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Fatty Acids of *Treponema pallidum* and *Borrelia burgdorferi* Lipoproteins

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A fundamental ultrastructural feature shared by the spirochetal pathogens *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) and *Borrelia burgdorferi*, the etiological agents of venereal syphilis and Lyme disease, respectively, is that their most abundant membrane proteins contain covalently attached fatty acids. In this study, we identified the fatty acids covalently bound to lipoproteins of *B. burgdorferi* and *T. pallidum* and examined potential acyl donors to these molecules. Palmitate was the predominant fatty acid of both *B. burgdorferi* and *T. pallidum* lipoproteins. *T. pallidum* lipoproteins also contained substantial amounts of stearate, a fatty acid not typically prevalent in prokaryotic lipoproteins. In both spirochetes, the fatty acids of cellular lipids differed from those of their respective lipoproteins. To characterize phospholipids in these organisms, spirochetes were metabolically labeled with [^{3}H]palmitate or [^{3}H]oleate; *B. burgdorferi* contained only phosphatidylglycerol and phosphatidylcholine, while *T. pallidum* contained phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cardiolipin. Although palmitate predominated in the lipoproteins, there were no apparent differences in the incorporation of these two fatty acids into phospholipids (putative acyl donors). Phospholipase A_1 and A_2 digestion of phosphatidylcholine from *B. burgdorferi* and *T. pallidum* labeled with either [^{3}H]palmitate or [^{3}H]oleate also revealed that neither fatty acid was incorporated preferentially into the 1 and 2 positions (potential acyl donor sites) of the glycerol backbone. The combined findings suggest that fatty acid utilization during lipoprotein synthesis is determined largely by the fatty acid specificities of the lipoprotein acyl transferases. These findings also provide the basis for ongoing efforts to elucidate the relationship between lipoprotein acylation and the physiological functions and inflammatory activities of these molecules.

A fundamental ultrastructural feature shared by the spirochetal pathogens *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) and *Borrelia burgdorferi*, the etiological agents of venereal syphilis and Lyme disease, respectively, is that their most abundant membrane proteins contain covalently attached fatty acids (8, 9, 44, 49). Included among these lipoproteins are outer surface proteins A to D of *B. burgdorferi* (5, 8, 31, 57) and the 47-, 34-, 17-, and 15-kilodalton antigens of *T. pallidum* (1, 10, 33, 49, 53). The predominance of lipoproteins in these bacteria implies the existence of an intimate relationship between protein acylation, molecular architecture, and membrane physiology. Nucleotide sequence analyses of cloned spirochetal lipoprotein genes have identified leader peptides terminated by consensus tetrapeptides for lipoprotein processing and modification (20), and these findings have been confirmed by metabolic labeling of native and cloned spirochetal lipoproteins with ^{3}H-fatty acids (1, 5, 22, 31, 33, 44, 50, 53, 57). Recent in vitro studies also have demonstrated that the lipoproteins of both *T. pallidum* and *B. burgdorferi* have inflammatory activities that may be clinically relevant (1, 28, 36, 38). Of particular importance, there is increasing evidence that the immunopotentiating activities of bacterial lipoproteins are conferred by their lipid constituents (1, 6, 12, 14, 19, 21, 25, 29, 37, 47, 56). These findings provided an impetus for defining the acyl moieties of the native spirochetal lipoproteins.

While lipoproteins have been identified in members of numerous bacterial genera (20), the fatty acid constituents have been characterized for relatively few of these molecules. The murein lipoprotein of *Escherichia coli*, whose structure is the paradigm for bacterial lipoprotein structure, possesses ester-linked fatty acids in proportions similar to those of the cellular phospholipids, whereas the amide-linked fatty acid of the amino-terminal cysteine is predominantly palmitate (17). Other bacterial lipoproteins have displayed acylation profiles dominated by palmitate (30, 58) or oleate and hydroxystearate (54). Presently it is believed that the specificity of acylation for bacterial lipoproteins is influenced by the distribution of fatty acids in the cellular lipids (17), by the positional distribution of fatty acids on the phospholipid acyl donors (23, 41), and/or by the specificities of the various acyltransferases involved in lipoprotein modification (16). In this study, we identified the fatty acyl moieties of *T. pallidum* and *B. burgdorferi* lipoproteins and examined factors that might influence the incorporation of fatty acids into these immunogens. These investigations have provided a foundation for future structure-function studies of the lipoprotein constituents of these important spirochetal pathogens.

MATERIALS AND METHODS

Bacterial strains. The *B. burgdorferi* avirulent (high-passage) strain B31 (ATCC 35210) was supplied by Alan Barbour (San Antonio, Tex.). The virulent (low-passage) strain 297 was obtained from Russell Johnson (Minneapolis, Minn.). The

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virulence of strain 297 was tested in a mouse infectivity model by recovering organisms from the urinary bladder 7 to 14 days after intradermal challenge with 10^7 organisms (45). *B. burgdorferi* strains were grown in vitro in BSK II medium (2) at 34°C.

