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Specific Adherence of *Borrelia burgdorferi* Extracellular Vesicles to Human Endothelial Cells in Culture

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***Borrelia burgdorferi* produces extracellular vesicles which contain some of the outer surface proteins of the bacterium (e.g., OspA and OspB). Borrelial vesicles, isolated by differential centrifugation and filtration, were tested for the ability to bind to cultured human umbilical vein endothelial (HUVE) cells in culture. The recently described lipoprotein OspD was expressed on vesicles. Vesicles exhibited differential expression of OspB and OspD in a relationship with passage number and medium serum supplement type, respectively. Qualitative immunoblotting analyses demonstrated dose-dependent, passage number-dependent adsorption of vesicles by HUVE cells. This adsorption was demonstrated to be dependent upon a borrelial component of the vesicle and not due to the presence of minor contamination with intact spirochetes. Quantitative experiments examining inhibition of *B. burgdorferi*-HUVE association as a function of prior vesicle-HUVE association demonstrated dependence upon (i) a borrelial component(s) in the vesicle, (ii) low passage number, and (iii) vesicle protein concentration. However, vesicle pretreatment of the HUVE cell monolayer was not requisite for this inhibition. Vesicles from highly passaged borrelias were noninhibitory for *B. burgdorferi*-HUVE cell association, regardless of the serum used to supplement the medium. The use of vesicles as a tool for studying *B. burgdorferi* pathogenesis and/or physiology is proposed.**

Lyme borreliosis is an arthropod-borne disease of considerable importance in several areas of the world. The spirochetal agent of the disease, *Borrelia burgdorferi*, is carried by tick vectors, usually of the *Ixodes ricinus* complex (32), and is injected into a vertebrate host's vascular system during or after a blood meal. The symptoms of Lyme borreliosis are observed primarily in regions other than the vascular system (e.g., cutaneous erythema migrans lesions, arthritic syndromes, and cardiac and/or neurologic manifestations; 2, 10, 30).

The suggestion that the spirochetes must leave the circulatory system at some point prior to symptomatic disease has prompted several laboratories to study the interactions of *B. burgdorferi* with cultured endothelium in vitro. *B. burgdorferi* association with and penetration of human umbilical vein endothelial (HUVE) cells in monolayers has been described (7, 21, 33, 34). Fab fragments of monoclonal and polyclonal antibodies to outer surface protein A (OspA; 6) and intact monoclonal antibodies to OspB (34) have demonstrated inhibitory effects on the adherence of borrelias to HUVE cells. Also, borrelial pretreatment with intact monoclonal antibodies to OspA and flagellin resulted in the inhibition of borrelial adherence to HEp-2 cells in vitro (5). Borrelial adherence has been demonstrated to be dependent upon the passage number of the tested strain (33, 34), as low-passage strains were found to bind at up to 30-fold greater levels than high-passage strains (33). This is reflective of the finding of Schwan et al. that highly passaged strains showed markedly reduced virulence in animal model systems (29).

B. burgdorferi produces or liberates extracellular vesicles (also known as blebs) by some undescribed mechanism. Reports have described vesicle production in vitro (3, 12), and immunologic evidence indicates their production in infected hosts as well (9). These vesicles have been described as containing (i) a low-molecular-mass lipoprotein of ~6 to 10 kDa (by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [PAGE]; 16, 17), (ii) OspA and OspB (9) and (iii) an 83-kDa antigenic complex of rabbit immunoglobulin M (IgM) with OspA and OspB (molecular mass determined by SDS-PAGE; 8), distinct from the ~83-kDa Lyme disease patient serum-reactive antigen described by Lefebvre et al. (20). The previously described B-cell mitogen (27) produced by *B. burgdorferi* has also been reported to be enriched in vesicle preparations (37). Vesicles have also been demonstrated to contain both linear and circular plasmid DNAs in an intact and DNase I-resistant state, as assessed by transmission electron microscopy (12).

Because extracellular vesicles exhibit at least two of the major surface proteins, OspA and OspB, and because there is evidence suggestive of a role for these proteins in borrelia-HUVE cell interactions, we examined the potential ability of vesicles to interact with cultured HUVE cells. This study demonstrated that *B. burgdorferi* extracellular vesicles associated with cultured HUVE cells and that vesicles competed for the same HUVE cell receptor(s) as intact, viable borrelias. We propose that because of this vesicle-HUVE cell association reaction and the relatively simplistic protein profile of the vesicles, *B. burgdorferi* extracellular vesicles may serve as an important tool for describing or defining the *B. burgdorferi* adhesin(s), as well as offer a mechanism for the observed chronicity of the disease state in the apparent absence of cultivable organisms.

(Portions of these data have been presented at the Vth International Conference on Lyme Borreliosis, Arlington, Va., 30 May to 2 June 1992; the 1st Bristol-Myers Squibb Symposium on Infectious Disease Research, Monterey, Calif., 22 to 23 February 1993; and the 93rd General Meeting of the American Society for Microbiology, Atlanta, Ga., 16 to 20 May 1993.)

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MATERIALS AND METHODS

Bacterial strains and media. *B. burgdorferi* HB19, a human blood isolate (31), was propagated at 34°C in BSK II liquid medium (1) supplemented with 6% unheated, filter sterilized normal rabbit serum (NRS; GIBCO Laboratories, Grand Island, N.Y.) or 6% unheated, filter-sterilized normal human serum (NHS; prepared in our laboratory). By definition in our laboratory, strains passaged fewer than 11 times from the original isolate are considered to be low passage.

For some experiments, borrelias were radiolabeled by overnight incubation in BSK II plus 10 μCi of [^{35}S]methionine (Express^{35} Label; New England Nuclear Corp., Boston, Mass.) per ml. Borrelias were pelleted at 13,000 × *g* for 30 min at 25°C and washed once in M199 medium (Sigma Chemical Co., St. Louis, Mo.) containing 20% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, Utah). Washed, labeled borrelias were assessed for viability, quantitated by dark-field microscopy, and adjusted to 7.5 × 10^7/ml in the same medium.

Tissue culture. HUVE cells were isolated by the method of Jaffe et al. (15) and propagated at 37°C in 5% CO_2 in M199 (Sigma) containing 20% FCS, 100 μg of heparin (Sigma) per ml, 50 ng of endothelial cell growth supplement (Sigma) per ml, 200 U of penicillin (Sigma) per ml, and 0.2 mg of streptomycin (Sigma) per ml. Cells were removed from flasks by treatment with trypsin and EDTA and seeded at 5 × 10^4 per well of a 96-well tissue culture plate (Corning Glass Works, Corning, N.Y.) in 0.125 ml of M199 plus supplements as detailed above. HUVE cell cultures used in assays were passaged fewer than 15 times from primary isolation.

