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Characterization of Outer Membranes Isolated from *Borrelia burgdorferi*, the Lyme Disease Spirochete

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The lack of methods for isolating *Borrelia burgdorferi* outer membranes (OMs) has hindered efforts to characterize borrelial surface-exposed proteins. Here we isolated OMs by immersion of motile spirochetes in hypertonic sucrose followed by isopycnic ultracentrifugation of the plasmolyzed cells. The unilamellar vesicles thus obtained were shown to be OMs by the following criteria: (i) they contained OspA and OspB; (ii) they did not contain flagellin, NADH oxidase activity, or the 60-kDa heat shock protein; and (iii) their morphology by freeze-fracture electron microscopy was identical to that of OMs of intact organisms. Consistent with previous studies which employed immunoelectron microscopy and detergent-based solubilization of *B. burgdorferi* OMs, only small proportions of the total cellular content of OspA or OspB were OM associated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) fluorography of OMs from spirochetes metabolically radiolabeled with [^{3}H]palmitate or ^{35}S-amino acids demonstrated that the OMs contained both nonlipidated and lipidated proteins. This fractionation procedure was also used to isolate OMs from virulent and avirulent isolates of the well-characterized *B. burgdorferi* N40 strain. SDS-PAGE fluorography revealed that OMs from the two isolates differed with respect to both nonlipoprotein and lipoprotein constituents. When whole cells, protoplasmic cylinders, and OMs were immunoblotted against sera from mice persistently infected with *B. burgdorferi* N40, the majority of antibody reactivity was directed against intracellular proteins. The availability of isolated OMs should facilitate efforts to elucidate the complex relationship(s) between *B. burgdorferi* membrane composition and Lyme disease pathogenesis.

Lyme disease is a tick-borne multisystem infection caused by the spirochete *Borrelia burgdorferi* (51). Intensive effort in the last decade has focused on the identification of surface-exposed proteins for use as potential Lyme disease vaccinogens. During this same period, however, we have learned comparatively little about the membrane biology of this pathogen. *B. burgdorferi*, like all spirochetes, possesses an outer membrane (OM) which surrounds the periplasmic space, peptidoglycan-cytoplasmic membrane complex, and the protoplasmic cylinder (2). Although *B. burgdorferi* is often analogized to enteric gram-negative bacteria because of its double membrane structure, recent investigations indicate that its membrane composition differs substantially from that of gram-negative bacteria. In this regard, distinctive features of *B. burgdorferi* include an extraordinary abundance of membrane proteins covalently modified with lipids (12), the absence of lipopolysaccharide (LPS) (53) and phosphatidylethanolamine (7), the presence of glycolipid antigens other than LPS (7, 22, 55), and an OM which contains a relatively low density of transmembrane proteins (41, 54). In view of these differences, it seems likely that a comprehensive analysis of *B. burgdorferi* membrane architecture and constituents not only will yield insights into the unique physiological mechanisms by which the bacterium survives in diverse environments but also may elucidate pathogenic mechanisms operative in Lyme disease.

We previously used metabolic radiolabeling with [^{3}H]palmitate and ^{14}C-amino acids in conjunction with Triton X-114 phase partitioning to show that *B. burgdorferi* contains two classes of amphiphilic proteins: (i) immunogenic proteins membrane anchored by N-terminal lipids (i.e., lipoproteins) and (ii) poorly immunogenic nonlipidated proteins presumably membrane anchored by their polypeptide chains (12). Not surprisingly, the vast majority of borrelial membrane proteins characterized thus far, including all six outer surface proteins (Osps), are lipoproteins (8, 24, 31, 36). OM proteins with membrane-spanning domains have been visualized by freeze-fracture electron microscopy (41, 54) but, because of their poor immunoreactivity, have not been characterized. In fact, we have hypothesized that the spirochete exploits the poor immunogenicity of its surface-exposed nonlipoproteins as part of its strategy for evading host clearance mechanisms and establishing persistent infection (12, 40, 41).

Isolation of OMs has been an invaluable technique for characterizing surface-exposed protein and nonprotein constituents of gram-negative bacteria (32, 38). However, efforts to fractionate *B. burgdorferi* membranes by modifications of the classical method of Osborn et al. (38) have been unsuccessful (9, 43), presumably because of the lability of the *B. burgdorferi* OM (2, 13). Recently, Bledsoe et al. (9) adapted the method of Kotarski and Salyers (30) to separate inner and outer membranes of *B. burgdorferi* by sequential isopycnic centrifugation after disruption of spirochetes in a French pressure cell. Here we report an alternative, extremely gentle technique for bulk isolation of *B. burgdorferi* OMs based upon isopycnic centrifugation of spirochetes following plasmolysis in hypertonic sucrose. The analyses reported here indicate that isolated OMs will be useful reagents to further our understanding of *B. burgdorferi* membrane composition and its complex relationship(s) to Lyme disease pathogenesis.

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MATERIALS AND METHODS

Bacterial strains and cultures. The avirulent (high-passage) B31 strain of *B. burgdorferi* was provided by Alan Barbour (San Antonio, Tex.) and maintained in

BSKII medium (1). A virulent (low-passage) isolate of *B. burgdorferi* N40 was provided by Stephen Barthold (New Haven, Conn.) and cloned twice by limiting dilution (14). The virulence of the cloned isolate was confirmed by its ability to induce arthritis and carditis in 2-week-old C3H/HeJ mice following intradermal inoculation with 10^4 organisms (4). An avirulent N40 isolate that did not cause disease in C3H/HeJ mice was obtained by passaging the cloned virulent N40 isolate approximately 40 times in BSKII medium.

Fractionation of *B. burgdorferi*. Three-milliliter inocula of spirochetes were added to 500 ml of BSKII medium. In some experiments, spirochetes were labeled metabolically by adding 5 mCi of [^{3}H]palmitate (Amersham, Arlington Heights, Ill.) or ^{35}S-amino acids (Translabel; ICN Biochemicals, Costa Mesa, Calif.). After the cultures had reached the late logarithmic phase (determined by enumeration of organisms by dark-field microscopy), the spirochetes were harvested by centrifugation at 13,000 × *g* for 30 min and then resuspended in 2 ml of OM buffer (OMB) consisting of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 150 mM NaCl, and 1 mM $MgCl_2$ (pH 7.4). One-tenth of the resuspended bacteria was removed for subsequent analyses. The remainder (approximately 3×10^{10} to 5×10^{10} organisms) was centrifuged as described above and then resuspended in 2 ml of ice-cold OMB containing 20% (wt/vol) sucrose. After 1 h of incubation, 0.5-ml aliquots were gently loaded onto 14-ml linear gradients containing 20 to 60% sucrose in OMB with the protease inhibitors leupeptin (1.6 μg/ml), pepstatin (1.6 μg/ml), aprotinin (0.7 μg/ml), and phenylmethylsulfonyl fluoride (34.8 μg/ml). The tubes were centrifuged for 18 h at 160,000 × *g* at 4°C. Thereafter, the tubes were removed and warmed to room temperature. Fractions of 0.6 ml were then removed, starting from the top of the gradient. In some experiments, 50-μl portions were removed for refractometry. Individual fractions were dialyzed against OMB (1,000-molecular-weight exclusion) overnight at 4°C, following which they were stored at −70°C. In later experiments, fractions containing OMs and protoplasmic cylinders were pooled separately, dialyzed as described above, and stored at −70°C for subsequent analyses.