T. pallidum subsp. *pallidum* (hereafter referred to as *T. pallidum*) (Nichols strain) was cultivated in the tests of New Zealand White rabbits for 10 to 12 days as previously described (40). Bacteria were extracted from rabbit testes in sterile isotonic saline or in sterile treponemal labeling medium (48), separated from rabbit tissue by differential centrifugation (40), and enumerated by dark-field microscopy.

Intrinsic radiolabeling of spirochetal lipids. Spirochetes were labeled with either (9,10(*n*))-[^{3}H]palmitate (30 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or (9,10(*n*))-[^{3}H]oleate (10 Ci/mmol; Amersham). Each ^{3}H-fatty acid was used at a final concentration of 6.25 μM for both *B. burgdorferi* and *T. pallidum* cultures.

For intrinsic labeling of *B. burgdorferi* lipids, the appropriate aliquot of each ^{3}H-fatty acid was first dried under a stream of nitrogen, and the dried material was dissolved in 1 ml of BSK II medium. These preparations were subsequently filter sterilized and added to 5-ml log-phase cultures of *B. burgdorferi* (4.5×10^7 organisms per ml). The bacteria were incubated at 34°C for approximately 72 h (about six generation times); strain B31 reached a final culture density of approximately 4×10^8 bacteria per ml, while strain 297 increased to about 2×10^8 organisms per ml. Radiolabeled spirochetes were harvested by centrifugation at 12,000 × *g* (20 min, 4°C), and the bacterial pellet was washed once gently without disruption by using about 5 ml of cold BSK II medium.

Intrinsic radiolabeling of *T. pallidum* lipids was carried out in the treponemal labeling medium of Stamm and Bassford (48) under conditions described by Chamberlain et al. (9); under these conditions, treponemes metabolize but do not replicate. Briefly, for each radioactive fatty acid, 7×10^9 bacteria were suspended in 4 ml of labeling medium containing the appropriate fatty acid and 100 μg of cycloheximide per ml to inhibit protein synthesis in contaminating testicular cells. *T. pallidum* cultures were incubated for 20 h (34°C). Treponemes were harvested by centrifugation and the cell pellet was gently washed as described above for the borreliae, except that treponemal labeling medium rather than BSK II medium was used. Because treponemal suspensions typically contained low levels of contaminating rabbit testicular tissue, the potential for incorporating radioactive fatty acids into rabbit cells during the labeling of treponemes also was assessed separately; normal rabbit testes were extracted and processed in a manner identical to that used for the isolation of treponemes from infected rabbit tissue. This testis material (tissue and serum) was added to treponemal labeling medium (containing cycloheximide) with radioactive palmitate, and the sham preparation was incubated for 20 h at 34°C.

Isolation of spirochetal lipoproteins. Spirochetal lipoproteins were isolated by extraction with 2% Triton X-114 in phosphate-buffered saline; extraction was followed by phase partitioning as previously described (35). The detergent phase was dialyzed overnight against cold H_2O to remove salts and then lyophilized to reduce the total volume. Proteins were subsequently recovered by precipitation with 10 volumes of cold acetone. A small amount of [^{3}H]palmitate was added to the sample as a tracer to ensure that subsequent removal of noncovalently associated fatty acids (i.e., delipidation) was complete. Noncovalently associated lipids then were removed by washing the protein pellet twice with chloroform-methanol (2:1) acidified with 6 N HCl to a final pH of 1 to 2; the washing was followed by four or more additional extractions with nonacidified chloroform-methanol (2:1). Sham-extracted rabbit testicular material (described above) was treated in an identical fashion. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as a reference standard.

Extraction of lipids. Lipids and fatty acids were isolated from spirochetes, rabbit testicular material, or BSK II medium by extraction of lyophilized samples with chloroform-methanol (2:1) (15). The organic material was dried under nitrogen, dissolved in 6 ml of chloroform-methanol (2:1), and washed with 1 ml of H_2O (15). Washed lipid material was dried and weighed.

