodies we compared whole cells of W, M, and R by ELISA. The aim of the experiment was to determine whether the total amount of bound antibody would be detectably lower when the flagella-less mutant was used as an antigen than when its flagella-bearing counterparts were used. Sera from patients and controls were examined for their reactivities against whole cells of W, M, and R in an ELISA when equivalent amounts of total cellular protein were used as antigen. In these experiments we found that the flagella-less cells were equal if not superior to flagella-bearing spirochetes in an immunologic assay that measures total IgG against *B. burgdorferi.*

The mean absorbance values (±SEM) for the 17 patient sera were 1.09 (±0.09) for W, 1.31 (±0.08) for M, and 1.15 (±0.09) for R cells. Analogous determinations for 18 control sera were 0.19 (±0.03), 0.18 (±0.03), and 0.20 (±0.03), respectively. Fig. 5 shows the pairwise comparisons for W, M, and R cells when patient and control sera were used in the ELISA. Whereas similar results were obtained with W and R cells for patient sera and all three isolates with control sera, the flagella-less M cells had an equal or higher absorbance reading for each patient serum when compared with W and R cells. Many of the values with M cells in the ELISA were $\geq$ 1.500, the maximum absorbance reading. When sera were further diluted to 1:1,000, consistently higher absorbance values were still obtained with M cells (data not shown). Similar results were obtained with this collection of sera when different batches of antigens were prepared from W, M, and R cells.

Association with and penetration of endothelial cells. The association of intrinsically labeled borreliae with human endothelial cells was studied. The binding of the isolates W, M, and R were compared with each other and also with an early passage isolate of the parent strain HB19. The results are given in Table I. This experiment showed that ~ 20% fewer of the flagella-less M spirochetes than the W and R spirochetes bound to the cells. As had been demonstrated previously (39), the high-passage isolates bound to cells less well than the early-passage version of the HB19 strain.

In the next experiment we studied the penetration of the four isolates of HB19 through HUVE monolayers. The results are given in Table II. Neither the W nor R isolates of HB19 penetrated HUVE cells as well as the low-passage isolate of HB19. R cells, which penetrated the monolayer less well than W, had undergone two more clonings by colony plating or limiting dilution than W cells. The greatest difference between the four isolates, however, was between M cells and the three motile forms of HB19. About 95% fewer M cells than W or R penetrated the cell monolayer. This difference in penetration was greater than could be attributed to decreased adherence.

The penetration of HUVE cells by the W, M, and R spirochetes was also assessed using transmission electron microscopy. Fig. 6 shows a typical result when M and R organisms were incubated with HUVE cell monolayers. In cross-section both R and M cells had discernible protoplasmic cylinders and outer sheaths but M cells, unlike R cells, did not have axial flagella (*insets* in Fig. 6, *A* and *B*). Both R and M spirochetes adhered to the HUVE cell surface in a manner indistinguishable from that observed with an early passage isolate of strain HB19 (37). When the cytoplasms of HUVE cells were examined in thin sections, differences between R and M in the number of intracellular spirochetes were noted. Whereas R spirochetes were observed in 57 (95%) of 60 HUVE cells examined, only 2 (3%) of 65 HUVE cells showed evidence of containing an intracellular M cell.

Discussion

These nonmotile "spirochetes" are the first mutants of a pathogenic *Borrelia* sp. to be characterized structurally and functionally. The distinctive morphologic and behavioral phenotype of M cells is attributable to lack of the major structural protein, flagellin. We concluded this because the revertant R regained flagella, helical morphology, and motility at the same time. Another reason for crediting nonmotility to lack of flagellin expression was the apparent presence in M cells of all discernible *B. burgdorferi* proteins and flagellar components save flagellin.