Assay of gradient fractions. The protein content of a 10-μl portion of each fraction was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.). NADH oxidase activity in 100-μl portions of each fraction was assayed by the assay described by Osborn et al. (38), with minor modifications (35, 37). Assay results were adjusted to the total volume of each fraction.

Analysis of *B. burgdorferi* lipids. Lipids from *B. burgdorferi* whole cells and OMs were prepared by chloroform-methanol extraction by the method of Bligh and Dyer (10). For analysis of total lipids by autoradiography, approximately 10^6 dpm of [^{3}H]palmitate-labeled lipids was spotted onto silica gel plates (9.7 cm by 9.7 cm by 0.25 mm; Brinkmann Instruments, Inc., Westbury, N.Y.). The plates were then chromatographed in a solvent system of $CHCl_3$-methanol-H_2O (65:35:5). After drying for several hours, the plates were turned 90° and chromatographed in the second dimension with $CHCl_3$-methanol-H_2O (85:15:1.5). After drying, the plates were sprayed with En^{3}hance (NEN, Boston, Mass.) and placed on Kodak X-OMAT AR film at −70°C for 5 days. [^{3}H]palmitate-labeled phosphatidylcholine and phosphatidylglycerol were identified by chromatography of unlabeled standards (Avanti Polar Lipids, Birmingham, Ala.) followed by staining with primulin.

Immunoblot analysis of *B. burgdorferi* lipids was performed as follows. Approximately 15 nmol of phospholipid was chromatographed as described above, after which the plates were blocked in phosphate-buffered saline (PBS)–Tween with 10% fetal calf serum. Rabbit polyclonal anti-*B. burgdorferi* B31 antiserum or normal rabbit serum was then added at a ratio of 1:50 with rocking overnight. After incubation, the plates were washed with PBS-Tween and then incubated sequentially for 1 h each with goat anti-rabbit and rabbit anti-goat horseradish peroxidase conjugates (Zymed, South San Francisco, Calif.). Bound antibodies were visualized with the ECL blotting system (Amersham) as described in the manufacturer's instructions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, and immunoblot analysis of protein antigens. Samples were boiled for 5 min in final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% bromophenol blue prior to electrophoresis through 2.4% stacking gels and 10 or 12.5% separating gels. The gels were then either silver stained by the procedure of Morrissey (34), stained with Coomassie brilliant blue prior to preparation for fluorography, or transferred electrophoretically to 0.2-μm-pore-size nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) for immunoblotting. For fluorography, Coomassie blue-stained gels were soaked in En^{3}hance, dried, and exposed at −70°C for various periods with Kodak X OMAT-AR film. Immunoblots were incubated with 1:100 dilutions of sera from *B. burgdorferi*-infected mice and then incubated sequentially with 1:1,000 dilutions of goat anti-mouse and rabbit anti-goat horseradish peroxidase conjugates (Zymed). Immunoblots were developed with 4-chloro-1-naphthol as the substrate.

To determine the relative amounts of OspA in the protoplasmic cylinder and OM fractions, spirochetes radiolabeled with [^{35}S]methionine and [^{35}S]cysteine were fractionated as described above. Portions corresponding to 10^8 organisms were removed from the separately pooled protoplasmic cylinders and OMs and separated on an SDS–12.5% polyacrylamide gel. After Coomassie blue staining, the gel was dried under vacuum, exposed overnight on a phosphor screen, and scanned with a PhosphorImager with ImageQuant software (Molecular Devices, Sunnyvale, Calif.). The OspA bands in the cylinder and OM lanes along with a blank segment of the gel (used as background) were circumscribed identically within a rectangular box. The relative image intensities of the circumscribed OspA bands were then determined by volume integration.

Fixation, embedding, and negative staining. *B. burgdorferi* whole cells and OMs were processed for embedding in Epon-Araldite and ultrathin sectioning as described previously (13). For negative staining, 5-μl portions of suspensions containing whole cells, protoplasmic cylinders, or OMs were placed onto glow-discharged 200-mesh copper grids (Energy Beam Sciences, Agawam, Mass.) for 1 min, washed three times, and then stained with 1% uranyl acetate.

Freeze-fracture EM. Motile *B. burgdorferi* organisms in the mid-logarithmic to late-logarithmic phase or freshly isolated OMs were processed for freeze-fracture electron microscopy (EM) as described previously (11). Fifty-milliliter cultures were fixed by the addition of 4 ml of 25% (vol/vol) glutaraldehyde (2% final concentration). After 2 h at room temperature, the cells were collected in a microcentrifuge, and the pellet was resuspended in 1 ml of 30% glycerol in PBS. The cells were collected again in a microcentrifuge, and 2-μl drops were placed into gold support pins (Balzers, Basel, Switzerland) and then frozen by immersion for 5 to 6 s in ethane cooled with liquid nitrogen. OMs from approximately 5×10^{10} spirochetes in 25 μl of OMB were fixed by the addition of 2 μl of 25% glutaraldehyde. After 2 h at room temperature, the membranes were collected in a Beckman (Palo Alto, Calif.) Airfuge, and the pellet was resuspended in 10 μl of 30% glycerol in OMB for 30 min. Two-microliter portions were transferred to gold support pins and frozen in ethane as described above. Freeze fracture was performed routinely at a temperature of −105°C; coating with platinum at 45°C and carbon at 90°C followed immediately. The replicas were cleaned overnight with undiluted bleach (Clorox), washed in double-distilled water, and transferred to 200-mesh copper grids. Replicas were examined and photographed with a JEOL 100SX EM at 80 kV of accelerating voltage. To ensure correct identification of fracture faces, all specimens were photographed with 6° tilts of the goniometer; the resulting images were examined as stereo pairs.

Particle counts and statistical analysis. Photomicrographic enlargements of freeze-fractured whole cells or isolated OMs were overlaid with a grid divided into 1-cm squares. For each fracture face, the numbers of particles were counted within 50 grid squares on 8 to 10 different organisms or membrane vesicles. Particle densities were then calculated by the following formula: particles/μm^2 = (total particles counted × total magnification^2)/(50×10^8 $\mu m^2/cm^2$). The differences between mean particle densities were analyzed for statistical significance with the Mann-Whitney nonparametric comparison test (18).