Fatty acid analysis. The fatty acid compositions of lipid (10 μg) and lipoprotein (150 μg) preparations were determined by gas chromatography. Preparations were aliquoted into glass culture tubes (13 by 100 mm) and then dried under nitrogen. The samples then were hydrolyzed and methylated with 300 μl of 3 M methanolic HCl (Supelco Inc., Bellefonte, Pa.) (80°C) overnight under a nitrogen atmosphere (24); the resulting methyl esters were recovered by partitioning between hexane and water. Methyl esters in hexane were subsequently separated on a DB-225 capillary column (J and W Scientific, Folsom, Calif.) by using a model 5890A gas chromatogram (Hewlett-Packard, Palo Alto, Calif.) with a flame ionization detector. Fatty acid methyl ester peaks were identified and quantitated by using 10 nmol of known fatty acid standards derivatized in parallel with the unknown samples; heptadecanoic acid (17:0) was used as an internal standard. Values for each fatty acid were corrected by subtraction of background values obtained from processing volumes of BSK II medium or rabbit testicular extract equivalent to those found in spirochetal preparations. To first determine the amount of BSK II medium in pellets of *B. burgdorferi* cells, suspensions of the organism were assayed for BSA content by an enzyme-linked immunosorbent assay using a monoclonal antibody against BSA (B2901; Sigma Chemical Co.); results were compared with a standard curve derived from assaying dilutions of BSK II medium. To prepare rabbit testicular material equivalent to that contaminating *T. pallidum* preparations, noninfected rabbit testicles were sham extracted and processed in a manner identical to that used to isolate treponemes. Finally, in separate experiments, the fatty acid compositions of BSK II medium and rabbit testicular tissue were determined by using greater quantities of these materials.

TLC of spirochetal lipids. Total lipids of spirochetes labeled with either [^{3}H]palmitate or [^{3}H]oleate, or normal rabbit tissue labeled with [^{3}H]palmitate, was suspended in 200 μl of chloroform-methanol (1:2). Ten microliters of each sample was mixed with standards (described below) and applied to Polygram Silica-G thin-layer chromatography (TLC) plates (Brinkmann Instruments, Westbury, N.Y.) for two-dimensional TLC. Chromatography of the first dimension was performed in a solvent system of $CHCl_3$-CH_3OH-NH_4OH (65:35:7.5). After chromatography in the first dimension, the plate was dried for several hours, turned 90°, and developed in a solvent system of $CHCl_3$-CH_3COCH_3-CH_3COOH-CH_3OH-H_2O (50:20:15:10:5). The lipids were visualized by treating the plate with iodine vapors, and the positions of the developed spots were marked on the back of the plate. The plate was then sprayed with En^{3}Hance (DuPont/New England Nuclear Research Products, Wilmington, Del.), dried, and exposed to preflashed X-ray film (−70°C). Phospholipid standards consisted of phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PtdIns), phosphatidylcholine (PtdCho), and phosphatidylglycerol (PtdGro) (Serdary Research Labo-

ratories, London, Ontario, Canada) and cardiolipin (CL) obtained as antigen for the Venereal Disease Research Laboratory (VDRL) test (0.03% CL, 0.9% cholesterol, 0.21% PtdCho; Baxter, McGaw Park, Ill.). Standards (25 μg of each lipid per plate) were chromatographed either separately or mixed with spirochetal samples.

Alkaline hydrolysis of spirochetal PtdCho. To identify ester-linked acyl chains on spirochetal phospholipid, PtdCho was purified from [^{3}H]palmitate- or [^{3}H]oleate-labeled lipid extracts of *B. burgdorferi* or *T. pallidum* by two sequential isolations by preparative one-dimensional TLC; the solvent system was $CHCl_3$-CH_3OH-NH_4OH (100:54:11.5). PtdCho was removed from the silica by sequential extraction with chloroform-methanol mixed 2:1 and chloroform-methanol mixed 1:2 (15). The PtdCho extract was dried under nitrogen, and contaminating silica was removed by biphasic partitioning as described by Folch et al. (15). Purified PtdCho was dried under nitrogen, resuspended in 0.5 ml of chloroform, mixed with an equal volume of 1 N NaOH, and incubated at room temperature for 30 min. The mixture was then extracted (7), and the chloroform phase was dried and analyzed by TLC on KC-18 RF silica plates (Whatman Inc., Clifton, N.J.) in a solvent system of CH_3CN-CH_3COOH (1:1). Radiolabeled myristate, palmitate, oleate, and stearate were chromatographed as reference standards in adjacent lanes. The plate was developed with iodine vapor, sprayed with En^{3}Hance, and exposed to X-ray film (−70°C).

