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Purification and Immunological Characterization of a Major Low-Molecular-Weight Lipoprotein from *Borrelia burgdorferi*

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Borrelia burgdorferi **resembles gram-negative bacteria in having both cellular and outer membranes. We previously showed that a lipopolysaccharide (LPS)-like material could be extracted from *B. burgdorferi* with phenol-chloroform-petroleum ether (PCP). The PCP extract of *B. burgdorferi* exhibited biological activity in several in vitro assays (e.g., mitogenicity, pyrogenicity, and cytokine release). These activities suggested the presence of endotoxin. The PCP extract of *B. burgdorferi*, however, also contained a small amount of protein. Preliminary studies showed that monoclonal antibody prepared against this protein inhibited the mitogenic activity of the PCP extract toward murine spleen cells. The current study was therefore undertaken to characterize this protein and to establish methods for its separation from the LPS. The PCP-extracted protein consisted of a single, low-molecular-weight lipoprotein (apparent M_r, 10,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (SDS-PAGE). By protein analysis, it accounted for 2% of the dry weight of defatted cells, thus making it a major constituent of the spirochete. It was purified from the LPS by initial extraction into 10% Triton X-100 followed by immunoaffinity chromatography in the presence of detergent. On removal of the LPS, the purified lipoprotein formed aggregates stable to SDS-PAGE which were detectable on Western blots (immunoblots) probed with either the monoclonal antibody or polyclonal antiserum. From a plot of the aggregate molecular weight versus aggregate size, a monomer molecular weight of 7,500 was obtained. Indirect immunofluorescence with the monoclonal antibody showed that the lipoprotein was exposed at the surface of the spirochete in only a small percentage of cells. The lipoprotein was present in several strains of *B. burgdorferi* but absent in other *Borrelia* spp., treponemes, and gram-negative human pathogens, indicating species specificity.**

Borrelia burgdorferi, the etiologic agent of Lyme disease, is transmitted through the bite of an infected *Ixodes* tick (20). Disease symptoms include erythema migrans (a characteristic skin lesion emanating from the site of the tick bite), headache, fatigue, migratory joint and muscle pains, meningitis, arthritis of the large joints, and cardiac abnormalities (69). Little is known about how the spirochete is able to cause disease. Virulence factors for the organism have yet to be identified (72).

The trilaminar construction of the spirochetal cell envelope (inner membrane, peptidoglycan layer, outer membrane) resembles that of gram-negative bacteria (50). Reports differ as to whether spirochetes contain lipopolysaccharide (LPS) in their outer membrane. *Leptospira interrogans*, *Treponema phagedenis* (biotype Reiter), and *Serpula* (*Treponema*) *hyodysenteriae* apparently do contain an LPS-like substance (3, 21, 23, 28, 35, 36, 41, 63, 66, 76, 77), while *Treponema pallidum* apparently does not (3, 63). There is conflicting evidence regarding the presence of LPS in *Borrelia* spp. (8, 22, 26, 73).

Previous studies in this laboratory showed that a phenol-chloroform-petroleum ether (PCP) extract of *B. burgdorferi* exhibited many of the same activities as endotoxin from gram-negative organisms. Thus, PCP extract was mitogenic for murine spleen cells (37) and stimulated the release of interleukin 1 from human synovial fluid cells (7) and interleukin 6 from C6 rat glioma cells (40). It also produced fever in rabbits (8) and inflammatory skin lesions (38). These activities suggest the presence of an endotoxic activity in *B. burgdorferi*. Whether this endotoxic activity is attributable to LPS, an LPS-like substance, or another spirochetal component needs to be determined.

LPS is typically isolated from gram-negative organisms by extraction with either cold trichloroacetic acid (68), cold butanol-water (57), hot phenol-water (79), or PCP (29). The first three methods are used to extract smooth LPS, while PCP is used to extract rough LPS. LPS isolated by trichloroacetic acid or aqueous butanol generally contains more protein than LPS isolated by aqueous phenol. At least some of this protein appears to be coextracted outer membrane proteins (33, 58). While PCP-extracted LPS was initially thought to be free of protein (29), more recent studies indicate that it, too, may contain coextracted membrane proteins (70, 71).

The proteins which coextract with LPS have been variously termed endotoxin protein (34), lipid A-associated protein (58), and LPS-associated protein (33). Preliminary characterization of the endotoxin proteins from *Escherichia coli* and *Salmonella typhosa* indicate that they consist mainly of outer membrane proteins (33, 34, 58). Among those identified were *E. coli* porins, protein II*, and Braun's lipoprotein (33, 58). Many of the activities ascribed to endotoxin have similarly been ascribed to endotoxin protein (47).

Initial chemical characterization of the *B. burgdorferi* PCP extract indicated that it contained a small amount of protein (8, 37). In preliminary experiments, monoclonal antibody prepared against this protein was found to inhibit the mitogenic activity of the PCP extract toward murine spleen cells. The object of the current study was thus to characterize this

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protein better and to establish methods for its purification from the borrelial LPS. Future studies will then define the biological activities of this protein independent of the borrelial LPS.

In the present studies, a single low-molecular-weight lipoprotein (M_r, 10,000) was found to coextract with the borrelial LPS. Purification from the LPS was accomplished by initial extraction into 10% Triton X-100 followed by immunoaffinity chromatography in the presence of detergent. Indirect immunofluorescence studies showed that this lipoprotein is surface exposed in only a small percentage of cells.