Immunologic reagents. Immunoglobulin G1 monoclonal antibodies 8E7 and 1H4 were directed against OspA and flagellin, respectively. To produce polyclonal antiserum directed against the 60-kDa *B. burgdorferi* heat shock protein, the gene encoding the protein was PCR amplified from 100 ng of strain B31 DNA with the following primers: 5′-GGGGATCCATGGCTAAAGACATA TATTTTAA-3′ and 5′-CCGAATTCTTACATCATTCCCATTCC-3′ (*Bam*HI site plus nucleotides 633 to 655 and *Eco*RI site plus nucleotides complementary to bases 2253 to 2270, respectively [15]). The PCR product was digested with *Bam*HI and *Eco*RI and then cloned into the respective sites of pGEX-2T. The fusion of heat shock protein and glutathione *S*-transferase was purified with a glutathione affinity matrix (50). Four- to 6-week-old female Sprague-Dawley rats were then immunized intraperitoneally with 50 μg of antigen in complete Freund's adjuvant. This was followed 1 month later by 25-μg boosts in incomplete Freund's adjuvant administered by the same route over three successive 10-day intervals. To generate polyclonal antisera against *B. burgdorferi* B31, 4- to 6-week-old Sprague-Dawley rats were primed by intraperitoneal injection with 10^9 spirochetes in a 1:1 mixture of PBS (pH 7.4)-Freund's complete adjuvant followed by intraperitoneal boosts at 2-week intervals with 10^8 spirochetes in a 1:1 mixture of PBS-incomplete Freund's adjuvant.

Chronic *B. burgdorferi* infection of mice. Random-sex, virus antibody-free C3H/HeJ mice were purchased from Charles River Laboratories, Portage, Mich. Mice were shipped in filtered crates, maintained in isolator cages, and provided with food and water ad libitum. Eight 4-week-old C3H/HeJ mice were needle inoculated intradermally with 10^4 cloned, low-passage *B. burgdorferi* N40 organisms. At 8 weeks and 8 months following infection, four animals each were sacrificed by CO^2 narcosis and exsanguinated. Active *B. burgdorferi* infection at 4 weeks postinoculation was confirmed by recovery of spirochetes from ear punches (49) and at the time of sacrifice by recovery of spirochetes from blood and viscera (e.g., hearts, spleens, and urinary bladders).

RESULTS

Fractionation of *B. burgdorferi* B31. Our protocol for isolating *B. burgdorferi* OMs stemmed from the observation that borreliae become distended following immersion in hypertonic sucrose (data not shown). Because the peptidoglycan-cytoplasmic membrane complex of spirochetes is relatively rigid (27), we presumed that cell distention was due primarily to displacement and destabilization of the OM. Following suspension in hypertonic sucrose, plasmolyzed high-passage *B. burgdorferi*

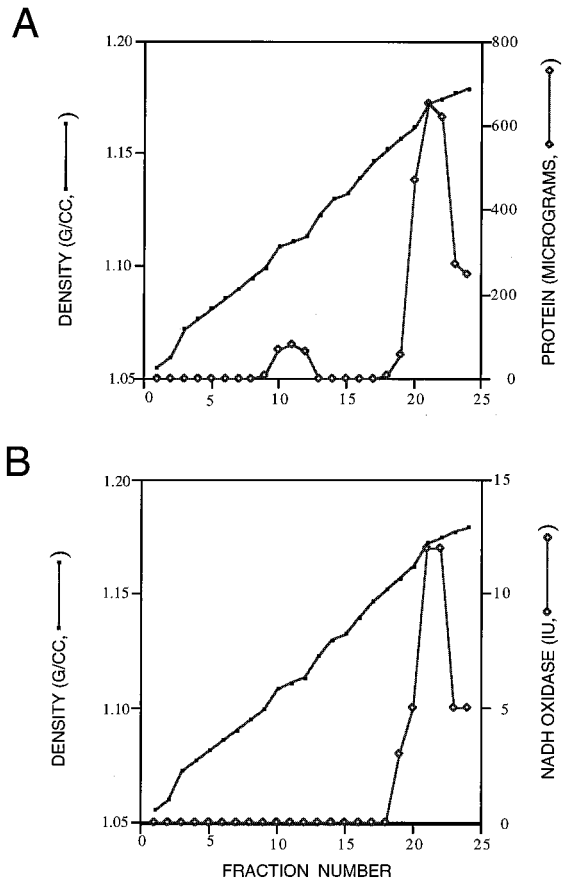

FIG. 1. Fractionation of *B. burgdorferi* B31. Assays of protein (A) and NADH oxidase activity (B) in individual fractions following density ultracentrifugation were performed.

B31 cells were loaded onto 20 to 60% linear sucrose density gradients for ultracentrifugation as described in Materials and Methods. Isopycnic centrifugation resolved the plasmolyzed cells into two distinct bands (specific gravities of 1.12 and 1.18 g/cm^2), which corresponded to the protein peaks shown in Fig. 1. Assay for NADH oxidase, a standard marker for gram-negative bacterial cytoplasmic membranes (38) which has also been detected in cytoplasmic membranes of *Treponema pallidum* (37), revealed that this enzymatic activity was associated exclusively with the lower (i.e., denser) band (Fig. 1). When examined by dark-field microscopy, the upper band contained only small particulates, while the lower band was densely packed with nonmotile spirochetes.

Identical volumes from each fraction were analyzed by SDS-PAGE and immunoblotting. Consistent with the protein assays in Fig. 1, Coomassie blue staining (Fig. 2A) revealed that the lower band contained the majority of borrelial proteins. Flagellin (Fig. 2B) and the 60-kDa heat shock protein (data not shown), a marker for both the borrelial cytoplasmic membrane and cytosolic compartments (47), were detected only in the denser band. OspA and OspB, in contrast, were identified in both bands (Fig. 2C). Interestingly, the Coomassie blue-stained gel (Fig. 2A) suggested that the majorities of both lipoproteins were associated with the heavier band. To determine the relative amounts of OspA in the two bands, pooled fractions from organisms metabolically radiolabeled with [^{35}S]cysteine or [^{35}S]methionine were analyzed with a PhosphorImager following SDS-PAGE. The lighter peak contained approximately 10% of the total OspA-associated disintegrations per minute. A comparable analysis for OspB was not performed because this lipoprotein, which contains a single cysteine and no methionine (8), labels poorly with ^{35}S-amino acids (see Fig. 5B).