Digestion of spirochetal PtdCho with phospholipases A_1 and A_2. Approximately 1×10^5 to 2×10^5 dpm of total spirochetal lipids labeled with either [^{3}H]palmitate or [^{3}H]oleate was mixed with 10 μg of unlabeled PtdCho (Serdary Research Laboratories) and separated by two-dimensional preparative TLC (described above). The radioactive spot representing PtdCho (identified by iodine staining of the PtdCho standard) was collected by scraping the silica from the appropriate area of the plate. PtdCho was removed from the silica (described above). The organic extract was dried under nitrogen, and the residue was dissolved in 0.2 ml of chloroform-methanol (2:1). A sample containing approximately 90,000 dpm was then divided into three aliquots which were dried under nitrogen and then treated with either *Rhizopus* phospholipase A_1 (Sigma Chemical Co.) (32) or cobra venom phospholipase A_2 (Serdary Research Laboratories) (32, 39) or not treated. The two phospholipase reaction buffers (0.5 ml) were prepared by mixing 100 μl of 0.32 M sodium acetate (pH 5 for the A_1 lipase or pH 7 for the A_2 lipase), 200 μl of 0.9% NaCl, 50 μl of a 0.5-mg/ml solution of fatty acid-free BSA (A7511; Sigma Chemical Co.), 12 μl of 1% Triton X-100, and 138 μl of water (all reagents were prepared with Chelex-treated deionized water). Forty microliters of each respective reaction buffer was added to each dried lipid sample, and the samples were sonicated in a water bath. Enzyme was added (4 U per reaction mixture), and the tubes were incubated at 37°C for 2 h. After incubation, 5 μg of unlabeled PtdCho was added to each sample to facilitate organic extraction. The samples were dried under nitrogen, dissolved in 0.6 ml of chloroform-methanol (2:1), and washed (phase partitioned) with 0.1 ml of water. The organic phase was collected, dried, dissolved in 20 μl of chloroform-methanol (2:1), and then applied to a silica gel G (Brinkmann Instruments, Inc.) plate. One-dimensional TLC was performed in a solvent system of $CHCl_3$-CH_3OH-NH_4OH (100:54:11.5). The plate was developed with iodine, sprayed with En^{3}Hance, and exposed to X-ray film (−70°C). The spots also were scraped from the plate, and radioactivity was quantitated by liquid scintillation.

TABLE 1. Long-chain fatty acids of spirochetal lipoproteins and cellular lipids

Preparation	Fatty acid[a,b]					
	14:0	16:0	16:1	18:0	18:1	18:2
B. burgdorferi B31						
Lipoproteins	1.9	78.3	2.7	7.1	5.1	4.9
Cellular lipids	1.5	30.8	2.2	19.0	21.0	25.4
B. burgdorferi 297						
Lipoproteins	2.5	81.6	3.4	2.3	6.5	3.6
Cellular lipids	2.1	40.4	2.5	16.0	23.5	15.4
BSK II medium	1.4	33.8	1.6	16.8	22.2	24.1
T. pallidum						
Lipoproteins	TR[c]	58.3	ND[d]	30.4	11.2	TR
Cellular lipids	1.8	52.5	ND	21.4	15.3	8.9
Rabbit testicular tissue	2.3	27.0	9.6	6.0	32.7	22.4

[a] Expressed as percentages of total fatty acids recovered.
[b] 14:0, myristate; 16:0, palmitate; 16:1, palmitoleate; 18:0, stearate; 18:1, oleate; 18:2, linoleic acid.
[c] TR, trace (<1% detected).
[d] ND, none detected.

RESULTS AND DISCUSSION

Fatty acid compositions of spirochetal lipoproteins and cellular lipids. Spirochetal lipoproteins were extensively extracted with organic solvents and then hydrolyzed to release covalently attached fatty acids. The released fatty acids were derivatized to methyl esters and then analyzed by gas chromatography. The overall distributions of fatty acids in lipoproteins from the avirulent and virulent *B. burgdorferi* strains were very similar; in each case, palmitate was the predominant fatty acid recovered (78.3 and 81.6%, respectively) (Table 1). Comparatively minor amounts of stearate, oleate, linoleate, palmitoleate, and myristate composed the remainder of the fatty acids recovered from borrelial lipoproteins (Table 1). Palmitate (58.3%) also was the major fatty acid recovered from *T. pallidum* lipoproteins. However, in contrast to *B. burgdorferi* lipoproteins, treponemal lipoproteins also contained substantial amounts (30.4%) of stearate (Table 1). Palmitate previously has been identified as the major fatty acid constituent of other bacterial lipoproteins (17, 30, 58). Stearate, on the other hand, is not typically prevalent in prokaryotic lipoproteins.