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MATERIALS AND METHODS

Bacteria and culture conditions. *B. burgdorferi* B31 (20), J31 (31), TI, Bep4, BP2, N/40 (6), ESP-1 (30), 20001, Aug, and HBD (9) were used. Strains B31, J31, TI, Bep4, BP2, and N/40 are American (*Ixodes dammini*) tick isolates; strains ESP-1 and 20001 are European (*Ixodes ricinus*) tick isolates; strains Aug and HBD are human blood isolates from Long Island, N.Y. Strain N/40 (6) was obtained from Stephen Barthold. Strains B31, J31, TI, Bep4, BP2, ESP-1, 20001, Aug, and HBD were obtained from Jorge Benach. All *B. burgdorferi* strains were grown in modified BSK medium without serum (10) at 32 to 34°C. *Borrelia anserina* and *Borrelia hermsii*, obtained from J. Benach from a previous gift of Russell C. Johnson, were grown at 32 to 34°C in modified BSK medium supplemented with 6% heat-inactivated normal rabbit serum. Cultures of *Acinetobacter calcoaceticus*, *Enterobacter aerogenes*, *E. coli*, *Plesiomonas shigelloides*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*, grown in Trypticase soy broth (BBL) at 37°C, were obtained from George Tortora.

Preparation of PCP extract. PCP extract was prepared by a modification of a previously described method (8). Spirochetes (5 liters of *B. burgdorferi* B31) were grown to late log phase and harvested by centrifugation (10 min at 5,000 × *g*). The cells were washed twice with cold sterile pyrogen-free water, and the final pellet was stored at −20°C. Sterile water was used instead of phosphate-buffered saline (PBS) to avoid potential loss of cell surface components by saline extraction (29). Before PCP extraction, the cells were defatted by sequential treatment with acetone and diethylether at 4°C. The defatted cells (typically 0.5 g) were then dispersed by sonication in 15 ml of PCP (2 ml of 89.3% aqueous phenol plus 5 ml of chloroform plus 8 ml of petroleum ether). The supernatant was collected by centrifugation (15 min at 3,000 × *g*). The residue was reextracted with PCP. The combined extracts were flash evaporated at 40°C to remove chloroform and petroleum ether. The remaining volume was transferred to dialysis tubing (pore size, 8,000 M_r) and dialyzed against distilled deionized water to remove phenol. The resultant precipitate was dispersed by sonication and stored as a milky white suspension at 4°C.

Purification of lipoprotein from PCP extract. Typically, 0.5 ml of PCP extract (approximately 0.2 mg of protein) was mixed with an equal volume of 20% Triton X-100, and aliquots were taken and analyzed for protein content. Sufficient 10% Triton X-100 was then added to yield a final protein concentration of 50 μg/ml. Cold acetone (10 volumes) was added, and the preparation was stored overnight at −20°C to allow precipitate formation. The precipitate was collected by centrifugation (4 h at 3,000 × *g* at 4°C), washed twice with cold acetone (40 ml per wash), and stored at 4°C. The maximum yield of precipitate as determined by Lowry protein assay was obtained with 4 h of centrifugation at this centrifugal force. Shorter times gave lesser yields.

The pellet was taken up in 1 ml of 10% Triton X-100 and vortexed vigorously until suspended. Insoluble material was removed by a brief centrifugation (5 min at 3,000 × *g*) to avoid application of this material to the immunoaffinity column. The soluble fraction was diluted 1:10 with radioimmunoprecipitation (RIPA) buffer (pH 8) (0.05 M Tris-HCl [pH 8], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M sodium chloride) and then applied to the column (see below). The column was washed with RIPA buffer (pH 8) to remove unbound material, and then the lipoprotein was eluted with 0.1 M glycine-HCl buffer (pH 2.5) containing 1% Triton X-100. The acid eluant was neutralized with 1.875 M Tris-HCl buffer (pH 8.8) (30 μl/ml of volume) and dialyzed overnight against 1% Triton X-100. Insoluble material was removed by centrifugation (20 min at 9,800 × *g* at 4°C). Cold acetone (10 volumes) was added, and the purified lipoprotein was allowed to precipitate overnight at −20°C. The precipitate was collected by centrifugation (4 h at 3,000 × *g* at 4°C), washed twice with cold acetone, and stored at 4°C. Silicon-treated glass conical centrifuge tubes (15 and 40 ml) were used throughout. An overview of the purification scheme is provided in Fig. 1.

Immunoaffinity column. Mouse monoclonal antibody (MAb 240.7) was covalently linked to protein G-Sepharose 4FF (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) by a modification of the method of Schneider et al. (65). Briefly, protein G-Sepharose 4FF (settled volume, 0.6 ml) was mixed with MAb 240.7 (10 mg) in 0.1 M Tris-HCl buffer (pH 6.8) containing 0.1% Triton X-100, 0.1% sodium deoxycholate, 0.01% SDS, and 0.1 M sodium chloride (total volume, 11 ml) and incubated overnight with gentle agitation at 4°C. The beads were pelleted (2 min at 1,500 × *g*) and washed three times with RIPA buffer (pH 6.8) (0.25 M Tris-HCl [pH 6.8], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M sodium chloride). The pH was brought to 8.2 during two washes with 0.2 M triethanolamine-HCl buffer (pH 8.2). Cross-linking reagent (10 ml of 20 mM dimethyl pimelimidate in 0.2 M triethanolamine buffer [pH 8.2]) was added, and the reaction was allowed to proceed for 45 min at room temperature with gentle agitation. The beads were recovered, 10 ml of 20 mM ethanolamine (pH 8.2) was added, and excess sites were blocked during 5 min of incubation at room temperature. The beads were then washed three times with 0.1 M sodium borate (pH 8.2) and stored at 4°C in borate buffer containing 0.02% sodium azide.