Although the actual genetic basis for this mutation in flagellin expression remains to be determined, the findings to date do suggest that some types of mutation are more likely than others. For instance, the transcription of the flagellin gene to its apparent full length is suggestive of a frame shift or nonsense mutation in the coding region of the flagellin gene or its ribosomal binding sequence, thereby producing translational failure or premature termination. If a large deletion involving the flagellin gene had occurred, we would not expect to see a full-length transcript. Alternatively, these data are consistent with a model in which the M cell mutation is not in the flagellin gene or its regulatory regions but in the gene for a putative minor protein that anchors the flagellin to the hook apparatus or in a gene affecting translation of the flagellin transcript. The latter type of genetic deficiency is the basis for a non-motile mutant of *S. typhimurium* that is characterized by the release of its major flagellar proteins into the medium (43). Although a defect in anchoring cannot at this time be ruled out, we think that either a frame shift or nonsense mutation is a more likely explanation of the flagella-less phenotype. For one thing it is hard to conceive how free flagellin protein, even in monomer form, could move across the outer sheath into the medium without leaving a trace in the periplasmic space. Furthermore, immunoprecipitation assays of concentrated spent medium from M cell cultures have revealed no evidence to date of excess free flagella in the medium (unpublished findings).

The apparent instability of the M mutant in medium containing Yeastolate has been a consistent finding in these and other experiments. When other ingredients of BSK II medium were deleted, there was not a significant difference between reversion frequencies (unpublished findings). It is possible that flagella-bearing cells have selective advantage over flagella-less cells in Yeastolate-containing medium. Another explanation is that the back-mutation rate is higher in the presence of a Yeastolate component or components. Finally, it is possible, as discussed above, that the actual mutation is not in one of the