Cellular lipids presumably function as acyl donors to lipoproteins in spirochetes. Thus, it was of interest to compare the fatty acid compositions of the spirochetal lipoproteins and cellular lipids from their respective organisms. With respect to *B. burgdorferi*, differences in the distributions of fatty acids in the avirulent and virulent strains were noted; the virulent strain contained more palmitate and less linoleic acid (Table 1). Although palmitate predominated among the fatty acids recovered from borrelial lipids, its relative abundance in the cellular lipids of each strain was considerably less than in the lipoproteins (Table 1). Palmitate, therefore, appears to be incorporated preferentially into borrelial lipoproteins at the expense of other saturated and unsaturated long-chain fatty acids. Of note, fatty acids within the total cellular lipids of both strains of *B. burgdorferi* closely paralleled those of BSK II medium, suggesting that fatty acids were incorporated relatively nonselectively into borrelial lipids. In contrast to observations with *B. burgdorferi*, the distributions of fatty acids in *T.*

pallidum cellular lipids and lipoproteins were relatively similar (Table 1), although palmitate and stearate appeared to be utilized preferentially, particularly at the expense of linoleic acid (Table 1). The observation that the fatty acid profile of the *T. pallidum* lipids was markedly different from that of rabbit testicular tissue (Table 1) argued against the presence of significant amounts of host contaminants in the preparations used for these analyses. However, the difference between the fatty acid profiles of the *T. pallidum* cellular lipids and rabbit testicular tissue, the presumed source of fatty acids for treponemal growth in vivo, suggested either that fatty acid analysis of rabbit testicular tissue is not an accurate indicator of fatty acid availability to *T. pallidum* during in vivo cultivation or that some degree of selectivity exists for fatty acid incorporation into treponemal cellular lipids. In any event, disparities between the fatty acid profiles of the treponemal cellular lipids and rabbit testicular tissue should not be interpreted as the result of fatty acid interconversion by *T. pallidum*, inasmuch as no evidence for this currently exists.

Analysis of spirochetal phospholipids. Phospholipids are the principal acyl donors to bacterial lipoproteins (16, 23, 41). We therefore characterized spirochetal phospholipids in order to identify putative acyl donors in these organisms. To accomplish this, *T. pallidum* and *B. burgdorferi* were metabolically radiolabeled with [^{3}H]palmitate or [^{3}H]oleate, two fatty acids highly abundant in the cellular lipids of both spirochetes (Table 1); following radiolabeling, cellular lipids were extracted and resolved by TLC.

Marked differences in the phospholipid compositions of *B. burgdorferi* and *T. pallidum* were noted. *B. burgdorferi* contained only PtdGro and PtCho (Fig. 1), the same two phospholipids previously identified in *B. hermsii* (27), an agent of relapsing fever. In *T. pallidum*, six phospholipids were identified as PtdGro, PtdCho, PtdEtn, PtdSer, PtdIns, and CL (Fig. 2). Additional attempts to characterize spirochetal lipids which did not comigrate with the phospholipid standards were made by using differential staining. α-Naphthol staining for glycolipids (46) revealed a species in the upper left corner of the TLC plate for *B. burgdorferi* (Fig. 1) which likely represented the glycolipid(s) recently reported for this bacterium (13, 55). Staining for choline-containing lipids (4) indicated that the two lipids in *T. pallidum* migrating to the right of PtdIns and above the origin (Fig. 2) were the lysoforms of PtdCho (data not shown). It is noteworthy that differences in the incorporation of palmitate and oleate into phospholipids were not observed for either spirochete (Fig. 1 and 2). Thus, it did not appear that selective incorporation of palmitate into a subset of phospholipids (putative acyl donors) could account for its relative abundance in spirochetal lipoproteins.

Inasmuch as metabolic alteration of the fatty acid labels would have complicated interpretation of these studies, we performed alkaline hydrolysis on the palmitate- or oleate-labeled PtdCho purified from *T. pallidum* and *B. burgdorferi*. In each case, the fatty acid released was that used for labeling (data not shown). These results also were consistent with previous studies demonstrating that treponemes and borreliae are fatty acid auxotrophs incapable of elongating, beta-oxidizing, or desaturating long-chain fatty acids (8, 9, 26, 27, 43). To ensure that the radiolabeled *T. pallidum* lipids (Fig. 2) did not include rabbit contaminants, parallel labeling experiments were conducted with suspensions of rabbit testicular tissue in treponemal labeling medium. Only one lipid migrating in proximity to PtdCho was barely detectable after prolonged exposure of the TLC plate (data not shown).