Monoclonal antibody to lipoprotein. PCP extract was used to immunize BALB/c mice for the preparation of monoclonal antibody. Male mice (3 months old) were injected intravenously at weekly intervals with 0.2 ml of PCP extract (20 μg [dry weight]) in sterile saline. Spleen cells were taken from one immune mouse 3 days after the final injection (5 weeks) and fused with the parent myeloma cell line, X63-Ag8.653. Standard procedures were used for the fusion, cloning, and subcloning of the antibody-producing cells. Clones were selected on the basis of the reactivity of their supernatants with PCP extract in an enzyme-linked immunosorbent assay (ELISA), and expanded, and the cells were used for mass production of monoclonal antibody in pristane-primed mice. Monoclonal antibody was partially purified from ascites fluid

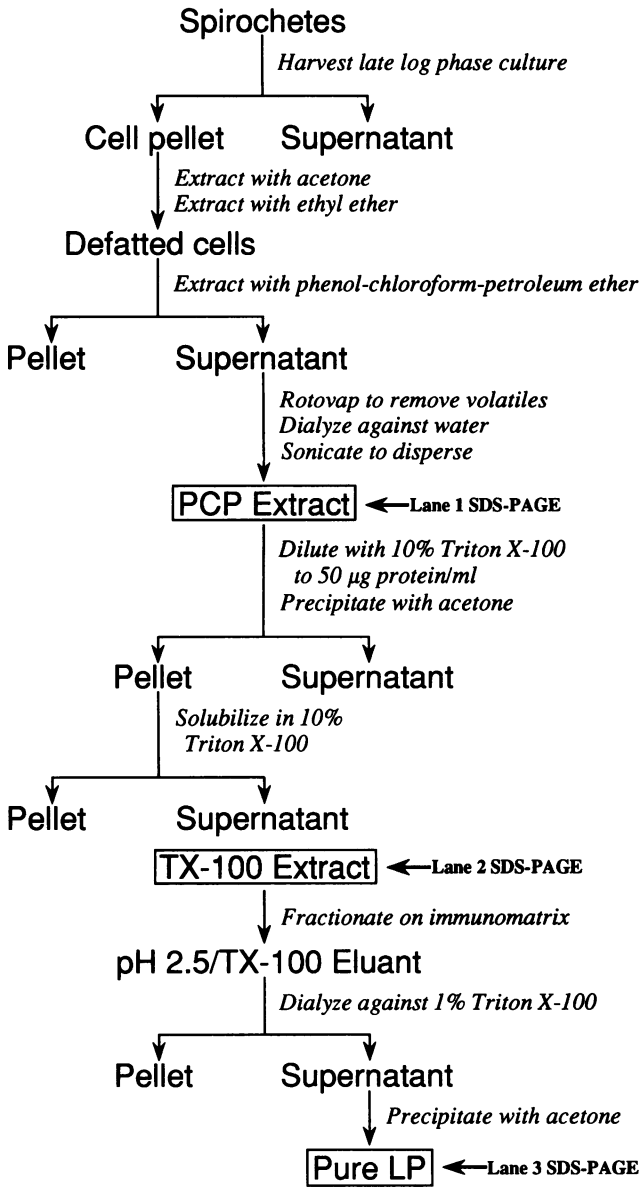

FIG. 1. Scheme for purification of the 10-kDa lipoprotein from *B. burgdorferi* B31. The labeled arrows indicate the samples taken for SDS-PAGE.

by three rounds of ammonium sulfate precipitation. Antibody concentration was estimated by A_{280}, assuming an extinction coefficient of 14.3 for a 1% solution. The isotype was determined by antigen-capture ELISA.

Rabbit antisera to PCP extract. New Zealand White rabbits (3 to 5 kg) were immunized by subcutaneous injection of PCP extract in complete Freund's adjuvant (primary injection) and incomplete Freund's adjuvant (boosts). Primary injections contained 0.5 mg of PCP extract (dry weight), while secondary injections contained 0.1 mg of PCP extract. Intravenous injections of PCP extract in saline were also given to boost the response. The presence of antibody was detected by ELISA.

Immunofluorescence. Immunofluorescent labeling of spirochetes was performed in two ways: (i) cells were fixed to a slide and antibody was added, or (ii) cells were suspended in medium and antibody was added. In either case, late-log-phase spirochetes were pelleted (10 min at 16,000 × *g*) and washed once with BSK medium to remove extracellular antigen. The resuspended spirochetes were then checked by dark-field microscopy, and only suspensions yielding fully (>99%) motile spirochetes were used.

In the fixed-cell assay, 5-μl aliquots of the resuspended spirochetes were applied to the wells (6 mm in diameter) of a Teflon-masked slide and allowed to air dry for 15 min. Primary antibody (43 μg of purified monoclonal antibody per ml of BSK medium) was then added (20 μl per well), and the slide was incubated in a moist chamber for 30 min at room temperature with gentle agitation. The slide was flushed with medium, immersed for 15 min in medium, and allowed to drain. Secondary antibody (75 μg of fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin G (IgG) [Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.] per ml of BSK medium) was added (20 μl per well), and the slide was incubated and washed with BSK medium as described before. BSK medium was used as the mounting fluid, and a coverslip was applied and sealed with nail polish. Attached cells were located by dark-field microscopy. Fluorescent cells were viewed under oil by using a 100× objective on a Nikon Labophot microscope equipped with an HBO 100-W mercury light source (Nikon Inc., Garden City, N.Y.).

