INFECTION AND IMMUNITY, Apr. 1990, p. 983–991
0019-9567/90/040983-09$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 58, No. 4

Immunogenic Integral Membrane Proteins of *Borrelia burgdorferi* Are Lipoproteins

MARY E. BRANDT,[1] BRYAN S. RILEY,[1] JUSTIN D. RADOLF,[1,2] AND MICHAEL V. NORGARD[1]*

Departments of Microbiology[1] and Internal Medicine,[2] University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

Received 27 September 1989/Accepted 28 December 1989

The pathogenic spirochete *Borrelia burgdorferi* contains a set of integral membrane proteins which were selectively extracted into the detergent phase upon solubilization of intact *B. burgdorferi* with the nonionic detergent Triton X-114. Virtually all of these hydrophobic proteins were recognized by antibodies in pooled sera from patients with chronic Lyme arthritis, demonstrating that proteins partitioning into the detergent phase of Triton X-114 encompass the major *B. burgdorferi* immunogens. Furthermore, most of these immunogenic proteins, including the previously characterized OspA and OspB membrane antigens, could be biosynthetically labeled when *B. burgdorferi* was incubated in vitro with [^{3}H]palmitate. The OspA and OspB antigens were radioimmunoprecipitated from [^{3}H]palmitate-labeled detergent-phase proteins with monoclonal antibodies, and [^{3}H]palmitate was recovered unaltered from these proteins after sequential alkaline and acid hydrolyses. The combined results provide formal confirmation that the major *B. burgdorferi* immunogens extracted by Triton X-114 are lipoproteins. The demonstration that *B. burgdorferi* integral membrane antigens are lipoproteins may explain the basis of their immunogenicity and may help to improve our understanding of the surface topology of *B. burgdorferi*.

Lyme disease, a tick-borne infection caused by the spirochete *Borrelia burgdorferi*, is a chronic disorder characterized by dermatologic, rheumatologic, cardiac, and neurological manifestations (54). First recognized in 1975, Lyme disease is now a major global public health problem (51). The spread of Lyme disease to areas beyond its endemic foci (38) and case reports documenting antibiotic treatment failures (26, 29) emphasize the need for continuing investigations into the molecular pathogenesis of this infection.

Lyme disease shares many features with syphilis, a sexually transmitted infection caused by the noncultivable spirochete *Treponema pallidum* (52). Following cutaneous inoculation, both *B. burgdorferi* (9, 53) and *T. pallidum* (52) become blood borne and invade many of the same organ systems, including the placenta (42, 50). Both organisms also persist for prolonged periods within the infected host in the face of vigorous humoral and cellular immune responses (23, 27, 30, 52). In the case of syphilis, the immune response in the majority of individuals eventually controls the infection and provides significant immunity against exogenous reinfection (52). Clinical studies suggest that an analogous course of events occurs in Lyme disease (55). While differences between these infections and between their causative organisms undoubtedly exist, it is plausible that *B. burgdorferi* and *T. pallidum* share some parasitic strategies and that common host immune mechanisms are operative in the containment of both chronic diseases.

In an attempt to understand better the interactions of pathogenic spirochetes with their hosts, selective solubilization with the nonionic detergent Triton X-114 has been employed previously to identify and characterize hydrophobic integral membrane proteins of *T. pallidum* (46, 47). We recently have shown not only that this family of integral membrane proteins comprises the dominant immunogens of *T. pallidum* (46) but also that these major immunogens are lipoproteins (18). Structural similarities between *T. pallidum* and *B. burgdorferi* prompted us to examine the hydrophobic integral membrane proteins of the Lyme disease spirochete and to search for lipoproteins analogous to those of *T. pallidum*. As in the case of *T. pallidum*, we have found that *B. burgdorferi* possesses a set of highly immunogenic integral membrane proteins readily identifiable by their phase-partitioning characteristics in Triton X-114. Furthermore, this group comprises at least 10 lipoproteins, including the abundant outer surface protein antigens OspA and OspB (2, 4, 6–8, 10, 32). Our findings support the contention that the extraordinary immunogenicity of the integral membrane proteins found in virulent spirochetes may be related directly to their lipoprotein structure(s).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* type strain B31 (ATCC 35210) (16) was supplied by Alan Barbour. Cells were grown in BSK II medium (1) at 34°C.

Immunologic reagents. Monoclonal antibodies (MAbs) directed against OspA (H5332, immunoglobulin G1 [IgG1]) (8), OspB (H6831, IgG2a) (7), and an epitope of the *B. burgdorferi* 41-kilodalton (kDa) endoflagellar subunit (H9724, IgG2a) (5) were provided by Alan Barbour. A pool of hyperimmune sera from four patients with confirmed Lyme arthritis was provided by Joseph Craft. A pool of human secondary syphilitic sera, which was highly reactive with *T. pallidum* antigens on Western blots (immunoblots), was previously described (46).

Triton X-114 phase partitioning. *B. burgdorferi* was grown in BSK II medium to a final concentration of approximately 10^9 organisms per ml. Extraction and phase separation of borrelial polypeptides by using Triton X-114 was performed essentially as described by Radolf et al. (46). Briefly, bacteria were collected by centrifugation, washed once in phosphate-buffered saline, suspended in ice-cold 2% (vol/vol) Triton X-114 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in phosphate-buffered saline at a ratio of 5 × 10^9 organisms per ml of detergent, and rotated end over end

at 4°C overnight. Insoluble material, shown by electron microscopy to consist of protoplasmic cylinders analogous to those of *T. pallidum* (data not shown), was removed by centrifugation, and the supernatant was phase separated by warming the solution for 15 min in a 37°C water bath and then centrifuging the solution for 15 min at 25°C in an Eppendorf centrifuge. The separated detergent and aqueous phases were each washed three times (46). The detergent phase was suspended to its original volume in cold (4°C) phosphate-buffered saline, while the aqueous phase was brought to a final concentration of 2% detergent by adding cold 10% Triton X-114. The solutions were then rewarmed and recentrifuged as described above. Protein concentrations were determined by the BCA method (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as a standard. *B. burgdorferi* cells (5×10^9) yielded approximately 500 μg of protein which partitioned into the detergent phase and about 375 μg which partitioned into the aqueous phase.

Intrinsic radiolabeling of *B. burgdorferi* proteins. *B. burgdorferi* proteins were labeled with either L-amino acid mixture (U-[^{14}C]) (ICN Radiochemicals, Irvine, Calif.; specific activity, 1.71 mCi/mg) or (9,10-(*n*)-[^{3}H])palmitate (Amersham Corp., Arlington Heights, Ill.; specific activity, 30 Ci/mmol). Tritiated fatty acids used to label *B. burgdorferi* were dried under a stream of nitrogen gas and suspended in a small volume of filter-sterilized 95% ethanol (final ethanol concentration was no higher than 2.0%).