Preparation of borrelial extracts. Washed whole-cell extracts (WCE) were prepared by centrifugation of the spirochetes from BSK II at 13,000 × *g* at 25°C for 30 min. Cell pellets were washed three times in phosphate-buffered saline (pH 7.35) plus 5 mM $MgCl_2$ (PBSM). Protein concentrations were determined by using a modified Bradford assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin (BSA) as the standard.

Vesicle preparation. *B. burgdorferi* extracellular vesicles were prepared by the method of Garon et al. (12). Briefly, 300 to 500 ml of borrelial culture at ca. 10^8 spirochetes per ml were centrifuged at 13,000 × *g* for 30 min at 25°C to remove the bacteria from suspension. Culture supernatants were clarified by filtration through 2.0-μm-pore-size polycarbonate membranes (Poretics Corp., Livermore, Calif.), and extracellular material was removed by ultracentrifugation at 208,000 × *g* for 90 min at 27°C in a Beckman Ti55.2 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The vesicle pellet was washed once with PBSM at 208,000 × *g* for 90 min at 4°C. Washed vesicles were resuspended in PBSM and stored at −20°C. Mock vesicles were prepared by incubating 300 ml of BSK II containing 6% NRS for 1 week at 34°C and processing it in an identical fashion. Protein concentrations were determined as described in the previous paragraph.

Antibodies. Monoclonal antibody supernatants 9B3D and 84C (anti-OspA and anti-OspB, respectively; 6) were prepared in this laboratory. Monoclonal antibody supernatants 1C8 (anti-OspD; 20a, 26) and H9724 (anti-flagellin; 4) were provided by Alan G. Barbour. Anti-*B. burgdorferi* HB19 WCE polyclonal rabbit serum was prepared by previously described protocols (18) in this laboratory.

Anti-rabbit IgM-horseradish peroxidase (HRP) (Cappel Research Products, Durham, N.C.) and anti-human IgM-HRP (HyClone) were purchased and used at 1:500 in PBSM. Anti-rabbit IgG-HRP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and anti-mouse IgG-HRP (Boehringer Mannheim and Cappel) were used at 1:1,000 in PBSM. A human IgM (hIgM) myeloma protein preparation was purchased (Pierce Chemical Co., Rockford, Ill.).

HUVE cell adsorption assays. Briefly, 5 × 10^4 HUVE cells were seeded into wells of a 96-well tissue culture plate and allowed to form a monolayer overnight at 37°C under 5% CO_2 with monolayer formation assessed visually by light microscopy. After establishment of monolayers, the medium was aspirated and replaced by 50 μl of a suspension containing a vesicle preparation diluted in M199 plus 20% FCS or a mock mixture containing PBSM in M199 plus 20% FCS. These primary treatment incubations were conducted at 37°C under 5% CO_2 for 2 to 4 h. To assess the potential role of the IgM molecule in HUVE cell association, one set of experiments utilized an hIgM myeloma protein preparation as a primary treatment.

Following incubation, the monolayers were washed three times with PBSM at room temperature (RT). Washed monolayers were solubilized with 50 μl of double-strength Laemmli sample buffer (0.125 M Tris HCl [pH 6.8], 2.5% SDS, 25% glycerol, 5 mM dithiothreitol, 0.006% bromophenol blue) (19) and incubation at 37°C for 30 min. Lysates were boiled for 5 min and analyzed by SDS-PAGE and Western immunoblotting as described below.

For quantitative assessment of the amount of vesicle adsorption to HUVE cells, an enzyme-linked immunosorbent assay (ELISA) was developed. Briefly, HUVE cell monolayers were established in 96-well tissue culture plates and treated in triplicate with vesicle suspensions diluted in M199 plus 1% FCS as described above. Following 2 h of incubation at 37°C under 5% CO_2, monolayers were washed three times at RT with M199 plus 1% FCS and then fixed with 50 μl of 0.25% glutaraldehyde (Sigma) in PBSM for 30 min at 37°C. Fixed monolayers were washed three times with PBSM–0.05% (vol/vol) Tween 20 (Sigma) at RT, and then wells were blocked with 100 μl of 5% (wt/vol) BSA (Fraction V; ICN Pharmaceuticals, Irvine, Calif.) for 30 min at 37°C. Primary probing was done with an anti-HB19 WCE rabbit serum (1:500 in PBSM–0.05% Tween 20; 50 μl) for 1 h at 37°C and followed by three washes with PBSM–0.05% Tween 20 at RT and secondary probing with 50 μl of an anti-rabbit IgG HRP-conjugated antibody preparation for 1 h at 37°C. Monolayers were washed three times with PBSM–0.05% Tween 20 at RT and developed with 50 μl of 0.04% *o*-phenylenediamine (Sigma) in 0.1 M citrate-phosphate buffer (pH 5.0) plus 1.2% H_2O_2 for 30 min at 37°C (36). Development was terminated with 50 μl of 1 N H_2SO_4. A_{490} was determined with a Biomek 1000 automated ELISA system (Beckman) and samples that were neat or diluted with citrate-phosphate buffer. Student's *t* test was used to compare the mean sample values.

Borrelial-HUVE cell association inhibition assay. A quantitative assay described by Thomas and Comstock (34) was used to examine the extent of borrelial association with HUVE cell monolayers. Briefly, monolayers were established in tissue culture plates and exposed to a primary treatment (e.g., vesicles or a mock medium control) as described in the previous paragraph. After incubation for 1 h at 37°C under 5% CO_2, 3.75 × 10^6 (50 μl; multiplicity of infection, 75) radiolabeled borrelias were added to the wells and the plate was reincubated for 4 h at 37°C under 5% CO_2. Following this incubation, monolayers were washed three times with PBSM at RT. The monolayer and associated materials were solubilized in 100 μl of 0.5% SDS for 30 min at 37°C. Lysates were analyzed by liquid scintillation spec-

trophotometry to determine the percentage of labeled borrelias that had remained associated with HUVE cells. All treatments were assayed in triplicate, and Student's *t* test was employed to compare the similarity or difference between the mean percentages of inhibition for different vesicle treatments.

A similar set of experiments was utilized to assess the importance of HUVE cell monolayer pretreatment with vesicles. Monolayers were either pretreated for 1 h with 2 mg of vesicle protein per ml as described above or subjected to simultaneous addition of labeled borrelias plus vesicles at 2 mg of protein per ml (final concentration). In both cases, 4 h of incubation followed the addition of labeled borrelias. Samples were processed for quantitative analysis as described above.

SDS-PAGE and Western immunoblotting. Discontinuous SDS–10% polyacrylamide gels (19) were used to resolve proteins in borrelial extracts and those recovered by HUVE cell adsorption. Gels were run at 20 mA of constant current and cooled by a circulating-water heat exchanger. Molecular weight standards were used in accordance with the suppliers' recommendations (Bio-Rad and Pharmacia Corp., Piscataway, N.J.).