In the suspended-cell assay, monoclonal antibody was added to resuspended spirochetes (final concentration, 85 μg/ml) and allowed to bind for 45 min at room temperature. The spirochetes were then pelleted, washed twice with BSK medium, and resuspended in BSK medium containing FITC-conjugated secondary antibody (final concentration, 75 μg/ml). The secondary antibody was allowed to bind for 1 h at room temperature. The spirochetes were pelleted, washed twice with BSK medium, and then mounted in BSK medium for viewing as described above.

In either assay, negative controls consisted of cells exposed to an irrelevant monoclonal antibody or to secondary antibody alone.

ELISA. A microplate modification of the method of Engvall and Perlmann (27) was used. Immulon 1 microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with PCP extract as follows. PCP extract was dissolved in 0.1 M sodium carbonate (pH 9.6) at 1 μg/ml, and 100 μl was applied to each well. The plates were incubated overnight at 4°C. The plates were emptied of liquid, and any plates not used immediately were stored at −70°C. Prior to use, the coated plate was washed three times with PBS-T (0.01 M sodium phosphate [pH 7.4], 0.15 M sodium chloride, 0.05% Tween 20). Hybridoma supernatant was added (100 μl per well), and the plate was incubated for 90 min at 37°C. The plate was then washed three times with PBS-T. Goat anti-mouse IgG Fc fragment (γ chain specific; Organon Teknika-Cappell, Durham, N.C.) at 10 μg/ml in PBS-T was added (100 μl per well), and the plate was incubated for 90 min at 37°C. The plate was washed three times with PBS-T. Rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) at 1 μg/ml in PBS-T was added (100 μl per well), and the plate was incubated for an additional 90 min at 37°C. The plate was then washed three times with PBS-T and then three times with distilled water. Sigma 104 phosphatase substrate was added according to the manufacturer's instructions (100 μl per well), and the plate was incubated for 60 min at room temperature. The reaction was stopped by the addition of 1 N sodium hydroxide (50 μl

per well), and the A_{410} was read on a Dynatech MR 600 microplate reader.

Radiolabeling of spirochetes with [^{3}H]palmitate. Five millicuries of [9,10-^{3}H]palmitic acid (specific activity, 30 Ci/mmol; DuPont NEN, Boston, Mass.) in 0.1 ml of ethanol was added to a 5-ml culture of *B. burgdorferi* B31, and the culture was further incubated at 32°C for 2 to 4 days to allow incorporation of the label.

Radioimmunoprecipitation. Radiolabeled spirochetes were harvested by centrifugation and washed twice with cold sterile water. The cell pellet from 5 ml of stationary-phase culture was taken up in a mix of detergents consisting of 50 μl of 10% Triton X-100, 50 μl of 5% sodium deoxycholate, and 5 μl of 10% SDS. This mixture was boiled for 10 min to aid dissolution and inactivate any potentially degradative enzymes. After boiling, 200 μl of 0.625 M Tris-HCl (pH 6.8), 50 μl of 1.5 M sodium chloride, and 145 μl of distilled deionized water were added, bringing the final volume to 0.5 ml and the final composition to that of RIPA buffer (pH 6.8). Insoluble material was removed by centrifugation (2 min at 8,700 × *g*). Aliquots (100 μl) of the supernatant fraction were mixed with either 20 μl of saline (control) or 20 μl of purified monoclonal antibody (4.25 mg/ml in saline). These mixtures were incubated for 2 h at room temperature with gentle agitation, after which 80 μl of a 20% suspension of protein G-Sepharose 4FF (Pharmacia) in 0.25 M Tris-HCl (pH 6.8) was added to each. These were further incubated for 1 h. The beads were collected by centrifugation (30 s at 8,700 × *g*), washed three times with RIPA buffer (pH 6.8), and suspended in distilled deionized water to 200 μl. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (see below), 100 μl of 3× Laemmli sample buffer (0.1875 M Tris-HCl [pH 6.8], 6% SDS, 30% glycerol, 15% β-mercaptoethanol, 0.003% bromophenol blue) was added. The samples were boiled for 5 min, the beads were pelleted, and then aliquots of the supernatant fraction were applied to the gel.

SDS-PAGE and Western blot (immunoblot) analysis. SDS-PAGE was carried out as described by Laemmli (53) but with a 16% running gel and a 3% stacking gel. LPS was visualized with periodate-silver by the method of Tsai and Frasch (75). Proteins were stained with Coomassie brilliant blue R-250 using 50% methanol-12% acetic acid as the fixative. Radiolabeled bands were detected by fluorography (13) using the fluorographic enhancer Amplify (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions.

Western blotting was done by a modification of the method of Sidberry et al. (67). Gels were electroblotted in Towbin's transfer buffer (74) onto 0.45-μm-pore-size nitrocellulose membranes (Hoeffer Scientific Instruments, San Francisco, Calif.). The blots were blocked in PBS containing 2% casein, incubated for 1 h each in primary antibody (2 μg of purified monoclonal antibody per ml or a 1:250 dilution of rabbit anti-PCP extract) and secondary antibody (2 μg of alkaline phosphatase conjugate of sheep anti-mouse IgG or goat anti-rabbit IgG per ml [Sigma]) and reacted with substrate (ρ-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate ρ-toluidine salt) according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, Calif.).