Positional distribution of fatty acids in spirochetal phospholipid. It has been proposed that in *Escherichia coli*, specific fatty acids (i.e., palmitate) are preferentially incorporated into the 1 position (donor site) of PtdEtn, a major acyl donor for lipoprotein synthesis (23, 41, 42, 52). Thus, we investigated the incorporation of fatty acids into the 1 and 2 positions of a potential acyl donor to lipoproteins. PdtCho from *B. burgdorferi* and *T. pallidum* labeled with either [^{3}H]palmitate or [^{3}H]oleate was digested separately with phospholipases A_1 and A_2. While comparable experiments with *E. coli* most frequently have been performed with PtdEtn (23, 41, 42, 52), the absence of this phospholipid in *B. burgdorferi* (Fig. 1) indicated that the Lyme disease spirochete uses alternative acyl donors. Given the paucity of data regarding the identities of lipoprotein acyl donors in pathogenic spirochetes, PtdCho was selected for positional analysis because of its abundance in both spirochetes (Fig. 1 and 2). It also has been found that in *E. coli* phospholipids other than PtdEtn may serve as lipoprotein acyl donors (16). In the cases of both spirochetes, [^{3}H]palmitate and [^{3}H]oleate were released from the 1 and 2 positions of PtdCho by digestion with phospholipases A_1 and A_2, respectively (Fig. 3). Moreover, for the PtdCho of each organism labeled with either [^{3}H]palmitate or [^{3}H]oleate, no significant differences in the disintegrations per minute released from either position were noted (data not shown). These data indicate that the two fatty acids were incorporated to similar extents into both acyl positions. Although the possibility still exists that fatty acids are asymmetrically distributed in other putative spirochetal phospholipid acyl donors, our combined results from intrinsic radiolabeling of spirochetal lipids and

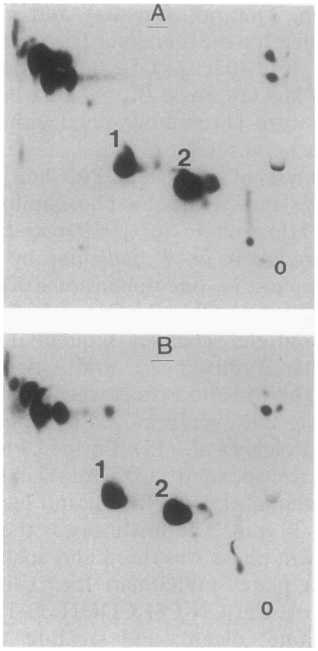

FIG. 1. Incorporation of palmitate and oleate into phospholipid (lipoprotein donor) pools of *B. burgdorferi*. Lipids extracted from 5 × 10^9 *B. burgdorferi* B31 organisms labeled with either [^{3}H]palmitate or [^{3}H]oleate were separated by two-dimensional TLC. Labeled phospholipids were detected by fluorography and identified by comparison with unlabeled phospholipid standards chromatographed concurrently with each sample. Chromatography for the first dimension was performed from bottom to top, while that for the second dimension was performed from right to left. (A) $CHCl_3$-extractable material (2 × 10^6 dpm) from strain B31 labeled with [^{3}H]palmitate. (B) $CHCl_3$-extractable material (2 × 10^6 dpm) from [^{3}H]oleate-labeled bacteria. 1, PtdGro; 2, PtdCho; 0, origin.

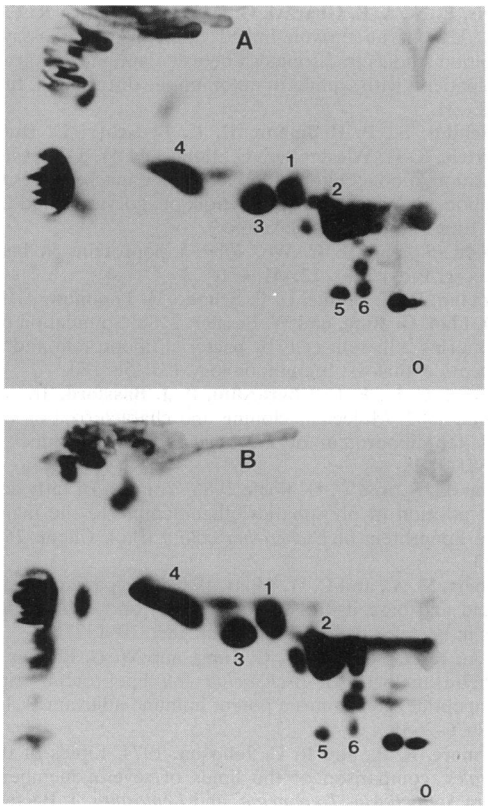

FIG. 2. Incorporation of palmitate and oleate into phospholipid (lipoprotein donor) pools of *T. pallidum*. Lipids extracted from 2×10^{11} *T. pallidum* organisms labeled with [^{3}H]palmitate (A) or [^{3}H]oleate (B) were separated by two-dimensional TLC. Chromatography was performed as described in the legend to Fig. 1. 1, PtdGro; 2, PtdCho; 3, PtdEtn; 4, CL; 5, PtdSer; 6, PtdIns; 0, origin.