Paired *B. burgdorferi* cultures were prepared for labeling with fatty acid and amino acids. For fatty acid labeling, *B. burgdorferi* was grown in BSK II medium at 34°C to a density of 10^8 organisms per ml. Radiolabeled palmitate was then added at a final concentration of 0.25 mCi/ml, and incubation was continued for approximately 40 h (about four generations). For amino acid labeling, *B. burgdorferi* was grown in BSK II medium modified by omitting the gelatin and decreasing the CMRL supplement to 1/10 of its original concentration. Five hundred microcuries of ^{14}C-labeled amino acid mixture was added to 5×10^8 bacteria, and these cells were also incubated for about four generations. Triton X-114 fractionation was then performed (described above) (46).

Radioimmunoprecipitation of [^{3}H]palmitate- or ^{14}C-amino acid-labeled *B. burgdorferi* proteins. MAbs directed against OspA, OspB, or the 41-kDa endoflagellar subunit of *B. burgdorferi* were used to radioimmunoprecipitate proteins from the detergent-phase preparations of ^{3}H-fatty acid- or ^{14}C-amino acid-labeled *B. burgdorferi* as described previously (35, 45, 57).

SDS-PAGE and immunoblotting. Samples to be analyzed by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were boiled for 5 min in final sample buffer (62.5 mM Tris hydrochloride, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol) and electrophoresed through 4% stacking and 12.5% resolving gels (36). Gels were stained with Coomassie brilliant blue, fixed, treated with En^{3}Hance (Dupont, NEN Research Products, Wilmington, Del.) for fluorography, and then vacuum dried. Relative molecular masses were determined by comparing the mobilities of the polypeptides to the mobilities of molecular weight markers (Amersham). Western blotting was performed as previously described (41). Blots were incubated at 25°C for 18 h with a 1:10 dilution of hybridoma clone supernatants containing either MAb H5332 (OspA), H6831 (OspB), or H9724 (41-kDa endoflagellar subunit). When antibody reactivities in human sera were examined, a 1:1,000 dilution of either pooled human hyperimmune Lyme disease or syphilitic sera was used. Blots were then incubated with horseradish peroxidase anti-IgG conjugates of the appropriate species specificities (Jackson ImmunoResearch Laboratories, West Grove, Pa.) and developed as previously described (41).

Lipid analysis of detergent-phase proteins excised from SDS-polyacrylamide gels. Tritiated fatty acids covalently bound to the OspA (31-kDa) and OspB (34-kDa) detergent-phase antigens were identified by the procedure of Casey and Buss (17). Detergent-phase proteins, prepared by Triton X-114 extraction of *B. burgdorferi* labeled with [^{3}H]palmitate, were precipitated with 10 volumes of acetone. Each acetone pellet was boiled in final sample buffer and electrophoresed through a SDS-12.5% polyacrylamide slab gel. After Coomassie blue staining and destaining, protein bands were excised with a scalpel and washed overnight with three 2-ml changes of 50% methanol. A blank gel slice also was removed and processed with the sample to establish background radioactivity. Sequential alkaline and acid hydrolyses were performed directly on each gel slice. Alkaline hydrolysis was performed by incubating each gel slice in 1.5 N NaOH under N_2 at 30°C for 3 h. The solution was then acidified to pH 1 to 2, and released fatty acids were extracted into chloroform by the method of Bligh and Dyer (14). The chloroform phases were pooled, dried under a stream of nitrogen, derivatized, and analyzed by high-pressure liquid chromatography (HPLC) (18). Each aqueous phase, still containing the gel slice, was dried under nitrogen, suspended in 6 N HCl, and incubated at 100°C for 4 h. The gel slices were extracted a second time, and fatty acids in the pooled chloroform phases were derivatized and identified by HPLC.

HPLC analysis of ^{3}H-labeled fatty acids. Tritiated fatty acids released from *B. burgdorferi* were derivatized to phenacylesters by using dibromoacetophenone in the presence of 18-crown-6 ether catalyst (Alltech Associates, Inc., Applied Science Div., Deerfield, Ill.) as described previously (18). As an internal control, 20 μl of 200 μM heptadecanoic acid (17:0) was added to each lipid extract before the samples were dried under nitrogen. Derivatized and nonderivatized fatty acids were separated by passing the samples through C_{18} Sep-pak cartridges (Waters Associates, Inc., Milford, Mass). Each derivatized sample was then chromatographed by using a C_8 reverse-phase HPLC column (Beckman Instruments, Inc., Fullerton, Calif.). Chromatography was performed isocratically with methanol-water (90:10, vol/vol) at a flow rate of 1 ml/min; A_{254} was monitored. Fractions were collected, dried, treated with fluor, and counted in a liquid scintillation counter (Beckman). Each sample was counted until a sufficient number of counts were recorded to ensure a less than 2% counting error. The identification of each fatty acid was based on the mobility of its phenacyl derivative relative to that of fatty acid standards derivatized and chromatographed concurrently. Disintegrations per minute were correlated with chromatographic peaks of A_{254}. For each time point, counts in the blank gel slice were subtracted from the counts in the sample gel slice.

RESULTS

Analysis of *B. burgdorferi* polypeptides identified by phase partitioning with Triton X-114. Figure 1 shows a Coomassie brilliant blue-stained SDS-polyacrylamide gel of whole cell lysates (lane 4), aqueous-phase and detergent-phase proteins (lanes 2 and 3, respectively), and Triton X-114 insoluble material corresponding to protoplasmic cylinders (lane 1) of 5×10^7 *B. burgdorferi*. The aqueous and detergent phases

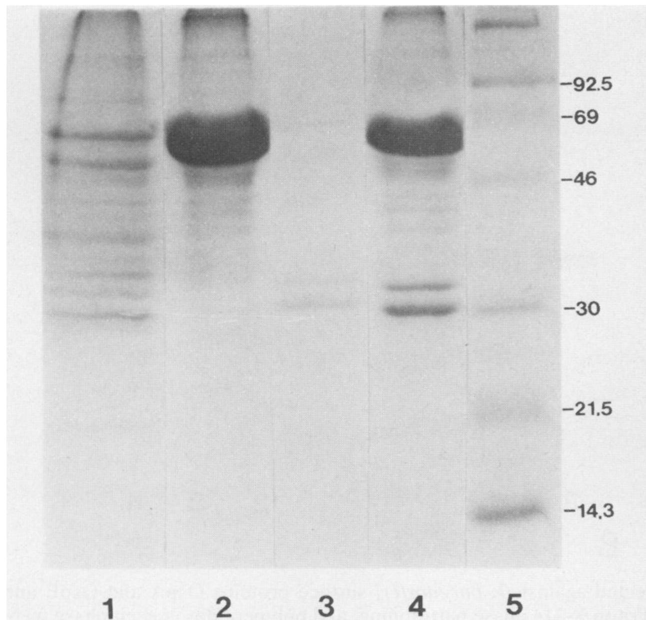

FIG. 1. Triton X-1114 phase partitioning of *B. burgdorferi* polypeptides. *B. burgdorferi* (5×10^7 cells) (lane 4) was extracted with Triton X-114, insoluble protoplasmic cylinders were removed (lane 1), and proteins in solution were partitioned into aqueous phase (lane 2) and detergent phase (lane 3). Polypeptides were separated by SDS-PAGE and stained with Coomassie brilliant blue. Molecular weight standards are shown (lane 5). Molecular masses of proteins are expressed in kilodaltons.

have distinct polypeptide profiles; the two principal polypeptides observed in the detergent phase (Fig. 1, lane 3) possessed apparent molecular masses of 31 and 34 kDa, suggesting that they were the OspA (8, 10) and OspB (7) antigens, respectively. The prominent polypeptide shown in lanes 2 and 4 was albumin from the BSK II medium. Bacteria were not washed before the detergent extraction in order to prevent damage to the fragile outer membranes, which could lead to a loss of some membrane proteins. In our experience, even one wash by centrifugation in phosphate-buffered saline is sufficient to disrupt the outer membranes, as demonstrated by negative-stain electron microscopy, which showed blebbing and extrusions of periplasmic endoflagella (not shown).