Protein methods. Protein was assayed by a modified Lowry method (62) with bovine serum albumin as the standard. Cyanogen bromide cleavage of PCP extract was done by using 1 mg of cyanogen bromide per ml in 70% formic acid with overnight incubation at room temperature as detailed by Matsudaira (55). Proteinase K digestion of PCP extract was done by a modification of the method of Hitchcock and Brown (45). Samples were mixed with 3× Laemmli sample buffer (2 parts to 1 part) and boiled for 10 min. Proteinase K (final concentration, 0.4 mg/ml) in 1× Laemmli sample buffer was then added, and the digestion was allowed to proceed for 18 h at 37°C. Sequencing of SDS-polyacrylamide gel-purified lipoprotein electroblotted onto Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) was done by using standard techniques (54).

Molecular weights were estimated by the method of Weber and Osborn (78). Low-molecular-weight unstained standards were used for Coomassie blue-stained gels, and low-molecular-weight prestained standards were used for Western blots (both from Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.).

RESULTS

SDS-PAGE analysis of *B. burgdorferi* B31 PCP extract. When the PCP extract of *B. burgdorferi* was subjected to SDS-PAGE analysis and the gel was stained with the periodate-silver method designed to detect LPS, two bands were seen, one diffuse gray-staining band at 3 kDa and one sharper brown-staining band at 10 kDa (Fig. 2A, lane 1). When a duplicate gel was stained with Coomassie blue, only the 10-kDa band was detected (Fig. 2B, lane 1). Two bands (3 and 10 kDa) were seen in Western blots probed with polyclonal antiserum raised against the PCP extract (Fig. 2C, lane 1). Western blots probed with monoclonal antibody (see below) showed a single band at 10 kDa (Fig. 2D, lane 1). These data suggested that the PCP extract contained two major components: a low-molecular-weight LPS-like material and a low-molecular-weight protein. Previously, we reported that *B. burgdorferi* PCP extract contained low-molecular-weight LPS-like material; the low-molecular-weight protein, which stains poorly with silver, was not detected (8).

Preliminary characterization of the 10-kDa protein. Overnight incubation of the PCP extract with proteinase K resulted in complete loss of the 10-kDa band as determined by Coomassie blue staining and Western blot analysis with the polyclonal anti-PCP extract used as the probe. The same treatment had no effect on the low-molecular-weight silver-staining material. Overnight incubation with cyanogen bromide destroyed the 10-kDa band while leaving the 3-kDa band intact, thus indicating that the former consisted of protein containing one or more methionine residues. A control incubated in 70% formic acid but without cyanogen bromide showed no change. Attempted sequence analysis of the 10-kDa protein showed a blocked N terminus.

To determine if the 10-kDa protein might contain lipid, we grew strain B31 in medium containing [^{3}H]palmitate to incorporate label into lipoproteins. Labeled spirochetes were harvested and boiled with sample buffer, and the supernatant fraction was subjected to SDS-PAGE and fluorography. The fluorograph showed several radiolabeled bands, including one at approximately 10 kDa (Fig. 3A). The two bands at 31 and 33 kDa were likely OspA and OspB (15).

Radioimmunoprecipitation of the 10-kDa protein. Monoclonal antibody to the 10-kDa protein was prepared, and several positive clones were detected. One clone that produced antibody that stained the 10-kDa band in Western blots of PCP extract (Fig. 2D, lane 1) was selected for further study. Isotype analysis showed this antibody (MAb 240.7) to be an IgG1. We used this monoclonal antibody to immuno-

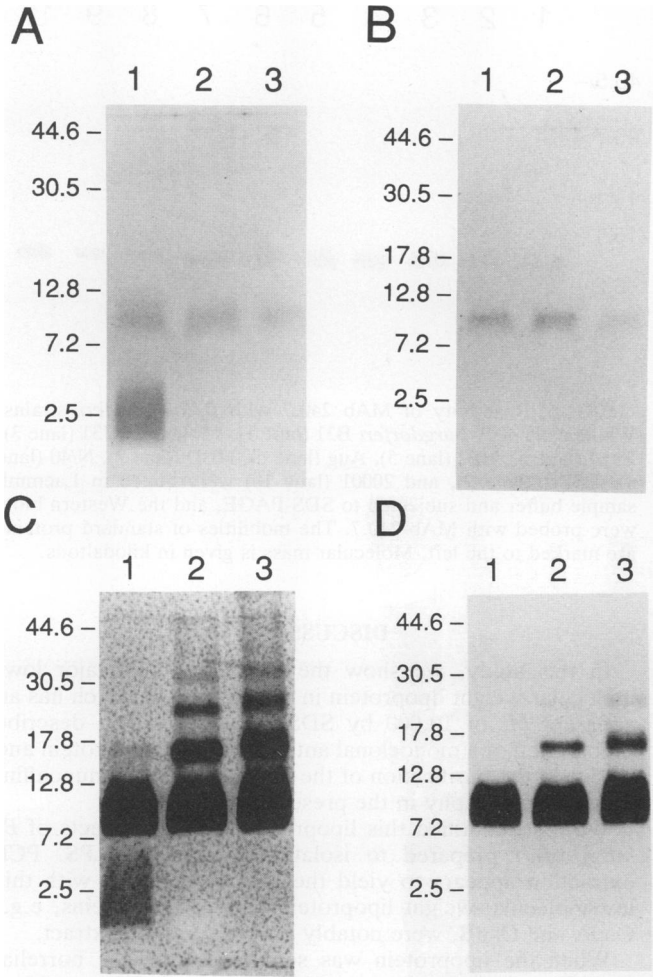

FIG. 2. SDS-PAGE and Western blot analysis of PCP extract, Triton X-100 extract, and pure lipoprotein. (A to D) Lanes: 1, PCP extract (0.5 μg); 2, Triton X-100 extract (0.5 μg); 3, purified lipoprotein (0.4 μg). The gels were stained with periodate-silver (A) or Coomassie blue (B). Western blots were probed with rabbit anti-PCP extract (C) or MAb 240.7 (D). The mobilities of standard proteins are marked to the left of each panel. Molecular mass is given in kilodaltons.