Separately pooled fractions from the two protein peaks also were examined by EM. The upper band consisted of a homogeneous population of unilamellar vesicles (Fig. 3A and B) whose freeze-fracture EM morphology (Fig. 3C and E) was highly similar to that of OMs of intact organisms (Fig. 3D and

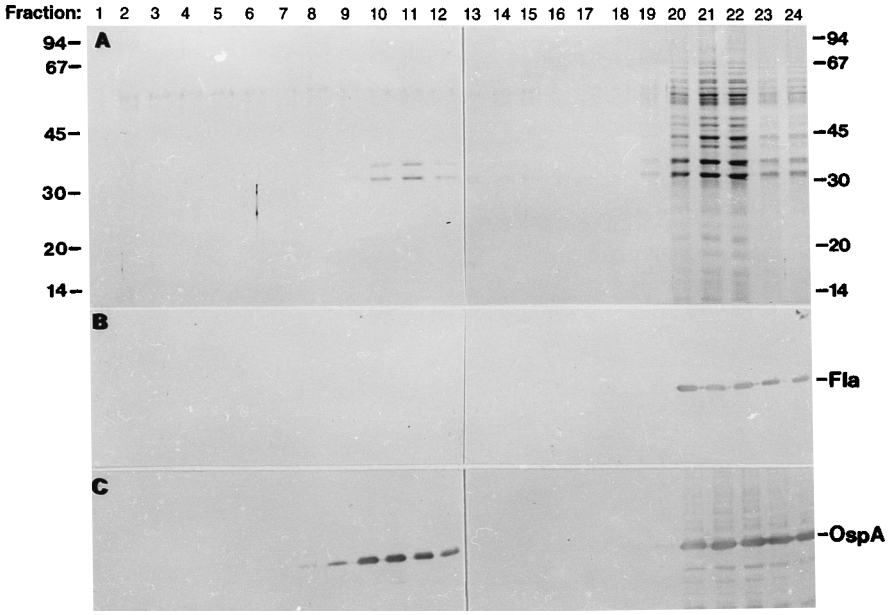

FIG. 2. Analysis of sucrose density gradient fractions by Coomassie blue staining of SDS-polyacrylamide gels (A) or by immunoblotting with monoclonal antibodies 8E7 (B) and 1H4 (C) directed against flagellin (Fla) and OspA, respectively. Molecular mass markers (in kilodaltons) are shown on the left.

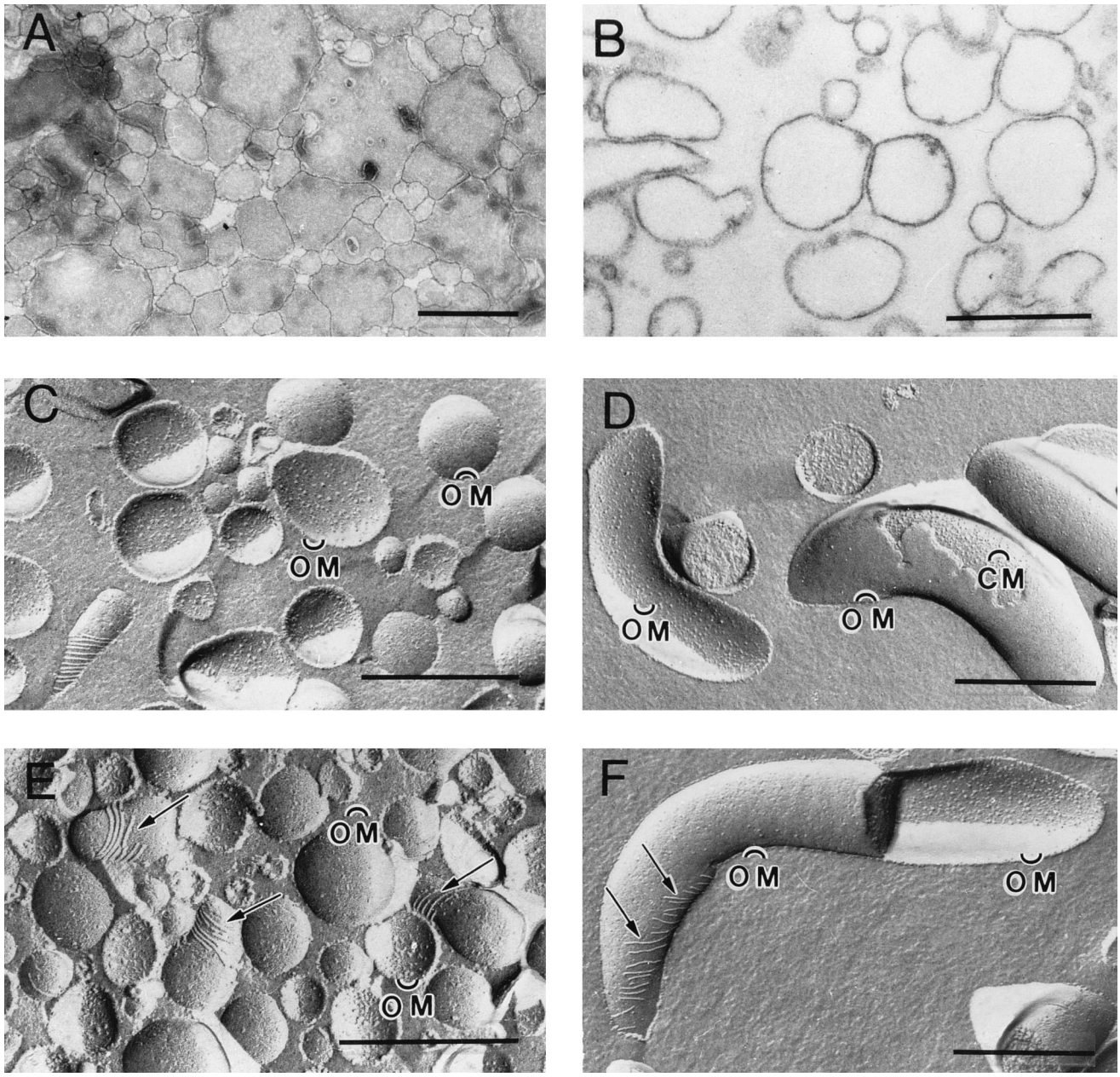

FIG. 3. EM analysis of pooled OM fractions by negative staining (A), ultrathin sectioning after plastic embedding (B), and freeze fracture (C and E). For comparison purposes, freeze-fractured *B. burgdorferi* B31 are shown in panels D and F. Arrows in panels E and F indicate linear bodies. $\breve{\text{OM}}$ and $\hat{\text{OM}}$ indicate OM concave and convex leaflets, respectively, in spirochetes and membrane vesicles. Bars, 0.5 μm.