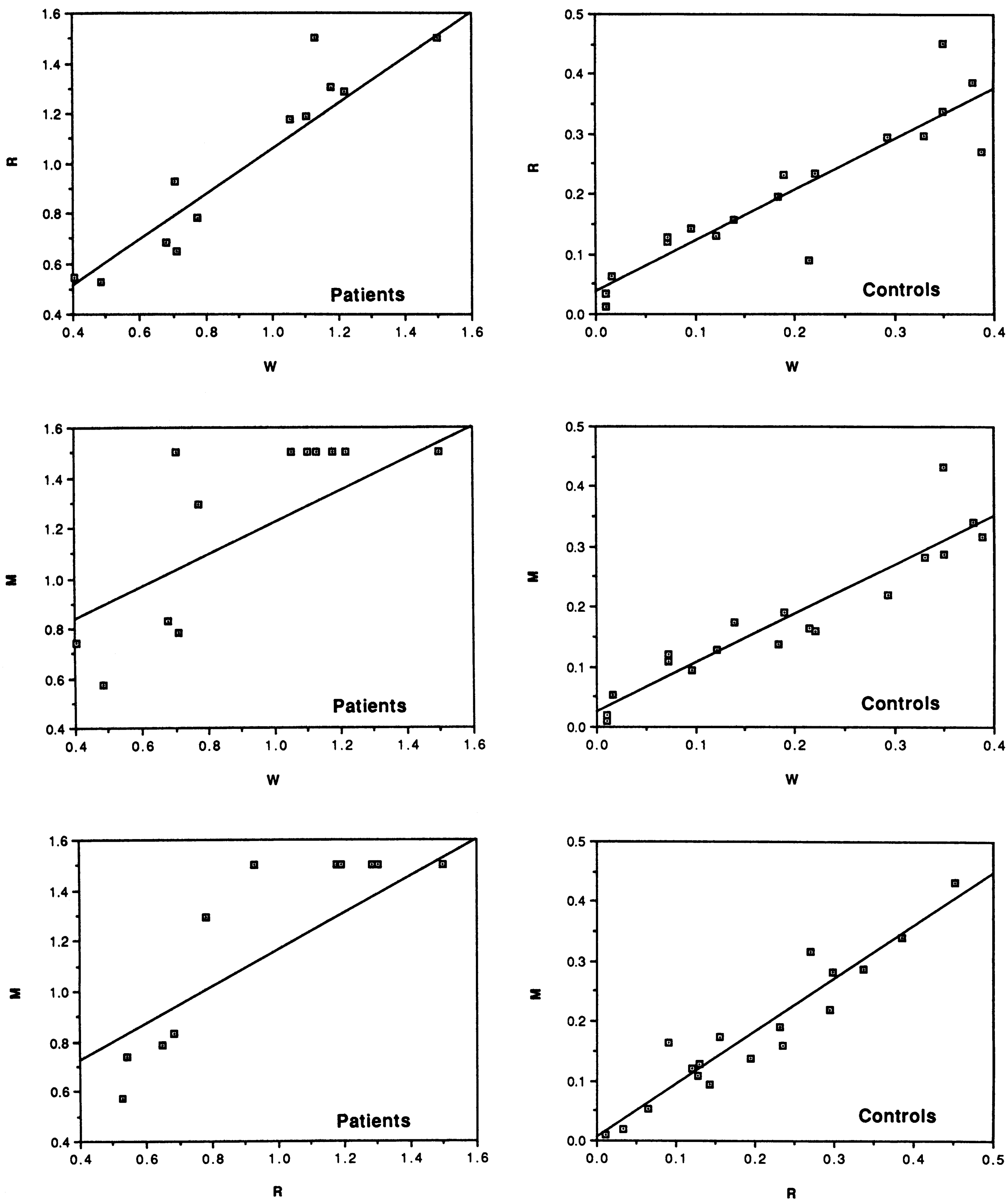

Figure 5. Comparison of W, M, and R cells of *B. burgdorferi* strain HB19 in an ELISA with Lyme disease patient and control sera. The *x*- and *y*-axes are the absorbance values from the assays. Points denote results with individual sera in the pairwise comparison. When absorbance results sera were ≥ 1.500 with both antigens in a comparison, a single point is shown for two or more sera. A simple best-fit curve was calculated for each pairwise comparison.