Resolved proteins were electrotransferred by the method of Towbin et al. to nitrocellulose (BA83; Schleicher & Schuell, Inc., Keene, N.H.) or to PolyScreen polyvinylidene difluoride (New England Nuclear Corp.) membranes (35). Transfer was conducted for 3 h at RT under 300 mA of constant current. Posttransfer, membranes were washed in PBSM and blocked with 5% low-fat dry milk in PBSM for 30 min at RT. Primary screening with mono- or polyclonal antibodies was done overnight at RT and followed by three 15-min washes with PBSM at RT. Secondary screening was performed with HRP-conjugated secondary antibodies diluted in PBSM and incubated for 1 to 2 h at RT and followed by three final washes with PBSM at RT. Colorimetric detection with imidazole, 4-chloro-1-naphthol, and H_2O_2 as peroxidase substrates (Sigma) was performed at RT (6). Development was terminated by extensively washing the membrane with distilled water and air drying it at RT.

Electron microscopy. Negatively stained vesicle preparations were examined by transmission electron microscopy as previously described (14). Briefly, vesicle suspensions (10 μl) were spotted onto carbon-treated, Formvar-coated grids and allowed to adsorb for 1 to 2 min at RT. Residual fluid was wicked away with filter paper. Grids were stained with 20 μl of 1% phosphotungstic acid (Electron Microscopy Sources, Ft. Washington, Pa.) for 10 s at RT. Residual stain was wicked away with filter paper, and the grids were air dried for 30 min at RT. Stained grids were examined with a JEOL JEM-1200EX transmission electron microscope.

RESULTS

Characterization of extracellular vesicle preparations. Figure 1A exhibits the Coomassie blue-stained protein profiles of *B. burgdorferi* HB19 WCE and vesicles from low-passage borrelias grown in BSK II supplemented with either NHS or NRS and a mock vesicle preparation from uninoculated medium supplemented with NRS. Some of the prominently stained bands in the vesicle preparations (lanes 2, 4, and 5) included the 83-kDa antigen (8, 9), the ~66-kDa BSA band (a medium component) and OspA and OspB in the two borrelial vesicle lanes (lanes 2 and 4), and an unidentified band of ~27 kDa (lane 4). Lanes containing WCE exhibited prominent bands for OspA and OspB, as well as the 37.5-kDa flagellin band (lanes 1 and 3).

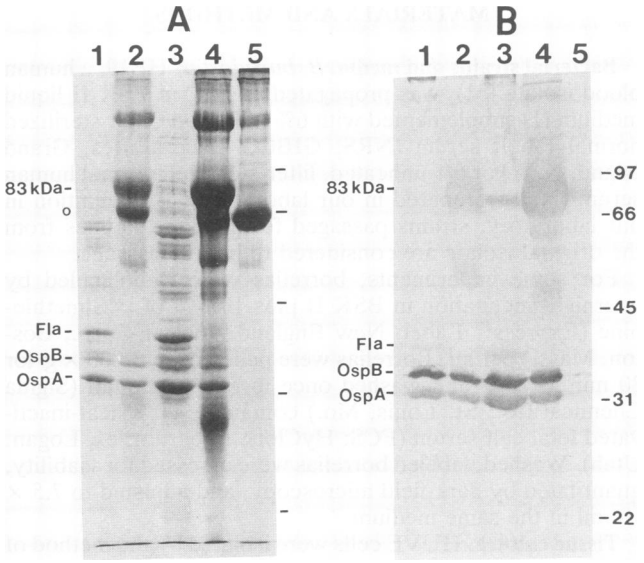

FIG. 1. Analysis of *B. burgdorferi* HB19 washed WCE and extracellular vesicle preparations by Coomassie blue staining (A) and Western immunoblotting (B). Five micrograms of WCE proteins or 25 μg of vesicle proteins per lane was resolved on SDS–10% polyacrylamide gels and electrotransferred to a nitrocellulose membrane. Lanes (all samples were of *B. burgdorferi* HB19 (passage 10), and the species of serum used to supplement the medium is given in parentheses): 1, WCE (NRS); 2, vesicles (NRS); 3, WCE (NHS); 4, vesicles (NHS); 5, mock vesicles (NRS). Primary immunologic screening was performed with the following monoclonal antibody supernatants: anti-OspA (9B3D; 6), anti-OspB (84C; 6), and anti-Fla (H9724; 4). Secondary probing was performed with anti-mouse IgG, anti-rabbit IgM, and anti-human IgM HRP-conjugated sera. Major *B. burgdorferi* antigens and BSA (○) are indicated on the left, and positions of molecular size standards (in kilodaltons; Bio-Rad) are on the right.

The corresponding Western immunoblot (Fig. 1B) confirmed the presence of rabbit IgM as an 83-kDa antigen as previously described (lanes 2 and 5; 8) and also demonstrated the presence of hIgM in the 83-kDa antigen band of vesicles prepared from medium supplemented with NHS (lane 4). Both types of vesicle preparations also contained a lesser amount of free H_μ chain (~60 kDa; lanes 2, 4, and 5).

As previously described, rabbit IgM was not detected in the WCE preparation from NRS-supplemented medium (lane 2; 8). However, hIgM was detected as an ~83 kDa antigen in the WCE preparation from NHS-supplemented medium (lane 3). As expected, flagellin was found in both of the WCE preparations (lanes 1 and 3) but was not detected in either of the extracellular vesicle preparations (lanes 2 and 4), as has been reported previously (9).

Figure 1 demonstrates that the mock vesicle preparation also contained an anti-IgM-reactive band of 83 kDa and BSA, indicating that an 83-kDa band may be formed in the absence of borrelial components (lane 5 in both figures). This has also been reported previously but was not discussed by the investigators (8). The traces of OspA and OspB observed in Fig. 1B (lane 5) are believed to be due to overloading of the adjacent vesicle lane.

Passage number-dependent expression of OspB was observed for vesicles (Fig. 2). Figure 2A is a Coomassie