Specimens identical to those used in Fig. 1 were immunoblotted with pooled human syphilitic sera (Fig. 2A), pooled sera from patients with chronic Lyme arthritis (Fig. 2B), MAbs directed against OspA and OspB (Fig. 3A and B), and a MAb directed against the 41-kDa endoflagellar protein of *B. burgdorferi* (Fig. 3C). A number of immunogens of *B. burgdorferi* whole cells (Fig. 2B, lane 4) bound antibodies from pooled human Lyme sera intensely (83 [obscured by albumin], 58 to 53 [doublet], 37, 34 [OspB], 31 [OspA], 21, 19, and 15 kDa); virtually all of these partitioned into the detergent phase of Triton X-114 (Fig. 2B, lane 3). Immunoblotting with MAbs directed against OspA and OspB confirmed the identity of two of the proteins (31 kDa and 34 kDa; Fig. 3A and B, lanes 3). The detergent-phase proteins were not totally extractable, as quantities of these proteins, including OspA and B, remained with the protoplasmic cylinders (Fig. 2B, 3A, and 3B, lanes 1). The 41-kDa endoflagellar antigen partitioned into the aqueous phase of Triton X-114 (Fig. 3C, lane 2), although some was also detected in immunoblots of the protoplasmic cylinders (Fig. 3C, lane 1).

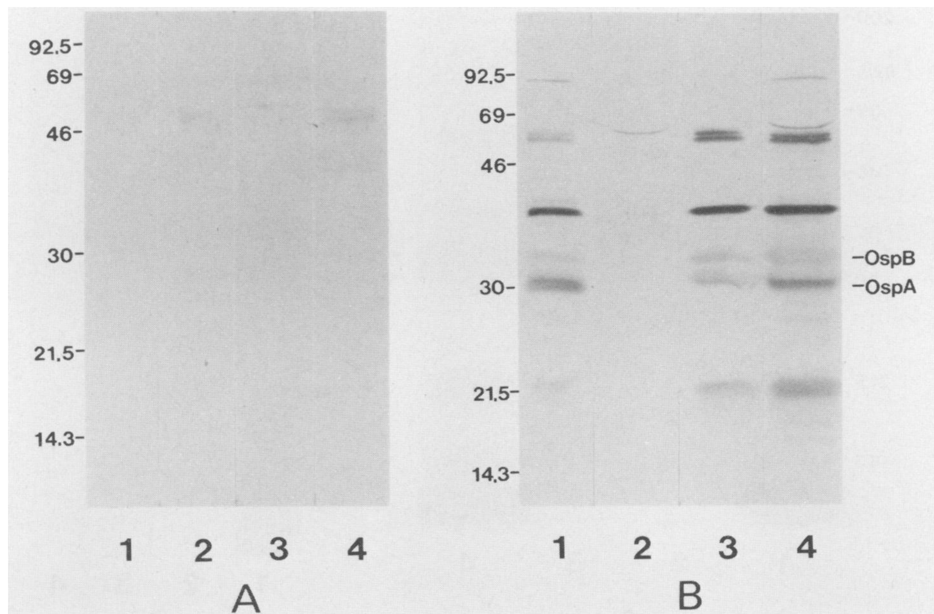

FIG. 2. Immunoblots of *B. burgdorferi* polypeptides probed with pooled human syphilitic (A) or Lyme disease (B) sera. *B. burgdorferi* (5×10^7 cells) (lane 4) was extracted with Triton X-114 and fractionated into protoplasmic cylinders (lane 1), aqueous-phase proteins (lane 2), and detergent-phase proteins (lane 3). SDS-PAGE-separated proteins were transferred to nitrocellulose and reacted with a 1:1,000 dilution of each serum pool. Blots were incubated with peroxidase-labeled anti-human IgG. The positions of proteins comigrating with OspA (31 kDa) and OspB (34 kDa) are shown. Molecular masses are in kilodaltons. The difference in migration of the molecular mass standards between panels A and B is a reflection of the slight variability between separate experiments.

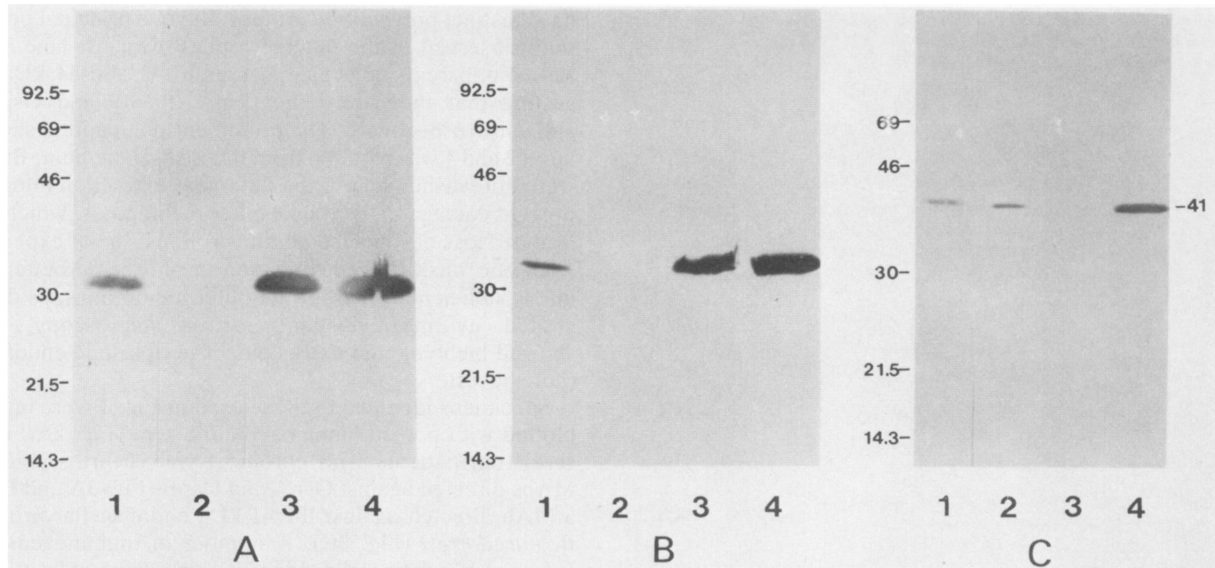

FIG. 3. Immunoblots of *B. burgdorferi* polypeptides with MAbs directed against *B. burgdorferi* surface proteins OspA and OspB and 41-kDa flagellar proteins. *B. burgdorferi* (5×10^7 cells) was subjected to Triton X-114 phase partitioning, and polypeptides in each phase were separated on SDS-PAGE. Lane 1, Protoplasmic cylinders; lane 2, aqueous-phase proteins; lane 3, detergent-phase proteins; lane 4, whole *B. burgdorferi* cells. Polypeptides were transferred to nitrocellulose and probed with a 1:10 dilution of hybridoma clone supernatants of H5332 (anti-OspA) (A), H6831 (anti-OspB) (B), or H9724 anti-41-kDa endoflagellar subunit) (C). Molecular masses of proteins are in kilodaltons.