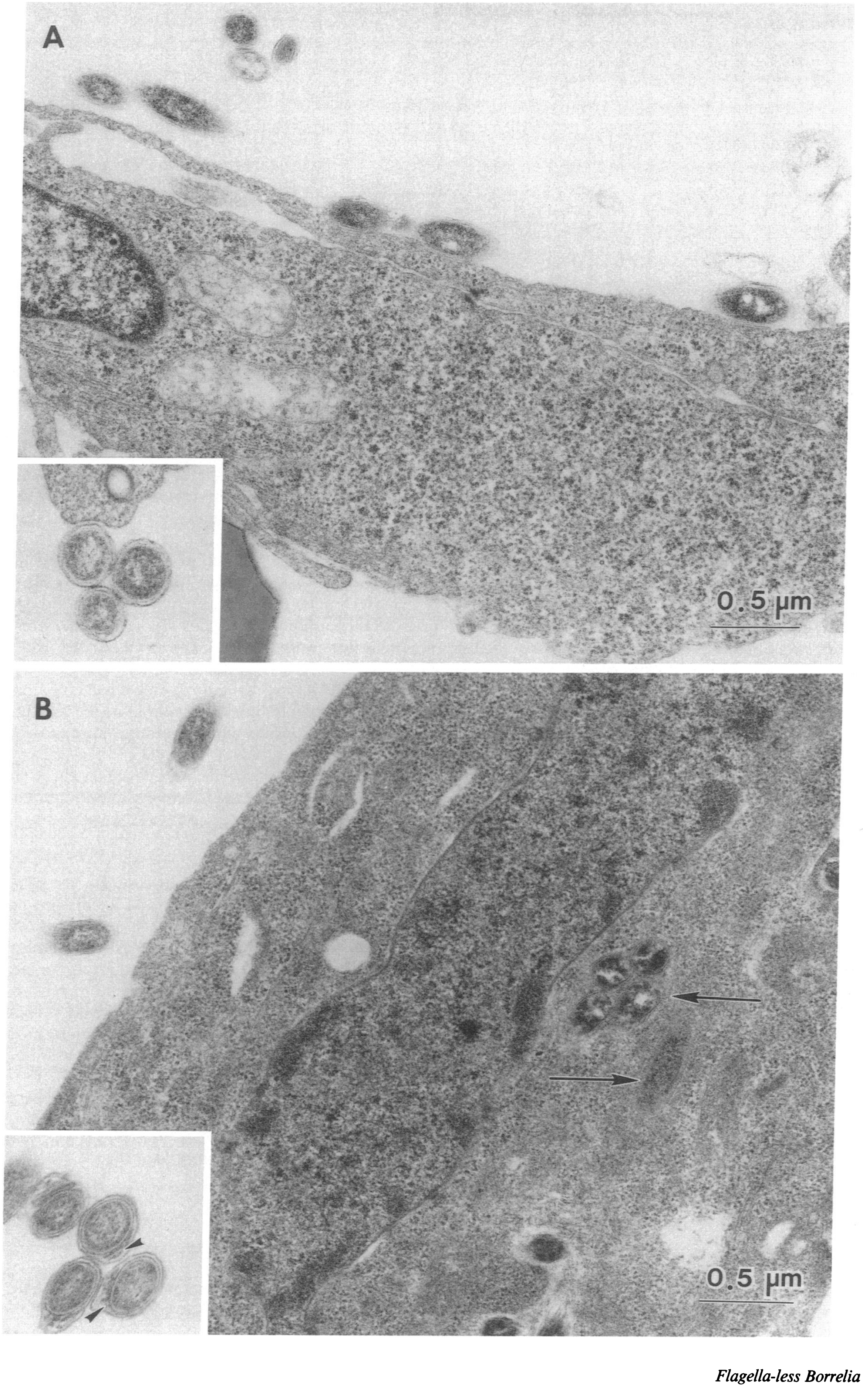
A
0.5 µm
B
0.5 µm

Table I. Host Cell Association Assay

Borrelia isolate	Mean counts per minute cell associated (±SEM)	Mean percentage of inoculum cell associated (±SEM)
W	6,880 (±342)	5.7 (±0.3)
M	5,173 (±276)	4.3 (±0.2)
R	6,256 (±336)	5.2 (±0.3)
Low passage HB19	8,208 (±425)	6.8 (±0.4)

Table II. Penetration of HUVE Cell Monolayers

Borrelia isolate	Mean counts per minute in lower chamber (±SEM)	Mean percentage of inoculum in lower chamber (±SEM)
W	33,504 (±1,144)	6.8 (±0.2)
M	887 (±283)	0.2 (±0.1)
R	12,810 (±569)	2.6 (±0.1)
Low passage HB19	41,387 (±1,709)	8.4 (±0.4)

structural genes in flagella synthesis cascade. The mutation may instead be in a gene that affects flagellin synthesis post-transcriptionally. According to this last model, the selection in Yeastolate's absence is not so much operative on the flagella phenotype per se as on other, unidentified phenotypic features of the genotype. The actual basis for Yeastolate's effect remains to be determined. Examination of its many components individually and in groups may be required; Yeastolate contains most of the amino acids and several water-soluble vitamins (unpublished findings, Difco Laboratories). This effort may be justifiable, though, because the reversion-enhancing properties of Yeastolate may have their equivalents in vivo.

Having characterized the mutant with regard to certain features of its morphology, protein constitution, and DNA organization, we next considered aspects of the immunology and function of the flagella. The contribution of the flagella to the antigenic make-up of *B. burgdorferi* was examined by comparing the total antibody responses of Lyme disease patients and controls to whole cells of W, M, and R. We reasoned that if flagella constituted a major part of the total antigenic mass recognized by patients' antibodies, there would be detectably less bound antibody when M cells were used in the assay instead of W or R cells. For these experiments the quantity of antigen and dilution of serum were those that gave the greatest discrimination between positive and negative standard sera for W cells in a preliminary assay. It is possible that at considerably different antigen and antibody concentrations the distinction by ELISA between M cells and either W or R cells would not have been as marked. Nevertheless, with this group of patient and control sera and under these test conditions, there was no apparent benefit to inclusion of flagella in the assay with regard to specificity or sensitivity. The greater amount of antibody binding to M cells perhaps was due to the correspondingly greater amount of nonflagellar antigens on the plates when flagella were not present.

Some investigators have proposed that isolated flagella are a sufficient basis for an immunoassay for *B. burgdorferi* infection: Hansen and his co-workers found that the flagellar antigens of *B. burgdorferi* were useful for diagnosis of early Lyme disease (23, 24). Other investigators found that healthy individuals, as well patients with other infectious diseases, have detectable antibody to *B. burgdorferi* flagellar epitopes; Coleman and Benach (22) and Grodzicki and Steere (41), who each used different methods for flagellin preparation, did not find that isolated flagellin was sufficiently discriminatory or sensitive for use in the diagnosis of early stage Lyme disease. Antigenic crossreactions between *B. burgdorferi* and other bacteria are not surprising given the conservation of primary sequence between flagellins of different types of bacteria (8). From the present study and a review of the literature we conclude that the contribution of flagellar antigens, in either monomer or polymer form, to an immunassay's sensitivity may not sufficient to offset the loss of specificity that comes with inclusion in the assay of a cross-reactive antigen of this type. Indeed, there is much to be said for deleting flagella from whole-cell or subunit antigen preparations when greater test specificity is the goal.