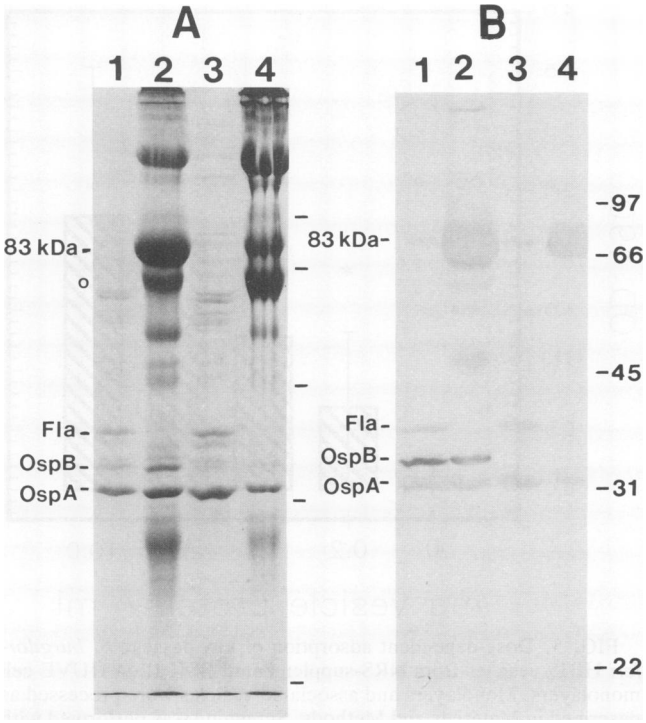

FIG. 2. Analysis of *B. burgdorferi* HB19 washed WCE and extracellular vesicle preparations by Coomassie blue staining (A) and Western immunoblotting (B). Five micrograms of WCE proteins or 25 μg of vesicle proteins per lane was resolved on SDS–10% polyacrylamide gels and electrotransferred to a PolyScreen membrane. Lanes (all samples were of *B. burgdorferi* HB19 propagated in BSK II supplemented with NHS): 1, WCE (passage 10); 2, vesicles (passage 10); 3, WCE (passage 53); 4, vesicles (passage 53). Primary immunologic screening was performed with the following monoclonal antibody supernatants: anti-OspA (9B3D; 6), anti-OspB (84C; 6), and anti-Fla (H9724; 4). Secondary probing was performed with anti-mouse IgG and anti-human IgM HRP-conjugated sera. Major *B. burgdorferi* antigens and BSA (○) are indicated on the left, and positions of molecular size standards (in kilodaltons; Bio-Rad) are on the right.

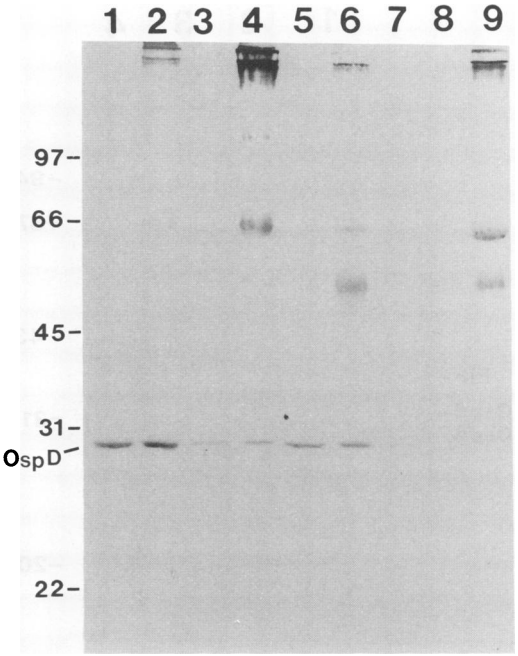

FIG. 3. Analysis of *B. burgdorferi* HB19 WCE and extracellular vesicle preparations by Western immunoblot. Five micrograms of WCE proteins or 25 μg of vesicle proteins per lane was resolved on SDS–10% polyacrylamide gels and electrotransferred to a PolyScreen membrane. Lanes (the type of preparation and passage number are given, and the species of serum used to supplement the medium is in parentheses): 1, WCE, passage 10 (NHS); 2, vesicles, passage 10 (NHS); 3, WCE, passage 53 (NHS); 4, vesicles, passage 53 (NHS); 5, WCE, passage 10 (NRS); 6, vesicles, passage 10 (NRS); 7, WCE, passage 50 (NRS); 8, vesicles, passage 50 (NRS); 9, mock vesicles (NRS). Primary immunologic screening was performed with an anti-OspD monoclonal antibody supernatant (1C8), and secondary probing was done with goat anti-mouse IgG HRP-conjugated serum. The positions of OspD and molecular size standards (in kilodaltons; Bio-Rad) are indicated on the left.

blue-stained gel of WCE and vesicles from strain HB19 at passage 10 or 53 propagated in BSK II supplemented with NHS. Western immunoblotting analysis (Fig. 2B) demonstrated OspA and OspB in both sets of WCE preparations, regardless of passage number (lanes 1 and 3). A markedly reduced level of OspB was expressed in the high-passage vesicles (lane 4), while OspB was detected at a WCE-comparable level in the low-passage vesicle preparations (lane 2). Similarly, this pattern of differential expression for OspB was observed with low- and high-passage WCE and borrelia vesicles propagated in BSK II supplemented with NRS (data not shown).

Vesicle preparations were also assayed by Western immunoblotting for the presence of OspD, a 28-kDa lipoprotein normally expressed only in low-passage isolates (23). Both WCE and vesicles of low-passage borrelias expressed OspD, regardless of the serum source used to supplement the growth medium (Fig. 3, lanes 1, 2, 5, and 6). OspD was also observed in the WCE preparations of high-passaged borrelias grown in either NHS or NRS (lanes 3 and 7) and in the vesicles of high-passage borrelias grown in NHS (lane 4). However, OspD was not expressed in the vesicles of high-passage borrelias propagated in the presence of NRS (lane 8). Other bands of ~54, 66, 115, and >150 kDa were observed in some of the vesicle lanes, including the mock vesicle lane. All of these reactive bands had undefined identities. Since they were observed in the mock vesicle preparation as well, they are not discussed further in this report.

Transmission electron microscopy of four different negatively stained vesicle preparations failed to demonstrate more than an occasional intact spirochete (i.e., less than one in over 50 fields; data not shown). These data were further confirmation that the protocol used to prepare vesicles preferentially enriched for extracellular materials and yielded intact borrelias or protoplasmic cylinders only at immunologically subdetectable levels, as reported previously (12).

Adsorption of extracellular vesicles to HUVE cells. After vesicles had been incubated with HUVE cell monolayers for 4 h and washed with PBSM, apparent vesicle binding was observed (Fig. 4). Three bands of moderate-to-intense reactivity were recognized by a polyclonal anti-HB19 WCE serum (lane 1). These bands included OspA and OspB and a band of ~20 kDa with an undescribed identity or function.