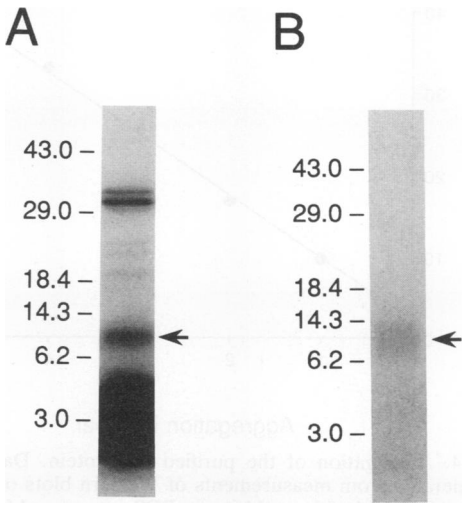

FIG. 3. Fluorographs of SDS-polyacrylamide gels showing radiolabeling of the 10-kDa lipoprotein by [^{3}H]palmitate. (A) Labeled whole spirochetes were boiled in Laemmli sample buffer and subjected to SDS-PAGE. (B) A detergent extract of labeled whole spirochetes was mixed with MAb 240.7, and the immunoprecipitate was boiled in Laemmli sample buffer and subjected to SDS-PAGE. Arrows indicate the position of the 10-kDa band. The mobilities of standard proteins are marked to the left of each panel. Molecular mass is given in kilodaltons.

precipitate the 10-kDa band from labeled cells and subjected this immunoprecipitate to SDS-PAGE and fluorography. The fluorograph showed a single 10-kDa band (Fig. 3B), indicating that the immunoreactive 10-kDa protein present in PCP extract is a lipoprotein.

Immunomatrix purification of the 10-kDa lipoprotein. Figure 1 gives an overview of the purification scheme. When the protocol was optimized, 70% of the initial protein was recovered after Triton X-100 extraction and 12% was recovered after immunoaffinity chromatography. The yield of purified lipoprotein from 90 mg of defatted cells (1 liter of culture) was 140 μg.

Aliquots were subjected to SDS-PAGE at three points during the purification: (i) PCP extract, (ii) Triton X-100 extract, and (iii) pure lipoprotein (Fig. 1). The samples were assayed for protein, and the same amount of protein was applied to the gel in each case. Pure lipoprotein gave a single band (M_r, 10,000) on Coomassie blue-stained gels (Fig. 2B, lane 3) and showed no evidence of contamination with periodate-silver-staining material (Fig. 2A, lane 3).

Western blots probed with either the polyclonal antiserum or monoclonal antibody showed multiple bands for both Triton X-100 extract and pure lipoprotein (Fig. 2C and D, lanes 2 and 3, respectively). In the case of pure lipoprotein, up to six bands were seen (three bands are apparent in Fig. 2D, lane 3). Molecular weights (plus standard errors of the mean) were estimated for five of these bands: 9,600 (±100), 16,700 (±300), 25,500 (±500), 33,200 (±500), and 39,000 (±100). Because of the regular spacing, these bands most likely represent aggregates of the lipoprotein. A plot of the molecular weight versus an assumed aggregate size is given in Fig. 4. From the slope of this plot, the estimated monomer size is 7,500.

Purification did not appear to alter the molecular weight of the lipoprotein. Most of the LPS-like material was separated from the lipoprotein in the earlier detergent step; however, trace amounts remained with the lipoprotein and were later separated on the immunoaffinity column.

Possible surface location of the 10-kDa lipoprotein. Two approaches were taken to determine if the lipoprotein was surface available in the whole spirochete. First, cells were dried down on slides and reacted with monoclonal antibody and then with FITC-conjugated secondary antibody. This method showed uniform fluorescein labeling of all attached spirochetes (Fig. 5). The second approach taken was to label the spirochetes with primary and secondary antibody while they were still viable in culture. That is, the cells were pelleted, incubated with monoclonal antibody in medium, washed with medium, incubated with FITC-conjugated secondary antibody in medium, washed with medium again, and finally mounted in medium for viewing. This method showed very few fluorescein-labeled spirochetes (i.e., <1%). Also, most of the unlabeled cells were motile while the labeled cells were not.

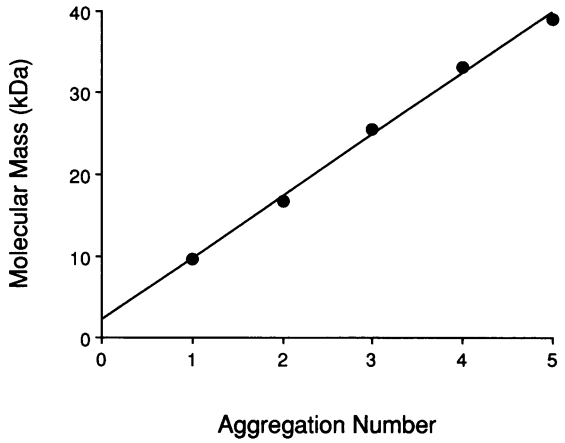

FIG. 4. Aggregation of the purified lipoprotein. Datum points were generated from measurements of Western blots of pure lipoprotein probed with either rabbit anti-PCP extract or MAb 240.7 in nine experiments. Six bands were detected; molecular weights were calculated for five of these. Error bars (standard errors of the means) are contained within the width of the points. Linear regression analysis (r^2 = 0.995) was used to draw the line.