None of the *B. burgdorferi* detergent-phase immunogens were recognized by human syphilitic sera (Fig. 2A).

The major integral membrane protein immunogens of *B. burgdorferi* are lipoproteins. We sought to extend to *B. burgdorferi* our previous finding that the major detergent-extractable immunogens of *T. pallidum* are lipoproteins (18). *B. burgdorferi* was cultivated in BSK II medium containing [^{3}H]palmitate. Radiolabeled proteins were separated by SDS-PAGE and visualized by fluorography (Fig. 4A). Whole cells of *B. burgdorferi* incorporated [^{3}H]palmitate into at

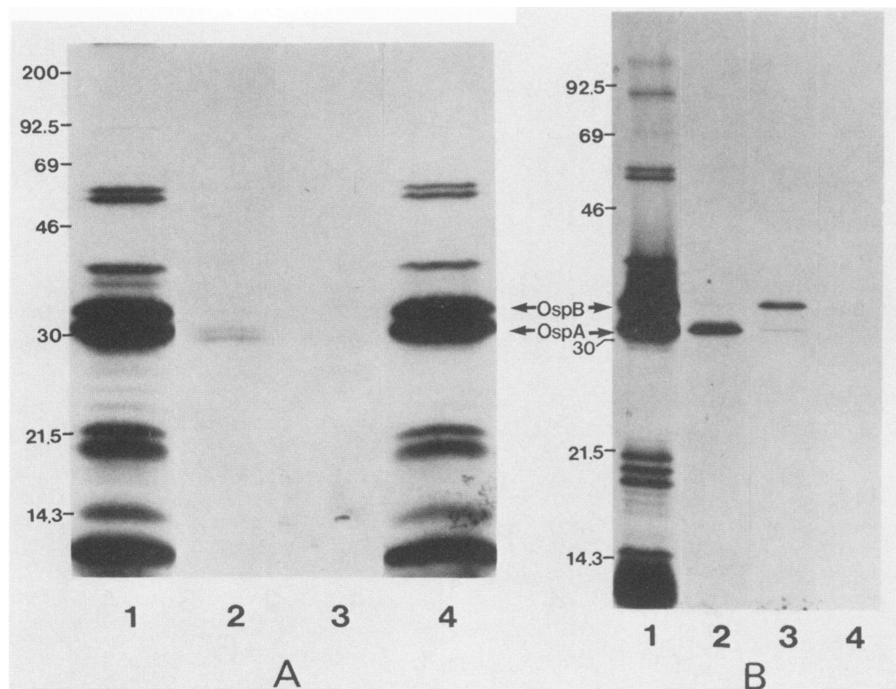

FIG. 4. Triton X-114 fractionation and radioimmunoprecipitation of *B. burgdorferi* proteins biosynthetically labeled with [^{3}H]palmitate. (A) Whole *B. burgdorferi* cells (5×10^8) (lane 1), protoplasmic cylinders (lane 2), and aqueous-phase (lane 3) and detergent-phase (lane 4) proteins were separated on SDS-PAGE and fluorographed. (B) Detergent-phase proteins from [^{3}H]palmitate-labeled *B. burgdorferi* (lane 1; same as panel A, lane 4) were radioimmunoprecipitated with MAbs H5332 (anti-OspA) (lane 2), H6831 (anti-OspB) (lane 3), and H9724 (anti-41-kDa endoflagellar subunit) (lane 4). Molecular masses of proteins are in kilodaltons.

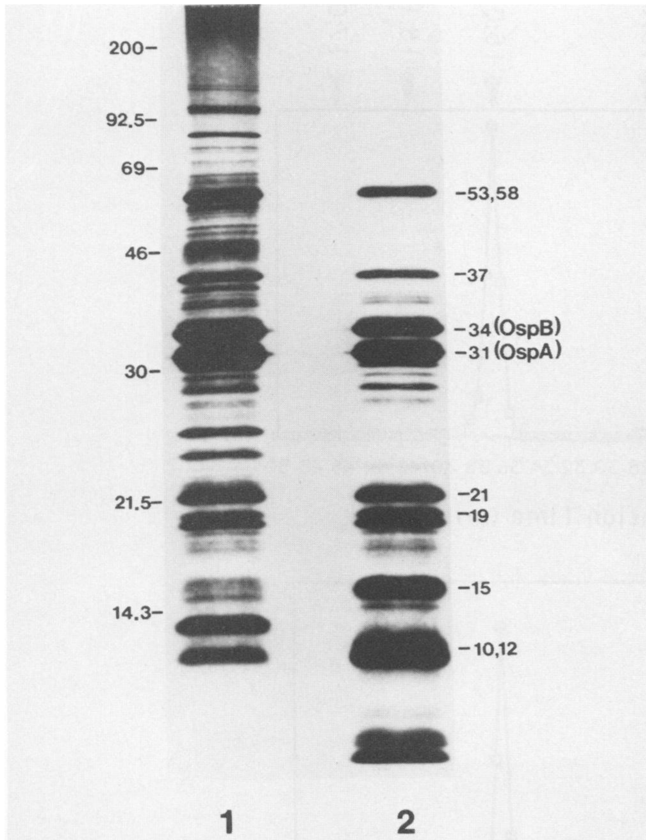

FIG. 5. Comparison of Triton X-114-extracted detergent-phase proteins from *B. burgdorferi* biosynthetically labeled with either ^{14}C-amino acids (lane 1) or ^{3}H-fatty acids (lane 2). Triton X-114 detergent-phase proteins (50 μg of protein) were separated by SDS-PAGE and visualized by fluorography.

least 10 major proteins having apparent molecular masses of 58 to 53 (doublet), 37, 34, 31, 21, 19, 15, and 12 to 10 (doublet) kDa (Fig. 4A, lane 1). The 19-kDa species sometimes resolved as a doublet (Fig. 4B, lane 1). All of the [^{3}H]palmitate-labeled polypeptides partitioned exclusively into the detergent phase of Triton X-114 (Fig. 4A, lane 4); their molecular masses corresponded well with those of the major *B. burgdorferi* immunogens shown earlier (Fig. 2B, lane 3). Radioimmunoprecipitations with the OspA and OspB MAbs confirmed the identities of the 31- and 34-kDa [^{3}H]palmitate-labeled proteins (Fig. 4B, lanes 2 and 3). In contrast, when a ^{14}C-amino acid mixture was used for biosynthetic labeling of *B. burgdorferi*, a large number of proteins partitioned into the detergent phase after Triton X-114 extraction (Fig. 5). No detergent-phase proteins appeared to become labeled exclusively with radioactive lipid.