As a positive control for adsorption, HB19 WCE also bound to HUVE cells (lane 2), with strong anti-HB19

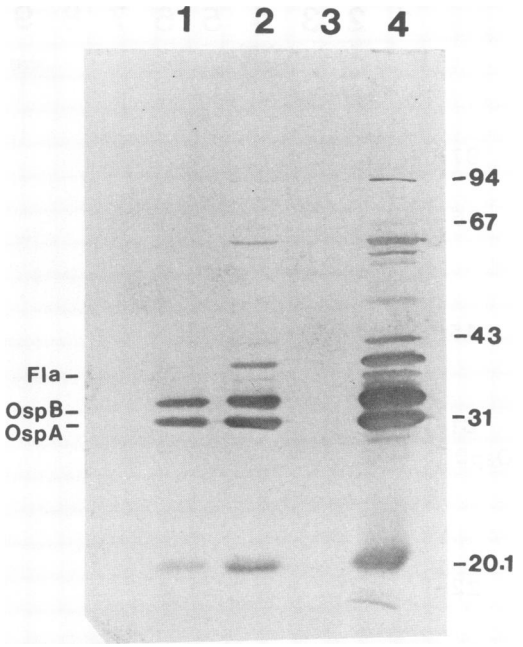

FIG. 4. Western immunoblot analysis of *B. burgdorferi* preparations following adsorption on HUVE cell monolayers and washing with PBSM. Monolayer lysates were resolved on SDS–10% polyacrylamide gels and electrotransferred to a nitrocellulose membrane. Anti-*B. burgdorferi* HB19 rabbit serum was used as the primary antiserum, and anti-rabbit IgG-HRP was used as the secondary antiserum. All samples were prepared from *B. burgdorferi* HB19 grown in BSK II supplemented with 6% NRS. HUVE cell monolayers were incubated with 0.77 mg of low-passage vesicles per ml (lane 1), 0.77 mg of low-passage WCE per ml (lane 2), or M199 medium plus 20% FCS (lane 3). Lane 4 contained a hybridization control containing 10 μg of HB19 WCE. The positions of some major *B. burgdorferi* surface proteins and molecular size standards (in kilodaltons; Pharmacia) and are indicated on the left and right, respectively.

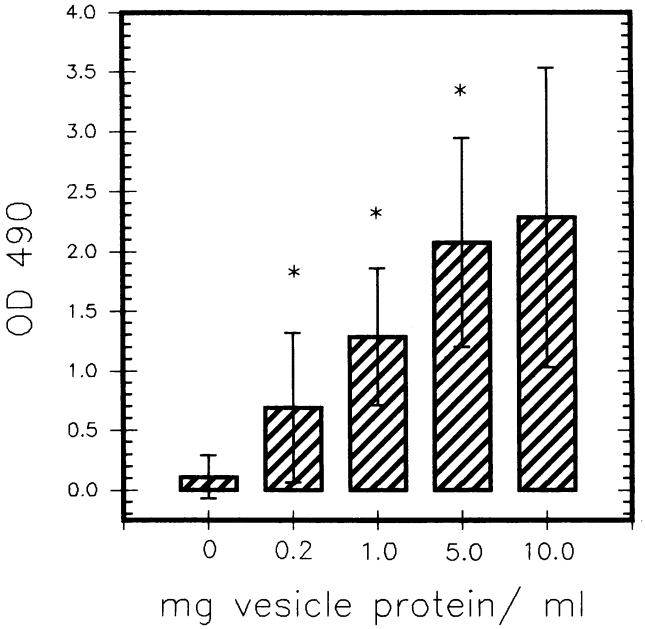

FIG. 5. Dose-dependent adsorption of low-passage *B. burgdorferi* HB19 vesicles from NRS-supplemented BSK II on HUVE cell monolayers. Monolayers and associated vesicles were processed as described in Materials and Methods. Screening was performed with rabbit anti-*B. burgdorferi* HB19 serum as the primary antiserum and goat anti-rabbit IgG-HRP as the secondary antiserum. The values shown are means of at least three experiments using triplicate samples read neat and diluted with citrate-phosphate buffer. Standard deviations are indicated by the error bars, and statistically significant ($P < 0.05$) values are indicated by asterisks. OD 490, optical density at 490 nm.

reactivity with flagellin, OspA and OspB, and the ~20 kDa bands. Weaker reactive bands of ~60, 42, and 36 kDa, all with undefined identities, were also observed. No signal was observed in a medium control adsorption (lane 3; M199 plus PBSM and 20% FCS), and lane 4 contained a positive immunoblotting control (10 μg of HB19 WCE), confirming the specificity of the bands observed in lanes 1 and 2.

Quantitative ELISA analysis demonstrated concentration-dependent association of vesicles with the HUVE cell monolayers (Fig. 5). An increased A_{490} signal was detected through 5 mg of vesicle protein per ml, indicating that a plateau had been reached between 5 and 10 mg of vesicle protein per ml ($P < 0.05$).

Vesicles prepared from borrelias grown in NHS-supplemented BSK II were also observed to associate with HUVE cells (data not shown). Adsorption or binding of vesicles to HUVE cells was also demonstrated to be passage number dependent by Western immunoblot analyses. Neither of the high-passage vesicle preparations (passage 50 grown in NRS-supplemented medium and passage 53 grown in NHS-supplemented medium) associated with HUVE cells (data not shown).

HUVE cell adsorption of vesicles as a function of the 83-kDa antigen (i.e., the IgM component) was examined through comparative adsorption of an hIgM myeloma protein and low-passage HB19 vesicles prepared from NHS-supplemented medium (Fig. 6). Western immunoblot analysis of HUVE cell-adsorbed proteins showed dose-responsive adsorption of vesicles (lanes 1 to 3) and virtually no adsorption of hIgM at equivalent protein concentrations (lanes 4 to 7). Similarly, when HUVE cells were incubated with mock vesicles, no adsorption was detected by immunoblot analysis or ELISA (data not shown). These data suggested that the adsorption of vesicles occurred by some mechanism other than HUVE cell-IgM complex formation and demonstrated dependence upon a borrelial component(s) in the vesicle.

Vesicle-mediated inhibition of borrelial-HUVE cell association. To assess the binding of vesicles to HUVE cells and the specificity of the interaction quantitatively, we examined the ability of vesicle preparations to inhibit the binding of viable, radiolabeled, low-passage HB19. Figure 7 demonstrates dose-responsive inhibition of *B. burgdorferi* HB19 spirochete binding as a function of prior vesicle adsorption. The inhibitory effect was approximately linear and nonsaturating through 2 mg of vesicle proteins per ml. By comparison, the ability of the mock vesicle preparation to inhibit HUVE cell association of HB19 was essentially zero (Fig. 7).

Further experiments confirmed the passage number dependency of the vesicle-HUVE cell interaction, as evidenced by the relative inability of high-passage vesicles to inhibit borrelia-HUVE cell association (Table 1). When low-passage vesicle preparations were compared with high-passage preparations, the mean percentages of inhibition were significantly different ($P < 0.001$). Also, when the