F). The mean particle densities in the concave and convex leaflets of the vesicles were not significantly different from those in the corresponding OM leaflets of intact spirochetes (Table 1). As with intact B31, the mean particle density of the vesicle concave leaflets was markedly greater than that of the convex leaflet (Table 1). Additionally, many vesicles contained linear bodies (Fig. 3E and F), distinctive structures described in a recent freeze-fracture EM study of *B. burgdorferi* OMs (41). Examination of the denser band by both whole-mount negative staining and ultrathin sectioning revealed that it consisted of protoplasmic cylinders devoid of OMs (Fig. 4). On the basis of these combined data, it was concluded that the vesicles in the lighter band were isolated OMs.

Protein and lipid constituents of *B. burgdorferi* OMs. The polypeptide constituents of the isolated OMs were examined initially by silver staining of SDS-polyacrylamide gels. Compared with whole cells and protoplasmic cylinders, the OMs contained a relatively small number of polypeptides including several which appeared to have distinct electrophoretic mobilities (Fig. 5A). On the basis of evidence obtained from freeze-fracture EM (41, 54) and by Triton X-114 phase partitioning of metabolically radiolabeled spirochetes (12), it has been proposed that the *B. burgdorferi* OM contains both lipid-modified and transmembrane integral membrane proteins. To confirm this, OMs were isolated from spirochetes labeled intrinsically with either [^{3}H]palmitate or ^{35}S-amino acids. SDS-PAGE flu-

TABLE 1. Mean particle densities of *B. burgdorferi* OMs and isolated OMs

Strain	Concave leaflets			Convex leaflets		
	Particles/μm^2 (mean ± SD)		*P* value[a]	Particles/μm^2 (mean ± SD)		*P* value[a]
	Whole cells	OMs		Whole cells	OMs	
B31	1,512 ± 153	1,419 ± 266	0.38	98 ± 139	139 ± 79	0.15
Low-passage N40	1,030 ± 103[b]	994 ± 140[c]	0.16	35 ± 15	41 ± 18	0.43
High-passage N40	1,699 ± 207[b]	1,560 ± 129[c]	0.16	46 ± 21	53 ± 25	0.61

[a] *P* values of ≤0.05 are considered significant.
[b] $P < 0.001$ for the difference in mean particle densities between concave OM leaflets of low-passage and high-passage N40 cells (45).
[c] $P < 0.001$ for the difference in mean particle densities for concave leaflets of low-passage N40 and high-passage N40 isolated OMs.

orography revealed that the OMs contained several ^{35}S-labeled proteins which were clearly distinct from the [^{3}H]palmitate-labeled OM proteins; the majority of these nonlipoproteins had apparent molecular masses greater than 35 kDa (Fig. 5B). It also was noteworthy that the protoplasmic cylinders contained lipoproteins not observed in the OMs (Fig. 5B).

The lipid composition of the isolated OMs was investigated subsequently. Except for minor differences in relative abundances, the lipid constituents in OMs from organisms labeled with [^{3}H]palmitate were essentially identical to those of whole cells (Fig. 5Ca and b). Immunoblot analysis with polyclonal rabbit antiserum generated against *B. burgdorferi* B31 whole cells revealed that the OMs also contained antigenic constituents (Fig. 5Cc and d) which, by α-naphthol staining (48), were determined to be glycolipids. Normal rabbit serum did not react with any of the lipid constituents of whole cells or isolated OMs (data not shown).

Comparison of OM proteins from virulent and avirulent *B. burgdorferi* N40 isolates. To identify proteins which correlate potentially with virulence expression, OMs were isolated from low-passage (virulent) and high-passage (avirulent) *B. burgdorferi* N40 clonal isolates. For both isolates, results for assays of total protein and NADH oxidase activity in individual fractions as well as immunoblot analyses with the anti-OspA, anti-flagellin, and anti-heat shock protein antibodies were essentially identical to those shown above for strain B31 (data not shown). As with OMs isolated from the high-passage B31 strain, the freeze-fracture EM ultrastructures of the putative OMs were highly similar to those of OMs on the corresponding intact spirochetes (Fig. 6). We reported previously that the mean particle density in concave OM leaflets of high-passage *B. burgdorferi* N40 was significantly greater than that in concave OM leaflets from the low-passage N40 isolate (41). Consistent with this observation, the mean particle density in the concave leaflets of OMs isolated from high-passage N40 was significantly greater than that in concave leaflets of OMs isolated from the low-passage clone (Table 1). The mean particle densities in the concave and convex leaflets of the isolated OMs

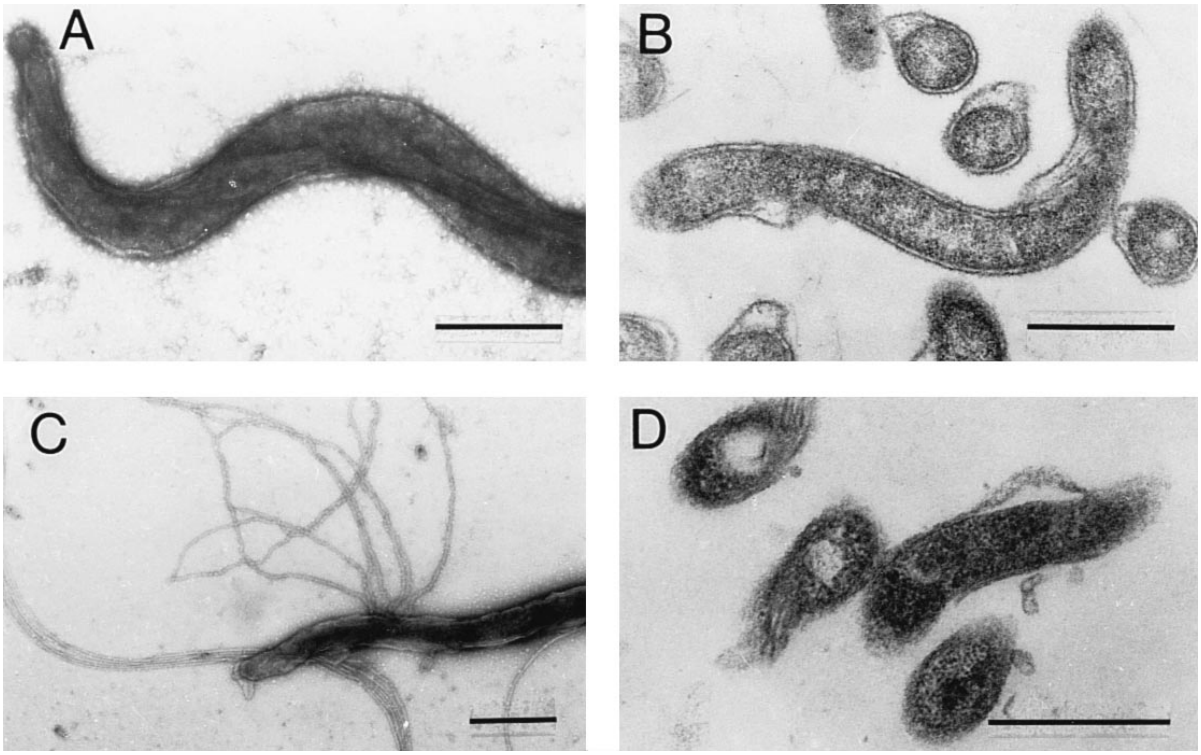

FIG. 4. EM characterization of protoplasmic cylinders. Material constituting the heavier peak from the sucrose density gradient was examined by negative staining (C) and ultrathin sectioning (D). For comparison purposes, intact spirochetes are shown in panels A and B.