A flagella-less strain of *B. burgdorferi* may be a safer starting point for a vaccine for protection against Lyme disease. Demonstrations of antigenic cross-reactions between flagellar-associated antigens and human tissues, including nerve and muscle (25, 26), suggest that vaccines containing even small amounts of flagellar materials may induce an autoimmune reaction in the recipient. Another possible advantage of an immunogen preparation lacking flagella is that antibodies to flagella, or, better, antibodies to pathogen-specific peptides of flagella, can be used to assess the efficacy of the vaccine. If an immunized person became ill with suspected Lyme disease, the presence of antibodies to pathogen-specific peptides of flagellin would indicate vaccine failure.

The penetration of human endothelial cells by the flagella-less M cells was significantly less than W and R borreliae in spite of approximately equal measures of cell association for W, M, and R cells. Whereas the penetration of R cells was also less than W cells, the differential between W and R cells was an order of magnitude less than that between W and M cells. R cells represented a population that had undergone additional rounds of cloning. As has been documented previously (37, 39), as well as presently by inclusion of an early version of strain of HB19, multiply-passaged isolates, such as W, associate with and penetrate human cells in lower numbers than their lower-passaged counterparts. Consequently, during in vitro cultivation there may be further selection against borreliae expressing determinants of penetration performance in these assays.

Figure 6. Thin-section electron photomicrographs of penetration of HUVE cells by M and R isolates of *B. burgdorferi* strain HB19. (*A*) Transverse section showing the M spirochetes attached to the surface of, but not within, a HUVE cell; bar, 1.0 μm. (*Inset*) Cross section of M spirochetes demonstrating lack of axial flagella (*arrows*). (*B*) Micrograph showing R spirochetes (*arrows*) within a HUVE cell. Bar, 1.0 μm. (*Inset*) Cross section of R spirochetes, which possess axial flagella (*arrows*).

A reasonable explanation for the significantly poorer penetration of the endothelial monolayer by M cells is that the motility conferred by flagella is an important factor for spirochetal invasion. It follows from this that we will find that a mutation in a gene concerned with flagella production is the proximate cause for a major loss of invasiveness. Alternatively, the poor invasiveness of the M isolate may be an effect of an undetected and unrelated second mutation in another gene. This latter explanation is unlikely, because the revertant isolate regained at the same time flagella and, to a large extent, its invasive properties. A third model specifies that the loss of invasiveness is the consequence of single but pleiotropic mutation in the M mutant. In other words, one or more other borrelia genes are silenced in expression by a putative "M" regulatory mutation. This third explanation cannot at this time be excluded, but even if it holds, an association between flagella and invasion still exists.

The present study demonstrates of the utility of isogenic mutants for studies of pathogenesis and immunity of spirochetal diseases. Among *Borrelia* spp. characterized mutants for these purposes had not hitherto been available. With advances in techniques of site-specific mutagenesis and for delivery of DNA into various type of organisms, it should be possible to be even more direct and precise in answering questions about the pathogenesis of Lyme disease by *B. burgdorferi.* Development of noninvasive or otherwise nonvirulent mutants of *B. burgdorferi* also makes possible the use of live organisms for immunization. Limited replication of an attenuated spirochete, like the flagella-less mutant described here, in the host may be necessary for optimum development of long-lasting immunity to these pathogens.

Finally, we consider the biological significance of the flagella variation phenomenon described here. From the spirochete's viewpoint the switching on-and-off of flagella synthesis —in our less teleologic terminology a "mutation" and "backmutation"—provides possible advantage in its vertebrate or arthropod host. For instance, a nonmotile variant need expend less energy than its flagella-bearing counterpart to survive. Once a borrelia has gained access via motility to certain niches in the host, such as the nervous system, the pressure for further migration in its environment may diminish, and consequently nonmotile variants in the population may fare better over the long term.

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