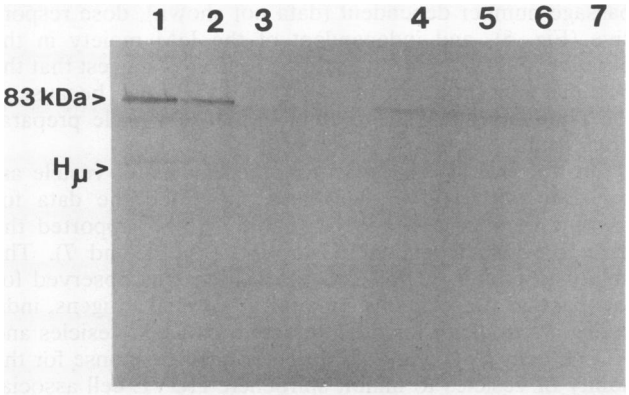

FIG. 6. Western immunoblot analysis of samples adsorbed to HUVE cell monolayers. hIgM or low-passage HB19 vesicles from BSK II supplemented with NHS were added to HUVE cell monolayers. Lysates of PBSM-washed monolayers were resolved on SDS–10% polyacrylamide gels and electrotransferred to a Poly-Screen membrane. Immunologic screening was performed with anti-human IgM-HRP. Lanes: 1, 2.0 mg of vesicle proteins per ml; 2, 0.4 mg of vesicle proteins per ml; 3, 0 mg of vesicle proteins per ml; 4, 2.0 mg of hIgM per ml; 5, 1.0 mg of hIgM per ml; 6, 0.4 mg of hIgM per ml; 7, 0.2 mg of hIgM per ml; 8, 0 mg of hIgM per ml. The positions of the 83-kDa antigen (8) and H_μ are indicated on the left.

abilities of low-passage vesicles prepared from NHS- or NRS-supplemented medium were compared, the NRS-prepared vesicles showed a significantly greater ability to inhibit spirochetal-HUVE cell association. Mock vesicle preparations demonstrated no inhibitory effect on the association of borrelias with HUVE cells, confirming that the inhibitory

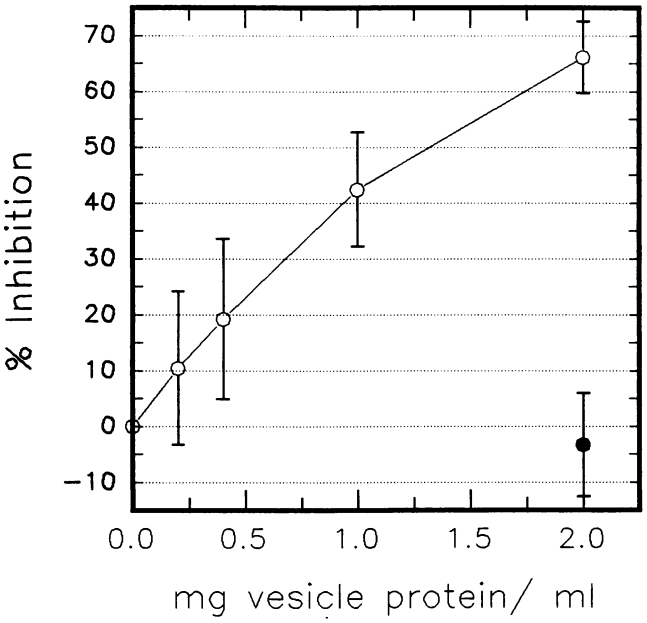

FIG. 7. Dose-dependent inhibition of *B. burgdorferi* HB19 binding to HUVE cell monolayers following pretreatment with low-passage HB19 vesicles (NRS; ○) or a mock vesicle preparation (NRS; ●). Datum points are means from three experiments (with triplicate samples) with three different vesicle preparations, and the resultant standard deviations are indicated.

TABLE 1. Comparison of the abilities of different *B. burgdorferi* vesicle preparations to inhibit the association of *B. burgdorferi* HB19 with HUVE cells

Vesicle preparation[a]	Mean % inhibition ± SD[b]	$P < 0.001$[c]
Mock, NRS	−3.34 ± 9.28	
Low passage, NRS	58.62 ± 10.00	+
Low passage, NRS	58.62 ± 10.00	
High passage, NRS	2.71 ± 9.15	+
Low passage, NHS	37.18 ± 18.46	
High passage, NHS	8.80 ± 28.19	+
Low passage, NRS	58.62 ± 10.00	
Low passage, NHS	37.18 ± 18.46	+

[a] Low passage indicates ≤10 passages from initial isolation; high passage indicates ≥50 passages from initial isolation; NRS, NRS-supplemented BSK II; NHS, NHS-supplemented BSK II.
[b] Means of at least three experiments with triplicate samples are shown.
[c] Student's *t* test was used to evaluate the means for statistical similarity. +, means are different with 99.9% confidence.

effect was due to some borrelial component(s) in the vesicle preparations.

Pretreatment of monolayers with vesicles was not a prerequisite for inhibition of borrelia-HUVE cell association. Experiments which compared the relative abilities of vesicles to inhibit the adherence of labeled borrelias to monolayers that had been pretreated for 1 h or had simultaneous addition of borrelias and vesicles indicated that borrelial association with pretreated monolayers was inhibited by 58.62% ± 10.00% (2 mg of vesicle protein per ml), while vesicles added concurrently with borrelias (final concentration, 2 mg of protein per ml) inhibited borrelia-HUVE cell association by 54.34% ± 16.56% ($P < 0.001$).

DISCUSSION

This study was prompted by a report describing rabbit IgM as a component of a *B. burgdorferi* vesicle antigen (8) and previous reports demonstrating that at least some of the major components of the *B. burgdorferi* outer surface were expressed in or on these extracellular vesicles, notably, OspA and OspB (9). Evidence from this and other laboratories has proposed the involvement of OspA and/or OspB in mediation of the association of *B. burgdorferi* with human cells in vitro (5, 6, 34). Therefore, it was of interest to test the ability of vesicles to associate with cultured HUVE cells for potential use in attempts to characterize borrelial association with mammalian cells.

Extracellular vesicles were prepared by the filtration and differential centrifugation protocol described by Garon et al. (12). Vesicle preparations were demonstrated to lack flagellin by Western immunoblot analysis (Fig. 1B and 2B), indicating a subdetectable amount of intact borrelias in the preparations. Additionally, electron microscopy of four different negatively stained vesicle preparations failed to demonstrate appreciable numbers of intact spirochetes, supporting the validity of this method of producing vesicles with minimal contamination with intact borrelias. The absence of viable or intact spirochetes was desirable in efforts to simplify the test system for HUVE cell association.

All of the vesicle preparations used contained OspA and the 83-kDa antigen (Fig. 1 and 2), two antigenic markers described for vesicles (8, 9), regardless of passage number or

medium serum supplement. While Coomassie blue staining suggested the presence of OspB in WCEs of both low- and high-passage HB19 (Fig. 2A, lanes 1 and 3), Western immunoblot analysis detected OspB in only the low-passage WCE (Fig. 2B, lane 2). Interestingly, the vesicles from high-passage HB19 exhibited a small amount of OspB (Fig. 2B, lane 4) while its corresponding WCE lacked detectable OspB (lane 3). Low-passage vesicles contained a level of OspB visually comparable to that of low-passage WCE (lanes 1 and 2). A similar pattern of OspB expression was observed in preparations from NRS-supplemented medium (data not shown). This is in partial agreement with the finding of Schwan and Burgdorfer, who saw a decrease in the amount of OspB produced by *B. burgdorferi* SH-2-82 with repeated in vitro subculture (28).