Analysis of fatty acids bound to *B. burgdorferi* lipoproteins. To verify that the ^{3}H radiolabeling of the detergent-phase proteins was due to the covalent attachment of radioactive fatty acids, [^{3}H]palmitate in either ester or amide linkage of OspA and OspB proteins (within gel slices) was released by sequential alkaline and acid hydrolyses. For OspA, 977,300 dpm was released after alkaline hydrolysis and 490,608 dpm was released after subsequent hydrolysis in acid. With OspB, 467,317 alkali-sensitive dpm and 261,031 acid-sensitive dpm were released. As predicted for conventional bacterial lipoproteins (31, 58), the ratio of alkali-labile (ester-linked) to acid-labile (amide-linked) radioactivity was thus about 2:1 for OspA (ratio of 1.99:1) and OspB (ratio of 1.79:1). HPLC analysis with derivatized fatty acid standards confirmed that the radioactivity released after alkali and acid hydrolyses comigrated with [^{3}H]palmitate (16:0) (Fig. 6 and 7); radioactivity was thus released unaltered from these lipoproteins.

DISCUSSION

Elucidation of the surface topology and antigenic composition of *B. burgdorferi* has been an ongoing activity in a number of laboratories. The major goal of such studies has been the characterization of diagnostically useful antigens and molecules that contribute to the immunopathogenesis of Lyme disease (2, 21, 22, 24, 39; for a review, see reference 3).

We previously employed Triton X-114 phase partitioning (15) to demonstrate the existence in *T. pallidum* of a group of highly immunogenic integral membrane proteins (46, 47); more recently, these immunogens were biochemically characterized as lipoproteins (18). In the present study, the same methods were used to demonstrate that the major immunogens of *B. burgdorferi*, including the well-characterized OspA and OspB (2, 4, 6–8, 10, 32), are also amphiphilic proteins that can be radiolabeled during in vitro incubation of organisms with [^{3}H]palmitate. Furthermore, for both OspA and OspB, sequential alkaline and acid hydrolyses confirmed that fatty acids are covalently linked to these two polypeptides in a 2:1 ester-to-amide linkage that is typical of most bacterial lipoproteins (31, 58). Fatty acids in ester linkage are sensitive to mild alkaline hydrolysis, whereas amide-linked fatty acids require acid hydrolysis for their removal (17). These biochemical results are consistent with the nucleotide sequences of the OspA and OspB genes (11). Both genes contain signal sequences with consensus sites (Leu-X-Y-Cys) (58) for processing by signal peptidase II, the bacterial enzyme that processes bacterial lipoproteins (58), and for the covalent addition of fatty acids to the resulting N-terminal cysteine.

Similar biochemical analyses of the other ^{3}H-lipid-labeled *B. burgdorferi* proteins were not performed. However, it is reasonable to assume that these molecules also contain covalently attached fatty acids, particularly since they remained radiolabeled after boiling in SDS and electrophoresis in SDS-polyacrylamide gels. Further biochemical analyses of these other borrelial lipoproteins are necessary to determine whether fatty acids are linked to some of them in ratios other than those described for conventional bacterial lipoproteins. In addition to lipoproteins with typical 2:1 ester-to-amide linkages, *T. pallidum* appears to contain at least one, the 47-kDa immunogen, with unconventional lipid linkages (18, 19). Putative unconventional lipoproteins in *Mycoplasma capricolum* have also been described (25).

It is noteworthy that no evidence for beta oxidation, desaturation, or chain elongation of radioactive fatty acids was found, since [^{3}H]palmitate was recovered unaltered from both Osp proteins after alkaline and acid hydrolyses. Similar results were found in previous analyses of three lipoproteins of *T. pallidum* (18; unpublished data), an organism in which the metabolic pathways described above have not been demonstrated (44, 49). It is interesting to speculate that *B. burgdorferi* may have similar metabolic limitations (4). Furthermore, in contrast to the large number of polypeptides labeled with ^{14}C-amino acids, only a discrete subset was labeled with [^{3}H]palmitate. This implies that radioactive fatty acid was not degraded before incorporation into these proteins.

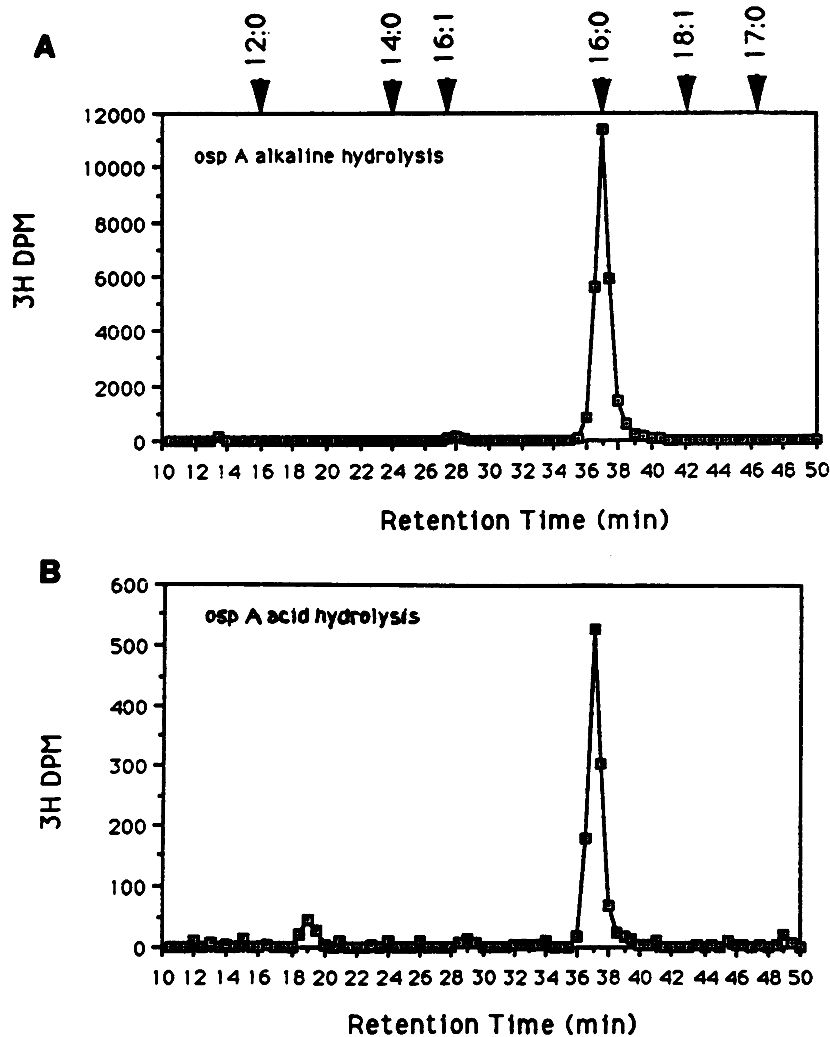

FIG. 6. HPLC of fatty acids from the *B. burgdorferi* OspA protein labeled with [^{3}H]palmitate. (A) Radioactivity recovered after alkaline hydrolysis of OspA protein in polyacrylamide gel slices; (B) radioactivity recovered after sequential acid hydrolysis of OspA gel slices previously depleted of alkali-labile radioactivity. Positions of fatty acid standards (arrowheads): 12:0, laurate; 14:0, myristate; 16:1, palmitoleic; 16:0, palmitate; 18:1, oleate; 17:0, heptadecanoic acid (internal standard).