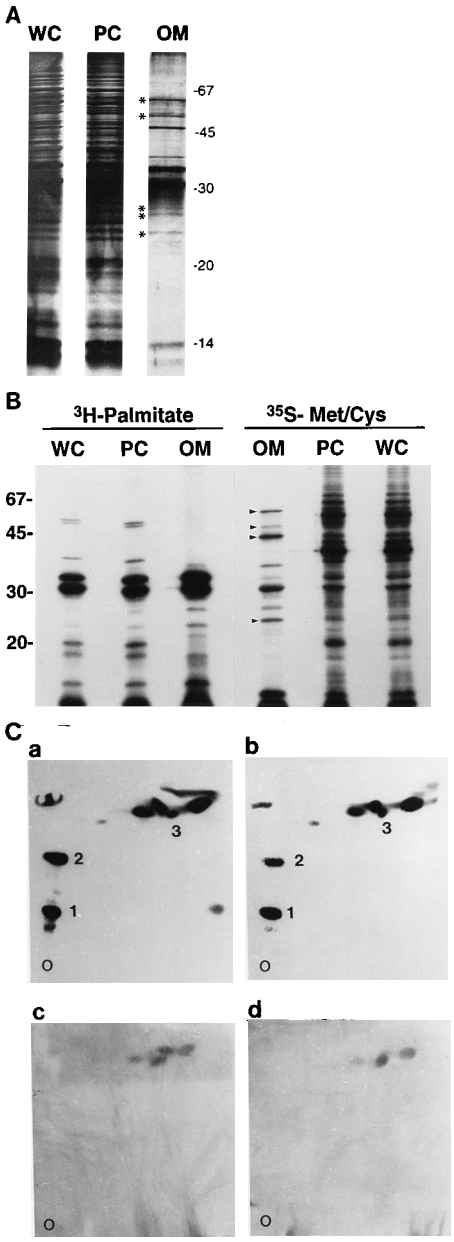

FIG. 5. Protein and lipid constituents of *B. burgdorferi* B31 OMs. (A) Silver-stained SDS-polyacrylamide gel of whole cells (WC), protoplasmic cylinders (PC), and pooled OM fractions. Asterisks denote polypeptides in OM preparations with electrophoretic mobilities distinct from those of proteins in whole cells and protoplasmic cylinders. (B) SDS-PAGE fluorography of the same fractions as those described for panel A from cells metabolically labeled with [^{3}H]palmitate or ^{35}S-amino acids. Lanes containing whole cells and protoplasmic cylinders were loaded with 10^7 organisms, while OM lanes contain membranes from 2×10^8 organisms. Arrowheads indicate ^{35}S-labeled OM polypeptides for which there were no corresponding [^{3}H]palmitate-labeled proteins (i.e., presumptive nonlipidated OM proteins). (C) Lipids extracted from *B. burgdorferi* whole cells (a and c) and isolated OMs (b and d) separated by thin-layer chromatography as described in Materials and Methods. Panels a and b are autoradiographs of [^{3}H]palmitate-labeled lipids. Panels c and d are immunoblots of unlabeled borrelial lipids immunoblotted with rat polyclonal antiserum directed against *B. burgdorferi* B31. Blots: 1, phosphatidylcholine; 2, phosphatidylglycerol; 3, glycolipids. Molecular mass markers (in kilodaltons) are indicated on the right and left sides of panels A and B, respectively.

were not significantly different from those in OMs of the corresponding intact spirochetes (Table 1).

SDS-PAGE revealed that, like the B31 strain, the OM fractions from the virulent and avirulent isolates contained a limited number of polypeptides; interestingly, the polypeptide profiles of the two OM preparations were similar but not identical (Fig. 7A). The lipoprotein and nonlipoprotein profiles of OMs from the two isolates were then compared. Consistent with the silver-stained gels, differences in the levels of expression of lipidated as well as nonlipidated proteins were observed (Fig. 7B). Regarding nonlipidated proteins, the much greater labeling of several proteins of 60 kDa in the low-passage clone was especially noticeable (Fig. 7B).

Humoral immune response against OM proteins during chronic Lyme disease. A poorly understood aspect of Lyme disease concerns the inability of host immune responses to eradicate persistent spirochetal infection. Although rising antibody titers to *B. burgdorferi* and expanding reactivity to spirochetal antigens have been well documented during persistent infection in a variety of mammalian hosts (39), it is not known how much of this antibody response is directed against surface-exposed borrelial proteins (and, therefore, is capable of contributing to bacterial clearance). To investigate this, C3H/HeJ mice were needle inoculated intradermally with low-passage N40 isolates (10^4 organisms) and then sacrificed at 8 weeks and 8 months postinfection. Pooled sera from the two groups of mice were then immunoblotted against proportional amounts of whole cells, protoplasmic cylinders, and OMs. The antibodies in both pools reacted predominantly with antigens associated with the protoplasmic cylinders (Fig. 8). Consistent with previous reports in which animals were needle inoculated with relatively small numbers of organisms (5, 45), no humoral response against either native or recombinant OspA and OspB was detected (Fig. 8).

DISCUSSION

Despite the considerable successes of the past decade in molecular characterization of *B. burgdorferi* surface antigens, systematic methods for identifying surface-exposed borrelial proteins are needed. Two novel strategies have recently evolved to address this need. The first involves the expression of export signals from borrelial polypeptides as fusions with an *Escherichia coli* alkaline phosphatase reporter lacking a signal peptide (16). A disadvantage of this molecularly based approach is that only a small proportion of the cloned export signals will be derived from surface-exposed OM proteins (26). The second, more direct approach, exemplified by this report and that of Bledsoe and coworkers (9), is to analyze protein and nonprotein constituents in isolated borrelial OMs based upon the presumption that a substantial proportion of OM-associated constituents are surface exposed.