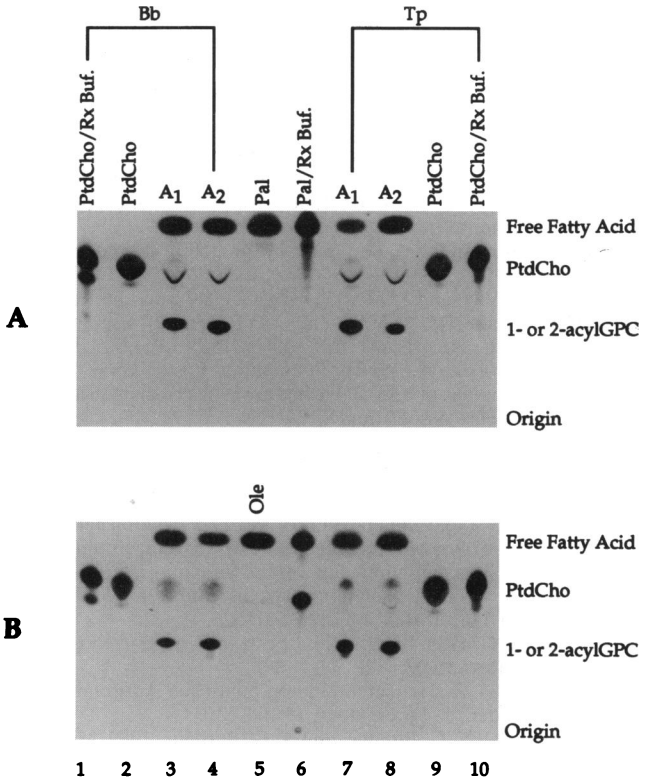

FIG. 3. Phospholipase A_1 or A_2 treatment of PtdCho isolated from either [^{3}H]palmitate- or [^{3}H]oleate-labeled *B. burgdorferi* or *T. pallidum*. PtdCho was purified from extracts of radiolabeled *B. burgdorferi* and *T. pallidum*, and aliquots of each labeled PtdCho were digested with either phospholipase A_1 or A_2. The released fatty acid and/or lyso-PtdCho (1- or 2-acyl glycerophosphocholine [GPC]) was identified by fluorography. (A) Phospholipase digests of [^{3}H]palmitate-labeled PtdCho from *B. burgdorferi* and *T. pallidum*. (B) Phospholipase digests of [^{3}H]oleate-labeled PtdCho from *B. burgdorferi* and *T. pallidum*. Lanes 1 and 10, spirochetal PtdCho in A_1 reaction buffer (Rx Buf.); lanes 2 and 9, spirochetal PtdCho spotted directly from $CHCl_3$-CH_3OH; lanes 3 and 7, spirochetal PtdCho digested with phospholipase A_1; lanes 4 and 8, spirochetal PtdCho digested with phospholipase A_2; lanes 5, [^{3}H]palmitate (Pal) or [^{3}H]oleate (Ole) used for labeling of spirochetes; lanes 6, [^{3}H]palmitate or [^{3}H]oleate in A_1 reaction buffer. The positions of free fatty acid, undigested PtdCho, lysophospholipids (1- or 2-acylGPC), and the origin of chromatography are indicated. The fatty acid migrating more slowly than PtdCho in B6 is an oxidation product of [^{3}H]oleate. Bb, *B. burgdorferi*; Tp, *T. pallidum*.

analysis of the positional distribution of labeled fatty acids in PtdCho suggest that fatty acid utilization during spirochetal lipoprotein synthesis is determined largely by the fatty acid specificities of the lipoprotein acyl transferases.

While the relevance of the differences in phospholipid composition of *B. burgdorferi* and *T. pallidum* with respect to their membrane biology is unclear, the exclusive presence of CL in *T. pallidum* may explain an important serological distinction between syphilis and Lyme disease. It has long been recognized that the antiphospholipid antibodies measured by the VDRL serological test are produced in response to infection with *T. pallidum*, while such antibodies are not commonly detected in the sera of Lyme disease patients. Clinically, VDRL antibody is detected by testing serum against a defined mixture of CL-cholesterol-PtdCho (18) in which CL is considered the major antigenic component (18). Although VDRL antibodies traditionally have been designated as nontreponemal in the belief that they are elicited by host lipids (11, 51), our data lead us to propose that they are induced by *T. pallidum* CL during syphilis and that their absence in Lyme disease is due to the fact that *B. burgdorferi* does not contain this phospholipid (3).

Previous work from our laboratories has supported the contention that clinical and pathogenic features common to syphilis and Lyme disease result from molecular and ultrastructural similarities shared by their respective etiological agents (34, 36). In this study, however, we identified differences in the lipids and lipoprotein acyl constituents of *T. pallidum* and *B. burgdorferi* that may contribute to the clinical features which distinguish syphilis from Lyme disease. Prior molecular cloning studies have revealed that the amino acid sequences of spirochetal lipoproteins are extremely heterogeneous (1, 5, 33, 44, 50, 53). The analyses presented here demonstrate that *T. pallidum* and *B. burgdorferi* also appear to generate diversity among their lipoproteins by altering the fatty acids which provide the membrane anchors for these molecules. Studies currently under way are intended to determine how variation of the lipoprotein acyl constituents impacts their physiological functions, their interactions with other membrane components, and their abilities to act as inflammatory mediators during spirochetal infection. Knowledge of the distinctive lipid compositions of these organisms will enable such studies to be

performed in artificial systems that closely reproduce the native lipid environments of the lipoproteins.