Presence of the lipoprotein in other bacteria. Various strains of *B. burgdorferi* (B31, ESP-1, N/40, HBD, Bep4, TI, Aug, J31, BP2, and 20001) and one strain each of *B. anserina*, *B. hermsii*, *T. pallidum*, and *T. phagedenis* (biotype Reiter) were tested for the presence of the lipoprotein. Western blots probed with the monoclonal antibody showed a cross-reactive band of the same molecular size (M_r, 10,000) in all strains of *B. burgdorferi* (Fig. 6) but none in the other spirochetes (Fig. 7A). Western blots probed with the polyclonal rabbit antiserum showed the same result (Fig. 7B). It should be noted, however, that a cross-reactive borrelial LPS was detected in all three *Borrelia* species. Various gram-negative organisms (i.e., *A. calcoaceticus*, *E. aerogenes*, *E. coli*, *P. shigelloides*, *P. vulgaris* and *P. aeruginosa*) were also tested with the monoclonal antibody, and none showed cross-reactive material.

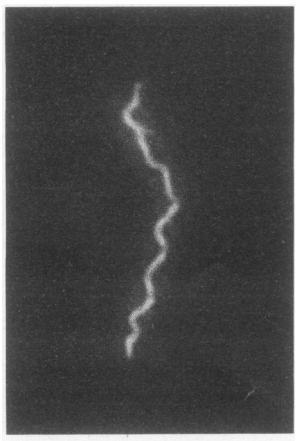

FIG. 5. Spirochetes labeled by indirect immunofluorescence using MAb 240.7 as primary antibody and FITC-conjugated secondary antibody. Magnification, ×1,400.

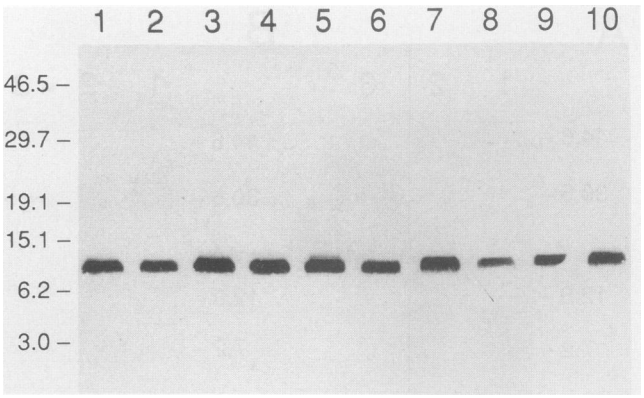

FIG. 6. Reactivity of MAb 240.7 with *B. burgdorferi* strains. Whole cells of *B. burgdorferi* B31 (lane 1), TI (lane 2), J31 (lane 3), Bep4 (lane 4), BP2 (lane 5), Aug (lane 6), HBD (lane 7), N/40 (lane 8), ESP-1 (lane 9), and 20001 (lane 10) were boiled in Laemmli sample buffer and subjected to SDS-PAGE, and the Western blots were probed with MAb 240.7. The mobilities of standard proteins are marked to the left. Molecular mass is given in kilodaltons.

DISCUSSION

In this study, we show the presence of a major low-molecular-weight lipoprotein in *B. burgdorferi* which has an apparent M_r of 10,000 by SDS-PAGE. We also describe preparation of a monoclonal antibody to this lipoprotein and its use in the purification of the lipoprotein by immunoaffinity chromatography in the presence of detergent.

We first identified this lipoprotein in PCP extracts of *B. burgdorferi* prepared to isolate the borrelial LPS. PCP extraction appears to yield the LPS in complex with this low-molecular-weight lipoprotein. Other lipoproteins, e.g., OspA and OspB, were notably absent from the extract.

When the lipoprotein was separated from the borrelial LPS, it formed stable aggregates detectable in Western blots probed with either monoclonal antibody or polyclonal antiserum. Aggregate molecular weight plotted against integral aggregate size gave a straight line (Fig. 4). The slope of this line indicates a monomer molecular weight of 7,500. This is in contrast to 9,600, which is the molecular weight of the fastest-running band. The reason for this discrepancy is not clear. Others have noted aggregate formation by low-molecular-weight bacterial lipoproteins on SDS-polyacrylamide gels (2, 18, 25, 46).

In addition to the propensity for aggregate formation, the borrelial lipoprotein resembles other gram-negative lipoproteins in both size and cellular location (see discussion below). The best studied of these is Braun's lipoprotein (16), which has an M_r of 7,200 (42) and is present in *E. coli* in both murein-linked (16) and free forms (51). The function of the bound form appears to be to anchor the outer membrane to the underlying cell wall (14). The function of the free form is unknown.

Whether the lipoprotein described here represents the borrelial equivalent of Braun's lipoprotein remains to be determined. Brandt and coworkers (15) also show the presence of a 10-kDa lipoprotein in *B. burgdorferi*.