An interesting pattern of serum source-dependent expression of OspD in vesicles for high-passaged borrelias (Fig. 3) was seen. Typically, OspD is not expressed by high-passaged borrelias, with HB19 being a notable exception (23). Therefore, this datum is not a contradiction of previously reported findings. The nature of the relationship between NHS supplementation of the medium and OspD expression in high-passage vesicles while high-passage vesicles from NRS-supplemented medium lack OspD is not known. This is the first description of this recently described lipoprotein (23) as a component of *B. burgdorferi* vesicles.

This is also the first report of hIgM involvement in the composition of *B. burgdorferi* vesicles. In agreement with a previous report (8), we were unable to detect the presence of rabbit IgM in WCE of borrelias grown in BSK II supplemented with NRS. However, in the present study, hIgM was detected as an 83-kDa band in the WCE of borrelias propagated in NHS-supplemented medium (Fig. 1). The reason for this difference is unknown. Possible explanations for this finding are that interactions between human IgM molecules and borrelias are of a higher affinity than those for borrelias and rabbit IgM and that the surface of borrelias grown in the presence of NHS is less prone to vesicle formation or blebbing, which may explain the absence of rabbit IgM in WCE preparations.

Interestingly, a mock vesicle preparation from uninoculated BSK II supplemented with 6% NRS also contained an anti-IgM antibody-reactive band migrating at ~83 kDa on SDS-PAGE (Fig. 1, lane 9). It is possible that IgM molecules likely exist in the medium as a pentameric complex which cosediments with borrelial vesicles at 208,000 × *g* and that the 83-kDa band has no functional or structural role in the vesicles. A previous report describing the putative association of rabbit IgM and OspA and OspB presented data correlating the 83-kDa band with rabbit IgM but did not present direct evidence (e.g., Western analysis data) for the existence of this heteromolecular complex, and the presence of the putative 83-kDa antigen band in the mock vesicle preparation used (i.e., in uninoculated medium) was not discussed (8). Therefore, there appears to be some controversy over the composition of this 83-kDa antigen.

Our initial finding that vesicles bound to HUVE cells was made by Western immunoblot analyses using specific antisera or monoclonal antibodies to detect the presence of borrelial antigens in lysates of washed HUVE cells that had been incubated with vesicle preparations (Fig. 4 and data not shown). While the presence of a small number of spirochetes in these reactions which had associated with the monolayer cannot be ruled out, the absence of detectable flagellin by the sensitive Western blotting technique argues strongly against this. Binding of vesicles to HUVE cells was observed to be passage number dependent (data not shown), dose responsive (Fig. 5), and independent of the IgM moiety in the putative 83-kDa antigen (Fig. 6). These data suggest that the borrelial adhesin is expressed on the vesicle and, because of the relatively simplistic protein profile of vesicle preparations, should be definable.

Quantitative assays measuring the amount of vesicle association with HUVE cells also supported the data for passage number dependence (Table 1) and supported the data for dose-dependent adsorption (Fig. 5 and 7). The ability to inhibit HUVE cell association was observed for low-passage preparations containing borrelial antigens, indicating a specificity for the interaction between vesicles and HUVE cells (Fig. 7 and Table 1). A linear response for the ability of vesicles to inhibit spirochete-HUVE cell association was also observed as a function of vesicle protein concentration (Fig. 7), and saturation or a plateau of inhibitory activity was not observed over the range of concentrations tested.

While the protein concentrations used in this study may seem to be very high, a recent report by Whitmire and Garon defined the relative concentrations of OspA and OspB, two of the major protein constituents of the vesicle, as being only 2.4 and 1.5% of the vesicular protein, respectively (37). The major component of the vesicle, BSA (66% of the vesicle protein), arose from the growth medium, while the percent contribution of the 83-kDa antigen band was unreported (37). Our data demonstrated that the mock vesicle preparation was neither adsorbed by HUVE cell monolayers (data not shown) nor inhibitory for the association of viable borrelias with HUVE cells (Table 1 and Fig. 7), suggesting that the active component(s) of the vesicle would indeed be present in minor concentrations.

The finding that only low-passage vesicle preparations inhibited borrelial-HUVE cell association (Table 1) further underscores previous findings that virulence in animal model systems was restricted to low-passage versions of a strain (29) and that the ability to associate with cultured cells in vitro was greatly diminished for high-passage strains (33, 34).

B. burgdorferi vesicles were demonstrated to bind to HUVE cells via both qualitative and quantitative techniques. Because HUVE cell treatment with vesicles resulted in decreased levels of radiolabeled borrelia-HUVE cell association, these data suggest that the vesicles (i) contained the *B. burgdorferi* HUVE cell adhesin(s) and (ii) competed for an HUVE cell receptor. We propose that the *B. burgdorferi* vesicles may provide an important tool for elucidation of the borrelial adhesion antigen(s) or structure.

Since the association of vesicles with HUVE cells in vitro is inhibitory for subsequent binding and assumed penetration by borrelias, vesicle production seems to present a paradoxically negative process for the bacteria when the same events are considered in vivo. Vesicle production likely occurs in the vascular system of an infected mammal, as blood samples taken from experimentally infected mice have been reported to contain vesicular materials (9). It thus seems likely that the vesicles confer some selective advantage (i.e., a beneficial function) to the borrelias, allowing survival in or perhaps successful egress from the circulatory system. Potential functions for vesicles are reduction of the antigen load on the borrelial surface (a mechanism for immune evasion) and delivery of borrelial antigens to the host to initiate pathogenic responses.

For example, *B. burgdorferi* vesicles contain a borrelial factor mitogenic for murine B lymphocytes that stimulates

IgM production exclusively by vesicle-challenged, naive murine B cells (37). Both viable and sonicated (i.e., disrupted) extracts of *B. burgdorferi* have been reported to be cytotoxic for primary rat brain tissues and astroglial cells (11). Both viable and antibiotic-killed borrelias have also been reported to induce interleukin 1 production in a murine macrophage cell line, as well as in human peripheral blood monocytes and histiocytes (13). In a more recent report, sonicated *B. burgdorferi* has been reported to stimulate interleukin 1β production by normal human peripheral blood monocytes (22). A mixture of purified OspA and OspB has also been demonstrated to stimulate production of tumor necrosis factor α by both fresh and transformed murine macrophages (24). Activation of human endothelial cells by *Treponema pallidum*, as well as by a purified *T. pallidum* lipoprotein, has been described previously (25). Therefore, data presented in the present study suggest that it is plausible that in a *B. burgdorferi* infection, vesicles are one means of delivering potential activating antigens to the endothelium or other target cells in a specific and efficient manner.

Data presented here also demonstrated passage number dependence and medium supplement dependence for expression of *B. burgdorferi* surface antigens. Reports from other laboratories have indicated that vesicles contain antigens such as a low-molecular-weight lipoprotein with undefined functions (16) and a variety of DNA molecules (12). Cumulatively, the data presented in this study suggest that *B. burgdorferi* vesicles may provide an important tool for future studies of *B. burgdorferi* pathogenesis and/or physiology.