In *Escherichia coli*, Braun lipoprotein serves as a physical link between the outer membrane and peptidoglycan, with the amino-terminal lipid portion inserted into the outer membrane and the COOH terminus covalently associated with peptidoglycan (31). Another group of peptidoglycan-associated lipoproteins are closely but noncovalently associated with peptidoglycan (28). Both gram-positive and gram-negative bacteria also contain membrane-bound lipoproteins that are subsequently secreted with the amino-terminal lipid either still attached, as with *Klebsiella pneumoniae* pullulanase (20), or proteolytically removed, as with *Bacillus licheniformis* penicillinase (37). In *B. burgdorferi*, it is difficult to speculate on the functional significance of the lipid moieties of the lipoproteins because the biological activities of these molecules, including OspA and OspB, have yet to be determined. Nevertheless, it is likely that the lipid moieties act as hydrophobic membrane anchors that enable hydrophilic polypeptides to be positioned on the functionally appropriate membrane face of the bacterium. This is supported by our recent nucleotide sequence analyses for three *T. pallidum* lipoprotein immunogens (33, 56; unpublished data) and the findings that all three of these proteins are hydrophilic when synthesized in an in vitro transcription-translation system where acylation does not occur (19, 56; unpublished data).

Comparison between the ^{14}C-labeled and ^{3}H-labeled detergent phases revealed that the lipoproteins represent only a subset of the total membrane proteins of *B. burgdorferi*. The poorly immunogenic, nonacylated ^{14}C-labeled polypeptides presumably include integral membrane proteins whose amphiphilic characters and membrane anchors arise from their amino acid constituents rather than from modifications such as the covalent addition of lipid. Recent freeze-fracture analysis of *B. burgdorferi* outer membranes revealed intramembranous particles (unpublished data), structures generally thought to represent transmembrane domains of membrane proteins (48). Proteins anchored in membranes exclusively via lipid moieties would not be expected to form intramembranous particles during freeze-fracture (48). Therefore, at least some of these poorly immunogenic amphiphilic proteins are likely to be located in the outer membrane and to be potential virulence factors.

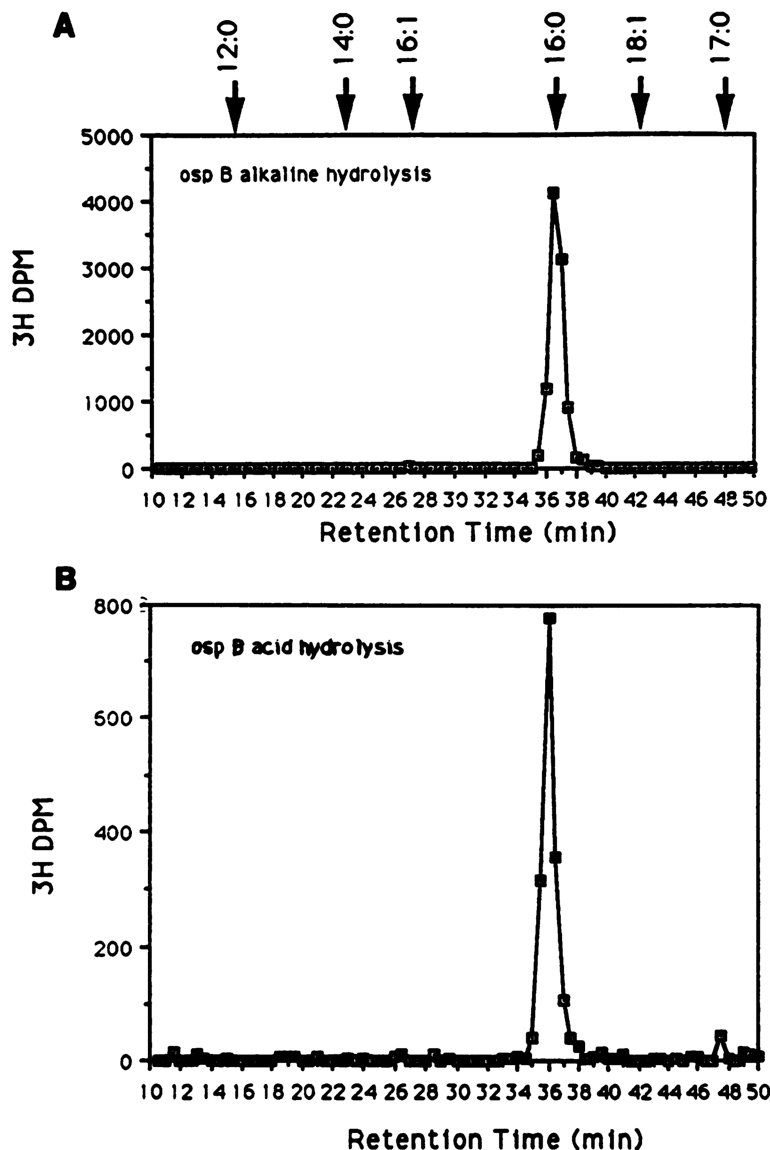

FIG. 7. HPLC of fatty acids from the *B. burgdorferi* OspB protein labeled with [^{3}H]palmitate. (A) Radioactivity recovered after alkaline hydrolysis of OspB protein in polyacrylamide gel slices; (B) radioactivity recovered after sequential acid hydrolysis of OspB gel slices previously depleted of alkali-labile radioactivity. Positions of fatty acid standards are as in Fig. 6.

Our immunoblot results essentially agreed with those from other laboratories; the human Lyme disease sera used in this study recognized from whole-cell lysates polypeptides with molecular masses similar to those reported in previous studies (2, 3, 21, 39). In addition, the use of Triton X-114 phase partitioning revealed that human antibodies recognized a detergent-phase immunogen possessing an apparent molecular mass of 37 kDa. The previously described 41-kDa endoflagellar antigen could not be detected when detergent-phase proteins were probed with a specific MAb. In view of the similar SDS-PAGE mobilities of these two proteins, it is possible that the amphiphilic 37-kDa protein may have been mistaken for the endoflagellar protein in previous immunoblotting analyses by others that employed total antigens of whole organisms. In support of this view, additional experiments have shown that when whole-cell lysates and detergent-phase proteins were coelectrophoresed and probed with hyperimmune Lyme sera, the predominant reactivity in this molecular mass region was against the 37-kDa detergent-phase protein, and little, if any, antibody was detected against the 41-kDa endoflagellar protein (data not shown). These two proteins thus migrate as close but distinct bands on immunoblots of whole *B. burgdorferi* cells; this minor difference may not be readily apparent when blots from separate experiments are compared.