Braun's lipoprotein is the most abundant protein in *E. coli* and accounts for approximately 3% of the total cellular dry weight (60, 61). PCP extracts of *B. burgdorferi* contained an average of 19 mg of protein per g of defatted cells (1.9%). Since spirochetes contain approximately 20% lipid by weight (52), the PCP-extracted lipoprotein accounted for about

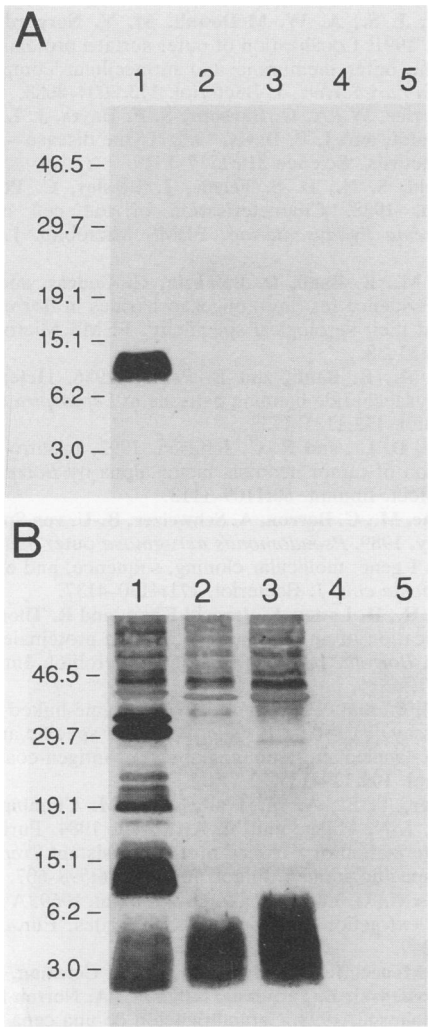

FIG. 7. Reactivities of MAb 240.7 (A) and polyclonal antiserum (B) with other spirochetes. Whole cells of *B. burgdorferi* B31 (lane 1), *B. anserina* (lane 2), *B. hermsii* (lane 3), *T. pallidum* (lane 4), and *T. phagedenis* (biotype Reiter) (lane 5) were boiled in Laemmli sample buffer and subjected to SDS-PAGE, and the Western blots were probed with either MAb 240.7 (A) or rabbit anti-PCP extract (B). The mobilities of standard proteins are marked to the left of each panel. Molecular mass is given in kilodaltons.

1.5% of the total cellular dry weight. Thus, like Braun's lipoprotein, the low-molecular-weight borrelial lipoprotein is a major constituent of the cell.

All 10 strains of *B. burgdorferi* tested contained the low-molecular-weight lipoprotein while two other *Borrelia* species, *B. anserina* and *B. hermsii*, did not. Two treponemes, three enterics, and three other gram-negatives also lacked immunoreactive lipoprotein. The three enterics are known to contain Braun's lipoprotein (44, 59); therefore, the epitope recognized by MAb 240.7 on the borrelial lipoprotein is not present in any of these variants of Braun's lipoprotein.

The low-molecular-weight lipoprotein differs from other, well-studied lipoproteins of *B. burgdorferi* (e.g., OspA, OspB, and OspC) in that its molecular size does not vary between strains (Fig. 6). Tick isolates from North America showed the same-size lipoprotein as those from Europe. Also, human blood isolates showed the same-size lipoprotein as tick isolates. Detection of the spirochete by means of this lipoprotein could, in the future, prove feasible (e.g., by antigen-capture ELISA).

Braun's lipoprotein is present in the outer membrane of enteric bacteria and yet is absent from the cell surface (14, 17). Thus, it is generally held that Braun's lipoprotein is located in the inner leaflet of the outer membrane (16). By using indirect immunofluorescence, we probed the question of whether or not the low-molecular-weight lipoprotein is exposed at the cell surface of *B. burgdorferi*. Others (4, 5) have tentatively localized OspA and OspB at the spirochetal cell surface by using this technique.

We obtained two results dependent on the method used. When spirochetes were fixed to the slide and probed with MAb 240.7, all of the cells fluoresced. However, when spirochetes were suspended in medium and probed with MAb 240.7, very few cells fluoresced and these were nonmotile. We interpret these results to mean that either fixing the cells to glass exposes the low-molecular-weight lipoprotein or cells that have surface-exposed lipoprotein preferentially bind to glass. Either way, it is possible that only dead cells fluoresce and that only in dead cells is the lipoprotein exposed at the surface.

Regardless of whether the spirochetes were bound to glass or free in medium, the labeled spirochetes showed bright, even fluorescence (Fig. 5). This is in contrast to the results obtained with OspA, which showed patching after the addition of the bivalent secondary antibody (5). Recent studies, however, question whether OspA and OspB are located in the outer membrane or are restricted to intracellular compartments (19).

Immunogold-labeling experiments performed on strain B31 with MAb 240.7 as the primary antibody showed dense labeling of surface-associated blebs (37). Presumably, these blebs represent sloughed outer membrane. Similar results were seen by Barbour and coworkers (4) with immunogold labeling of OspB. Blebs (32) from *B. burgdorferi* kindly provided by David Dorward contained the low-molecular-weight lipoprotein as determined by Western blot analysis using MAb 240.7 as the probe (data not shown). Taken together, these results suggest that the low-molecular-weight lipoprotein is in the outer membrane of the spirochete. Whether it is confined to the inner leaflet of the outer membrane or exposed at the cell surface is not yet clear.

Future studies will determine if the *B. burgdorferi* major low-molecular-weight lipoprotein is the borrelial equivalent of Braun's lipoprotein. Many of the activities traditionally ascribed to LPS have recently been found to be associated with Braun's lipoprotein or its lipopeptide derivatives (11, 12, 43, 48, 49, 56). Both intact organisms and soluble extracts of *B. burgdorferi* have been shown to induce mitogenesis as well as the elaboration of cytokines in a variety of mammalian cells (1, 7, 8, 24, 39, 40, 64). The biological activity of the purified lipoprotein of *B. burgdorferi* can now be studied to determine if it accounts for any of the activities of the intact organism or its extracts. Whether this lipoprotein acts as a virulence factor or elicits protective immunity or both can also now be studied.

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