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REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:521–525.
2. **Barbour, A. G.** 1988. Laboratory aspects of Lyme borreliosis. Clin. Microbiol. Rev. **1**:399–414.
3. **Barbour, A. G., and S. F. Hayes.** 1986. Biology of *Borrelia* species. Microbiol. Rev. **50**:381–400.
4. **Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier.** 1986. A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. **52**:549–554.
5. **Benach, J. L., J. L. Coleman, J. C. Garcia-Monco, and P. C. Deponte.** 1988. Biological activity of *Borrelia burgdorferi* antigens. Ann. N.Y. Acad. Sci. **539**:115–125.
6. **Comstock, L. E., E. Fikrig, R. J. Shoberg, R. A. Flavell, and D. D. Thomas.** 1993. A monoclonal antibody to OspA inhibits association of *Borrelia burgdorferi* with human endothelial cells. Infect. Immun. **61**:423–431.
7. **Comstock, L. E., and D. D. Thomas.** 1991. Characterization of invasion of endothelial cells by *Borrelia burgdorferi*. Microb. Pathog. **10**:137–148.
8. **Dorward, D. W., E. D. Huguenel, G. Davis, and C. F. Garon.** 1992. Interactions between extracellular *Borrelia burgdorferi* proteins and non-*Borrelia*-directed immunoglobulin M antibodies. Infect. Immun. **60**:838–844.
9. **Dorward, D. W., T. G. Schwan, and C. F. Garon.** 1991. Immune capture and detection of *Borrelia burgdorferi* antigens in urine, blood, or tissues from infected ticks, mice, dogs, and humans. J. Clin. Microbiol. **29**:1162–1170.
10. **Duray, P. H., and A. C. Steere.** 1986. The spectrum of organ and systems pathology in human Lyme disease. Zentralbl. Bakteriol. Hyg. A **263**:169–178.
11. **Garcia-Monco, J. C., B. F. Villar, A. Szczepanski, and J. L. Benach.** 1991. Cytotoxicity of *Borrelia burgdorferi* for cultured rat glial cells. J. Infect. Dis. **163**:1362–1366.
12. **Garon, C. F., D. W. Dorward, and M. D. Corwin.** 1989. Structural features of *Borrelia burgdorferi*—the Lyme disease spirochete: silver staining for nucleic acids. Scanning Microscopy **3**(Suppl.):109–115.
13. **Habicht, G. S., G. Beck, J. L. Benach, J. L. Coleman, and K. D. Leichtling.** 1985. Lyme disease spirochetes induce human and murine interleukin 1 production. J. Immunol. **134**:3147–3154.
14. **Haschemeyer, R. H., and R. J. Meyers.** 1972. Negative staining, p. 101–147. *In* M. A. Hayat (ed.), Principles and techniques of electron microscopy, volume 2. Van Nostrand Reinhold Co., New York.
15. **Jaffe, E. A., R. L. Nachman, C. G. Becker, and R. C. Minick.** 1972. Culture of human endothelial cells derived from human umbilical cord veins. Circulation **46**:211–253.
16. **Katona, L. I., G. Beck, and G. S. Habicht.** 1992. Purification and immunological characterization of a major low-molecular-weight lipoprotein from *Borrelia burgdorferi*. Infect. Immun. **60**:4995–5003.
17. **Katona, L. I., and G. S. Habicht.** 1993. Purification of the *Borrelia burgdorferi* low-molecular-weight lipoprotein by preparative isoelectric focusing. B-228, p. 67. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993. American Society for Microbiology, Washington, D.C.
18. **Kaul, R., and W. M. Wenman.** 1986. Cyclic AMP inhibits developmental regulation of *Chlamydia trachomatis*. J. Bacteriol. **168**:722–727.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lefebvre, R. B., G.-C. Perng, and R. C. Johnson.** 1990. The 83-kilodalton antigen of *Borrelia burgdorferi* which stimulates immunoglobulin M (IgM) and IgG responses in infected hosts is expressed by a chromosomal gene. J. Clin. Microbiol. **28**:1673–1675.

20a. **Luke, C., et al.** Unpublished data.

21. **Ma, Y., A. Sturrock, and J. J. Weis.** 1991. Intracellular localization of *Borrelia burgdorferi* within human endothelial cells. Infect. Immun. **59**:671–678.
22. **Miller, L. C., S. Isa, E. Vannier, K. Georgilis, A. C. Steere, and C. A. Dinarello.** 1992. Live *Borrelia burgdorferi* preferentially activate interleukin-1β gene expression and protein synthesis over the interleukin-1 receptor antagonist. J. Clin. Invest. **90**:906–912.
23. **Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour.** 1992. Low-passage-associated proteins of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect. Immun. **60**:4662–4672.
24. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. J. Immunol. **147**:1968–1974.
25. **Riley, B. S., N. Oppenheimer-Marks, E. J. Hansen, J. D. Radolf, and M. V. Norgard.** 1992. Virulent *Treponema pallidum* activates human vascular endothelial cells. J. Infect. Dis. **165**:484–493.
26. **Šadziene, A., P. A. Rosa, P. A. Thompson, D. H. Hogan, and A. G. Barbour.** 1992. Antibody-resistant mutants of *Borrelia burgdorferi*: in vitro selection and characterization. J. Exp. Med. **176**:799–809.
27. **Schoenfeld, R., B. Araneo, Y. Ma, L. Yang, and J. J. Weis.** 1992. Demonstration of a B-lymphocyte mitogen produced by the Lyme disease pathogen, *Borrelia burgdorferi*. Infect. Immun. **60**:455–464.
28. **Schwan, T. G., and W. Burgdorfer.** 1987. Antigenic changes of *Borrelia burgdorferi* as a result of in vitro cultivation. J. Infect. Dis. **156**:852–853.
29. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes

in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect. Immun. **56:**1831–1836.
30. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
31. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308:**733–740.
32. **Steere, A. C., and S. E. Malawista.** 1979. Cases of Lyme disease in the United States: locations correlated with the distribution of *Ixodes dammini*. Ann. Intern. Med. **91:**730–733.
33. **Szczepanski, A., M. B. Furie, J. L. Benach, B. P. Lane, and H. B. Fleit.** 1990. Interaction between *Borrelia burgdorferi* and endothelium in vitro. J. Clin. Invest. **85:**1637–1647.
34. **Thomas, D. D., and L. E. Comstock.** 1989. Interaction of Lyme disease spirochetes with cultured eucaryotic cells. Infect. Immun. **57:**1324–1326.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
36. **Voller, A., and D. Bidwell.** 1986. Enzyme-linked immunosorbent assay, p. 99–109. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
37. **Whitmire, W. M., and C. F. Garon.** 1993. Specific and nonspecific responses of murine B cells to membrane blebs of *Borrelia burgdorferi*. Infect. Immun. **61:**1460–1467.