Two bands with distinctly different specific gravities were obtained when plasmolyzed cells were subjected to isopycnic centrifugation on sucrose gradients. Compared with conventional gram-negative bacteria, markers for the various cellular and membrane compartments in *B. burgdorferi* are poorly defined. For this reason, we used several lines of evidence to prove that the membrane vesicles in the lighter band consisted of purified OMs. First, they contained OspA and OspB, two extremely well-characterized surface-exposed proteins of *B. burgdorferi* (2). Second, they contained minimal amounts of antigenic or enzymatic markers associated with the periplasmic space, cytoplasmic membrane, or cytosolic compartment. Last, their freeze-fracture EM morphologies were essentially identical to those of OMs on intact organisms. This was especially

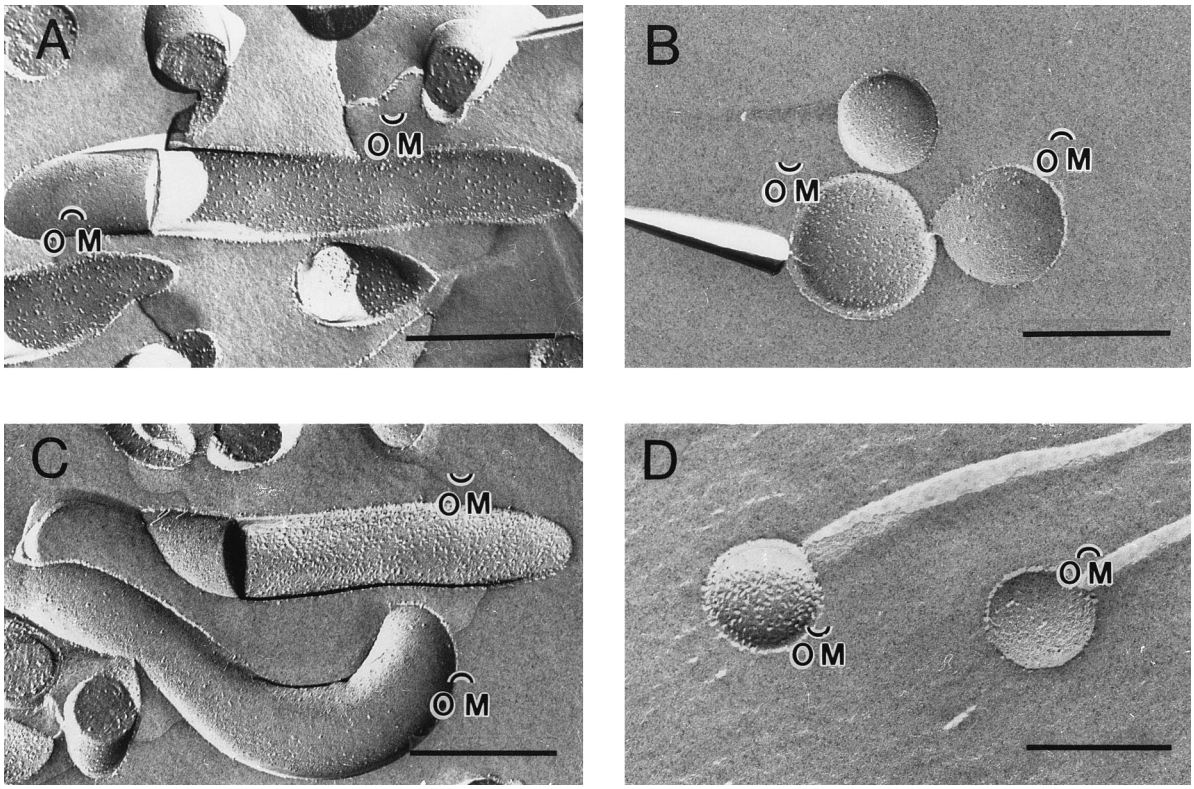

FIG. 6. Freeze-fracture EM of OMs isolated from low-passage (B) and high-passage (D) *B. burgdorferi* organisms. Shown for comparison purposes are freeze-fractured low-passage (A) and high-passage (C) *B. burgdorferi* N40 whole cells. $\breve{\text{OM}}$ and $\hat{\text{OM}}$ are as described in the legend to Fig. 3. Bars, 0.5 μm.

striking for the N40 isolates in which significant differences in particle densities in isolated OMs reproduced differences observed previously in whole cells (41). In a recent freeze-fracture EM study, we noted that borrelial blebs contain both outer and cytoplasmic membranes (41). Although it is possible that some of the vesicles in the OM fraction might have been blebs, structures with dual-membrane morphology were not observed despite meticulous examination of the replicas.

A particularly noteworthy observation was that isolated OMs contained only a small proportion of the total cellular content of OspA and OspB. Given the lack of an independent *B. burgdorferi* OM marker, it was essential to eliminate the obvious possibility that this finding reflected incomplete membrane fractionation. EM analysis of both negatively stained whole mounts and ultrathin sections revealed that the fractions with the preponderance of OspA and OspB consisted of protoplasmic cylinders without OMs. These results complement previous studies in which we showed, by both immunoelectron microscopy and detergent-based cell fractionation, that both OspA and OspB are predominantly cytoplasmic membrane associated (13). Moreover, to further assess the cellular distribution of OspA and OspB, we recently developed an immunofluorescence assay in which spirochetes are encapsulated in porous agarose beads (gel microdroplets) (20). A dramatic increase in labeling with polyclonal antisera to both OspA and OspB was observed among organisms incubated with concentrations of Triton X-100 which selectively exposed periplasmic antigens (19). Based upon these combined observations, we now propose that the majorities of OspA and OspB are anchored by their N-terminal lipids to the periplasmic leaflet of the cytoplasmic membrane and that small portions of these molecules are transported to the bacterial surface (13, 42). It is worth noting that *T. pallidum*, the agent of venereal syphilis, provides ample precedent for the notion that the large amounts of lipoproteins in a pathogenic spirochete may be cytoplasmic membrane associated; in fact, all lipoproteins in the syphilis spirochete appear to be subsurface (19, 40). At the same time, it must be acknowledged that this notion contradicts the general belief, also supported by experimental evidence (e.g., proteinase accessibility assays [3]), that OspA and OspB are exclusively OM associated. Further studies are warranted to resolve these discrepancies.