Cross-reactivity between antigens of *B. burgdorferi* and those of other spirochetes, especially *T. pallidum*, has been a major cause of false positivity in serological tests for Lyme disease that employ whole *B. burgdorferi* as the antigen (34, 40). The 41-kDa endoflagellar protein, in particular, has been shown to cross-react with antibodies to the endoflagella of other spirochetes (34, 40). None of the *B. burgdorferi* detergent-phase immunogens were recognized by human syphilitic sera. Because the endoflagella either remain associated with the protoplasmic cylinders or enter the aqueous phase during phase partitioning, this antigen is eliminated as

a source of nonspecific reactivity. These results suggest that the *B. burgdorferi*-specific immunogens within the Triton X-114 detergent-phase proteins could provide the basis for a more specific serologic test for Lyme disease than those currently available.

The precise role of lipid modification in conferring immunogenicity upon bacterial proteins is unclear. However, the lipopeptide from the outer membrane of *E. coli* (Braun lipoprotein) has been shown to constitute a potent B-lymphocyte mitogen and polyclonal B-cell activator in vitro (43). In other studies, the covalent attachment of lipid to protein has been shown to confer significant immunogenicity to such modified proteins or lipopeptides (12, 13). The in vivo correlates of these observations, particularly in the cases of Lyme disease and syphilis, remain to be elucidated.

ACKNOWLEDGMENTS

We thank Alan Barbour for providing MAbs and *B. burgdorferi*, Joseph Craft for antisera, Mark Swancutt for helpful discussions, Martin Goldberg for outstanding technical assistance, and Robert Munford and Alice Erwin for assistance with the fatty acid analyses.

This study was partially supported by grant I-940 from the Robert A. Welch Foundation, Public Health Service grants AI-16692 and AI-17366 from the National Institute of Allergy and Infectious Diseases to M.V.N., and Public Health Service grant AI-26756 from the National Institute of Allergy and Infectious Diseases to J.D.R. J.D.R. is a scholar of the American Foundation for AIDS Research. M.E.B. is a Robert A. Welch Foundation postdoctoral fellow.

LITERATURE CITED

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
2. **Barbour, A. G.** 1984. Immunochemical analysis of Lyme disease spirochetes. Yale J. Biol. Med **57:**581–586.
3. **Barbour, A. G.** 1988. Laboratory aspects of Lyme borreliosis. Clin. Microbiol. Rev. **1:**399–414.
4. **Barbour, A. G., and S. F. Hayes.** 1986. Biology of *Borrelia* species. Microbiol. Rev. **50:**381–400.
5. **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.
6. **Barbour, A. G., R. A. Heiland, and T. R. Howe.** 1985. Heterogeneity of major proteins of Lyme disease borreliae: a molecular analysis of North American and European isolates. J. Infect. Dis. **152:**478–484.
7. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect. Immun. **45:**94–100.
8. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and Ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41:**795–804.
9. **Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow.** 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. **308:**740–742.
10. **Benach, J. L., J. L. Coleman, and M. G. Golightly.** 1988. A murine IgM monoclonal antibody binds an antigenic determinant in outer surface protein A, an immunodominant basic protein of the Lyme disease spirochete. J. Immunol. **140:**265–272.
11. **Bergstrom, S., V. G. Bundoc, and A. G. Barbour.** 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochete *Borrelia burgdorferi*. Mol. Microbiol. **3:**479–486.
12. **Bessler, W. G., B. Suhr, H. J. Buhring, C. P. Muller, K. H. Wiesmuller, G. Becker, and G. Jung.** 1985. Specific antibodies elicited by antigen covalently linked to a synthetic adjuvant. Immunobiology **170:**239–244.
13. **Biesert, L., W. Schener, and W. G. Bessler.** 1987. Interaction of mitogenic bacterial lipoprotein and a synthetic analogue with mouse lymphocytes: isolation and characterization of binding proteins. Eur. J. Biochem. **162:**651–657.
14. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
15. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256:**1604–1607.
16. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease, a tick-borne spirochetosis? Science **216:**1317–1319.
17. **Casey, P. J., and J. E. Buss.** 1988. Chemical analysis of fatty acids covalently linked to proteins, p. 64–71. *In* U. Brodbeck and C. Bordier (ed.), Post-translational modification of proteins by lipids. A laboratory manual. Springer-Verlag, New York.
18. **Chamberlain, N. R., M. E. Brandt, A. L. Erwin, J. D. Radolf, and M. V. Norgard.** 1989. Major integral membrane protein immunogens of *Treponema pallidum* are proteolipids. Infect. Immun. **57:**2872–2877.
19. **Chamberlain, N. R., L. DeOgny, C. Slaughter, J. D. Radolf, and M. V. Norgard.** 1989. Acylation of the 47-kilodalton major membrane immunogen of *Treponema pallidum* determines its hydrophobicity. Infect. Immun. **57:**2878–2885.
20. **Chapon, C., and O. Raibaud.** 1985. Structures of two divergent promoters located in front of the gene encoding pullulanase in *Klebsiella pneumoniae* and positively regulated by the *malT* product. J. Bacteriol. **164:**639–645.
21. **Coleman, J. L., and J. L. Benach.** 1987. Isolation of antigenic components from the Lyme disease spirochete: their role in early diagnosis. J. Infect. Dis. **155:**756–765.
22. **Coleman, J. L., J. L. Benach, G. Beck, and G. S. Habicht.** 1986. Isolation of the outer envelope from *Borrelia burgdorferi*. Zentrabl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A **263:**123–126.
23. **Craft, J. E., R. L. Grodzicki, M. Shrestha, D. K. Fischer, M. Garcia-Blanco, and A. C. Steere.** 1984. The antibody response in Lyme disease. Yale J. Biol. Med. **57:**561–565.
24. **Cunningham, T. M., D. D. Thomas, S. D. Thompson, J. N. Miller, and M. A. Lovett.** 1988. Identification of *Borrelia burgdorferi* surface components by Triton X-114 phase partitioning. Ann. N.Y. Acad. Sci. **539:**376–378.
25. **Dahl, C. E., J. S. Dahl, and K. Bloch.** 1983. Proteolipid formation in *Mycoplasma capricolum*: influence of cholesterol on unsaturated fatty acid acylation of membrane proteins. J. Biol. Chem. **258:**11814–11818.
26. **Dattwyler, R. J., and J. J. Halperin.** 1987. Failure of tetracycline therapy in early Lyme disease. Arthritis Rheum. **30:**448–450.
27. **Dattwyler, R. J., D. J. Volkman, J. J. Halperin, B. J. Luft, J. Thomas, and M. G. Golightly.** 1988. Specific immune responses in Lyme borreliosis: characterization of T cell and B cell responses to *Borrelia burgdorferi*. Ann. N.Y. Acad. Sci. **539:**93–102.
28. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170:**489–498.
29. **Diringer, M. N., J. J. Halperin, and R. J. Dattwyler.** 1987. Lyme meningoencephalitis: report of a severe, pencillin-resistant case. Arthritis Rheum. **30:**705–708.
30. **Golightly, M., J. Thomas, D. Volkman, and R. Dattwyler.** 1988. Modulation of natural killer cell activity by *Borrelia burgdorferi*. Ann. N.Y. Acad. Sci. **539:**103–111.
31. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein: diglyceride and amide-linked fatty acid at the N-terminal end of the murine-lipoprotein of the *Escherichia coli* outer membrane. Eur. J. Biochem. **34:**284–296.
32. **Howe, T. R., L. W. Mayer, and A. G. Barbour.** 1985. A single recombinant plasmid expressing two major outer surface proteins of the Lyme disease spirochete. Science **227:**645–646.
33. **Hsu, P.-L., N. R. Chamberlain, K. Orth, C. R. Moomaw, L. Q. Zhang, C. A. Slaughter, J. D. Radolf, S. Sell, and M. V. Norgard.**