Analysis of isolated OMs has afforded novel insights into *B. burgdorferi* OM composition and molecular architecture. First, it is striking that all of the previously characterized *B. burgdorferi* surface antigens have been lipoproteins. In this study, we have confirmed the previous speculation (12, 40, 54) that the borrelial OM contains both lipidated and nonlipidated integral membrane proteins. Second, by comparing OMs from virulent and avirulent isolates of the same strain, potential virulence-associated OM proteins (nonlipidated as well as lipid modified) which can be targeted for further study were identified. Third, it was interesting to note that, compared with low-passage N40 OMs, particle densities were greater in high-passage N40 OMs despite their apparently smaller number of nonlipidated protein species (the presumed particle-formers). This result suggests that multiple factors, including the number of distinct proteins, their levels of expression, and, perhaps, their physical state (e.g., aggregation) in the OM, determine the freeze-fracture morphology of *B. burgdorferi* OMs. Fourth, the highly similar lipid compositions of OMs and whole cells suggest that bulk transfer of lipids occurs between cytoplasmic

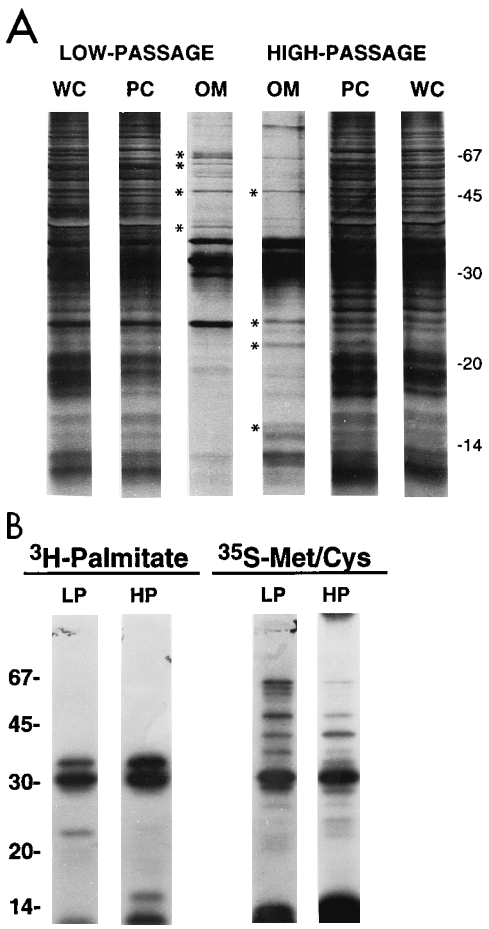

FIG. 7. Protein constituents of OMs from low- and high-passage *B. burgdorferi* N40 organisms. (A) Silver-stained SDS-polyacrylamide gel of 10^7 whole cells (WC), 10^7 protoplasmic cylinders (PC), and OMs from 2×10^8 low- and high-passage clonal isolates. Asterisks indicate OM proteins with electrophoretic mobilities distinct from those of the corresponding protoplasmic cylinders or whole cells. (B) Comparison of polypeptide constituents in OMs from low- and high-passage N40 clonal isolates metabolically labeled with [^{3}H]palmitate or ^{35}S-amino acids. Lanes contain OMs from 2×10^8 organisms. Molecular mass markers (in kilodaltons) are shown on the right and left of panels A and B, respectively.

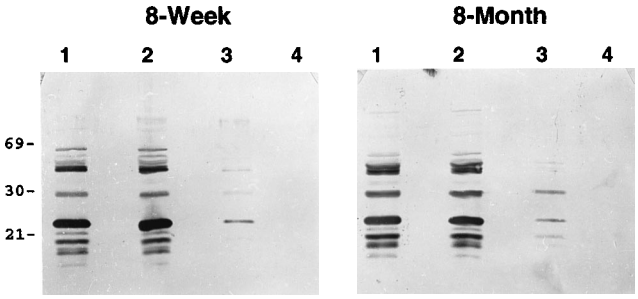

FIG. 8. Limited humoral immune response to *B. burgdorferi* OM proteins in mice with chronic Lyme disease. Pooled sera from C3H/HeJ mice needle inoculated intradermally with 10^4 low-passage *B. burgdorferi* N40 organisms 8 weeks or 8 months prior to sacrifice were immunoblotted against whole cells (lanes 1), protoplasmic cylinders (lanes 2), or isolated OMs (lanes 3) from 2×10^7 *B. burgdorferi* N40 organisms. Lanes 4 contain 2 μg each of recombinant, nonlipidated OspA and OspB. Molecular mass markers (in kilodaltons) are shown on the left.

membranes and OMs; this speculation is consistent with the highly fluid nature of the *B. burgdorferi* cell envelope (2). Lastly, antigenic glycolipids, which presumably correspond to the nonprotein antigens described by other investigators (6, 22, 55), were identified in the isolated OMs. It is not yet determined whether these molecules are surface exposed, but by analogy with LPS of gram-negative bacteria, the possibility exists that they may be targets for protective immunity.

Bledsoe and coworkers (9) recently described a protocol for fractionation of *B. burgdorferi* OMs and cytoplasmic membranes based upon sequential isopycnic centrifugation following disruption of spirochetes. As in the present study, they also observed that both OspA and OspB were associated predominantly with the cytoplasmic membrane fractions. Nevertheless, major differences were also noted between the two methods. In their hands, isolated OMs had a specific gravity of approximately 1.20 g/cm^2, a value similar to that of OMs from gram-negative bacteria (38). In contrast, OMs isolated from plasmolyzed spirochetes were considerably lighter (specific gravity, 1.12 g/cm^2), a result consistent with the relatively low protein content of *B. burgdorferi* OMs as determined by freeze-fracture EM studies (41, 54). Moreover, OMs isolated by their procedure contained many more polypeptides. One potential explanation for these differences is the methods used to disrupt the bacteria prior to isopycnic centrifugation. Disruption of spirochetes in a French pressure cell (9) creates a homogeneous suspension of all borrelial cell compartments and potentially complicates the subsequent separation steps; reassortment of both soluble and membrane-associated constituents from different compartments also could occur prior to and during separation. In contrast, our extremely gentle plasmolysis-based method for removal of OMs completely avoids disruption of the protoplasmic cylinders prior to isopycnic centrifugation. Because it involves only one ultracentrifugation, it has the additional advantage of being more rapid and simpler to perform.

The ability of *B. burgdorferi* to evade host immune defenses is one of the most enigmatic features of Lyme disease. On the basis of studies with tissue culture models, it has been proposed that residence within intracellular and/or other immunologically protected niches accounts for this phenomenon (17, 29, 33). However, other investigators have not found evidence for spirochete intracellularity in such in vitro systems (25, 52), while spirochetes visualized in tissue specimens from *B. burgdorferi*-infected humans and animals appeared to be extracellular (5, 21, 28). For this reason, we are attracted to the alternative hypothesis that *B. burgdorferi* evades host clearance mechanisms, at least in part, by limiting the antigenicity of its surface. This could occur as a result of (i) poor antibody responses against OspA and OspB (5, 23, 44, 46), (ii) the presence of poorly immunogenic nonlipidated OM proteins (12, 40), and (iii) a limited surface exposure of immunogenic lipoproteins. The net effect of these mechanisms, shown by the data in Fig. 8, is that only a fraction of *B. burgdorferi*-specific serum antibodies bind to OM constituents. The availability of purified *B. burgdorferi* OMs should facilitate efforts to elucidate the basis for immune evasion by the Lyme disease spirochete.

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