1989. Sequence analysis of the 47-kilodalton major integral membrane immunogen of *Treponema pallidum*. Infect. Immun. **57**:196–203.

34. **Hunter, E. F., H. Russell, C. E. Farshy, J. S. Sampson, and S. A. Larsen.** 1986. Evaluation of sera from patients with Lyme disease in the fluorescent treponemal antibody-absorption test for syphilis. Sex. Transm. Dis. **13**:232–236.
35. **Jones, S. A., K. S. Marchitto, J. N. Miller, and M. V. Norgard.** 1984. Monoclonal antibody with hemagglutination, immobilization, and neutralization activities defines an immunodominant, 47,000 mol wt, surface-exposed immunogen of *Treponema pallidum* (Nichols). J. Exp. Med. **160**:1404–1420.
36. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
37. **Lai, J.-S., M. Sarvas, W. J. Brammar, K. Neugebauer, and H. C. Wu.** 1981. *Bacillus licheniformis* penicillinase synthesized in *Escherichia coli* contains covalently linked fatty acid and glyceride. Proc. Natl. Acad. Sci. USA **78**:3506–3510.
38. **Lastavica, C. C., M. L. Wilson, V. P. Berardi, A. Spielman, and R. D. Deblinger.** 1989. Rapid emergence of a focal epidemic of Lyme disease in coastal Massachusetts. N. Engl. J. Med. **320**:133–137.
39. **Magnarelli, L. A., J. F. Anderson, and A. G. Barbour.** 1989. Enzyme-linked immunosorbent assays for Lyme disease: reactivity of subunits of *Borrelia burgdorferi*. J. Infect. Dis. **159**: 43–49.
40. **Magnarelli, L. A., J. F. Anderson, and R. C. Johnson.** 1987. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. J. Infect. Dis. **156**:183–188.
41. **Marchitto, K. S., S. A. Jones, R. F. Schell, P. L. Holmans, and M. V. Norgard.** 1984. Monoclonal antibody analysis of specific antigenic similarities among pathogenic *Treponema pallidum* subspecies. Infect. Immun. **45**:660–666.
42. **Markowitz, L. E., A. C. Steere, J. L. Benach, J. D. Slade, and C. V. Broome.** 1986. Lyme disease during pregnancy. J. Am. Med. Assoc. **255**:3394–3396.
43. **Melchers, F., V. Braun, and C. Galanos.** 1975. The lipoprotein of the outer membrane of *Escherichia coli*: a B-lymphocyte mitogen. J. Exp. Med. **142**:473–482.
44. **Nichols, J. C., and J. B. Baseman.** 1975. Carbon sources utilized by virulent *Treponema pallidum*. Infect. Immun. **12**:1044–1050.
45. **Norgard, M. V., N. R. Chamberlain, M. A. Swancutt, and M. S. Goldberg.** 1986. Cloning and expression of the major 47-kilodalton surface immunogen of *Treponema pallidum* in *Escherichia coli*. Infect. Immun. **54**:500–506.
46. **Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard.** 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. **56**:490–498.
47. **Radolf, J. D., and M. V. Norgard.** 1988. Pathogen specificity of *Treponema pallidum* subsp. *pallidum* integral membrane proteins identified by phase partitioning with Triton X-114. Infect. Immun. **56**:1825–1828.
48. **Satir, B. H., and P. Satir.** 1979. Partitioning of intramembrane particles during the freeze-fracture procedure, p. 43–49. *In* J. E. Rash and C. S. Hudson (ed.), Freeze fracture: methods, artifacts, and interpretations. Raven Press, New York.
49. **Schiller, N. L., and C. D. Cox.** 1977. Catabolism of glucose and fatty acids by virulent *Treponema pallidum*. Infect. Immun. **16**:60–68.
50. **Schlesinger, P. A., P. H. Duray, B. A. Burke, A. C. Steere, and M. T. Stillman.** 1985. Maternal-fetal transmission of the Lyme disease spirochete. Ann. Intern. Med. **103**:67–68.
51. **Schmid, G. P.** 1984. The global distribution of Lyme disease. Yale J. Biol. Med. **57**:617–618.
52. **Sell, S., and S. J. Norris.** The biology, pathology, and immunology of syphilis. Int. Rev. Exp. Pathol. **24**:203–276.
53. **Steere, A. C., N. H. Bartenhagen, J. E. Craft, C. J. Hutchinson, J. H. Newman, D. W. Rahn, L. H. Sigal, P. N. Spieler, K. S. Stenn, and S. E. Malawista.** 1983. The early clinical manifestations of Lyme disease. Ann. Intern. Med. **99**:76–82.
54. **Steere, A. C., S. E. Malawista, N. H. Bartenhagen, P. N. Spieler, J. H. Newman, D. W. Rahn, G. J. Hutchinson, J. Green, D. R. Snydman, and E. Taylor.** 1984. The clinical spectrum and treatment of Lyme disease. Yale J. Biol. Med. **57**:453–464.
55. **Steere, A. C., R. T. Schoen, and E. Taylor.** 1987. The clinical evolution of Lyme arthritis. Ann. Intern. Med. **107**:725–731.
56. **Swancutt, M. A., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1989. Molecular characterizaton of the pathogen-specific, 34-kilodalton membrane immunogen of *Treponema pallidum*. Infect. Immun. **57**:3314–3323.
57. **Swancutt, M. A., D. A. Twehous, and M. V. Norgard.** 1986. Monoclonal antibody selection and analysis of a recombinant DNA-derived surface immunogen of *Treponema pallidum* expressed in *Escherichia coli*. Infect. Immun. **52**:110–119.
58. **Wu, H. C.** 1987. Posttranslational modification and processing of membrane proteins in bacteria, p. 37–74. *In* M. Inouye (ed.), Bacterial outer membranes as model systems. John Wiley & Sons, Inc., New York.