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REFERENCES

1. **Akins, D. R., B. K. Purcell, M. Mitra, M. V. Norgard, and J. D. Radolf.** 1993. Lipid modification of the 17-kilodalton membrane immunogen of *Treponema pallidum* determines macrophage activation as well as amphiphilicity. Infect. Immun. **61:**1202–1210.
2. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
3. **Barbour, A. G.** 1988. Laboratory aspects of Lyme borreliosis. Clin. Microbiol. Rev. **1:**399–414.
4. **Beiss, U.** 1964. Zur papierchromatographischen Auftrennung von Pflanzenlipiden. J. Chromatogr. **13:**104–110.
5. **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 spirochaete *Borrelia burgdorferi*. Mol. Microbiol. **3:**479–486.
6. **Bessler, W. G., M. Cox, A. Lex, B. Suhr, K.-H. Wiesmuller, and G. Jung.** 1985. Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J. Immunol. **135:**1900–1905.
7. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
8. **Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect. Immun. **58:**983–991.
9. **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.
10. **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.
11. **Costello, P. B., and F. A. Green.** 1986. Reactivity patterns of human anticardiolipin and other antiphospholipid antibodies in syphilitic sera. Infect. Immun. **51:**771–775.
12. **Deres, K., H. Schild, K.-H. Wiesmuller, G. Jung, and H.-G. Rammensee.** 1989. *In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. Nature (London) **342:**561–564.
13. **Eiffert, H., K. Jarecki-Khan, and R. Thomssen.** 1991. Identification of an immunoreactive nonproteinaceous component in *Borrelia burgdorferi*. Med. Microbiol. Immunol. **180:**229–237.
14. **Erdile, L. F., M.-A. Brandt, D. J. Warakomski, G. J. Westrack, A. Sadziene, A. G. Barbour, and J. P. Mays.** 1993. Role of attached lipid in immunogenicity of *Borrelia burgdorferi* OspA. Infect. Immun. **61:**81–90.
15. **Folch, J., M. Lees, and H. S. Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.
16. **Gupta, S. D., W. Dowhan, and H. C. Wu.** 1991. Phosphatidylethanolamine is not essential for the *N*-acylation of apolipoprotein in *Escherichia coli*. J. Biol. Chem. **266:**9983–9986.
17. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein. Eur. J. Biochem. **34:**284–296.
18. **Harris, E. N., A. E. Gharavi, G. D. Wasley, and G. R. V. Hughes.** 1988. Use of an enzyme-linked immunosorbent assay and of inhibition studies to distinguish between antibodies to cardiolipin from patients with syphilis or autoimmune disorders. J. Infect. Dis. **157:**23–31.
19. **Hauschildt, S., P. Hoffmann, H. U. Beuscher, G. Dufhues, P. Heinrich, K.-H. Wiesmuller, G. Jung, and W. G. Bessler.** 1990. Activation of bone marrow-derived mouse macrophages by bacterial lipopeptide: cytokine production, phagocytosis, and Ia expression. Eur. J. Immunol. **20:**63–68.
20. **Hayashi, S., and H. C. Wu.** 1990. Lipoproteins in bacteria. J. Bioenerg. Biomembr. **22:**451–471.
21. **Hoffmann, P., S. Heinle, U. F. Schade, H. Loppnow, A. J. Ulmer, H.-D. Flad, G. Jung, and W. Bessler.** 1988. Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogs. Immunobiology **177:**158–170.
22. **Hubbard, C. L., F. C. Gherardini, P. J. Bassford, Jr., and L. V. Stamm.** 1991. Molecular cloning and characterization of a 35.5-kilodalton lipoprotein of *Treponema pallidum*. Infect. Immun. **59:**1521–1528.
23. **Jackowski, S., and C. O. Rock.** 1986. Transfer of fatty acids from the 1-position of phosphatidylethanolamine to the major membrane lipoprotein of *Escherichia coli*. J. Biol. Chem. **261:**11328–11333.
24. **Lambert, M. A., and C. W. Moss.** 1983. Comparison of the effects of acid and base hydrolyses on hydroxy and cyclopropane fatty acids in bacteria. J. Clin. Microbiol. **18:**1370–1377.
25. **Lex, A., K.-H. Wiesmuller, G. Jung, and W. G. Bessler.** 1986. A synthetic analogue of *Escherichia coli* lipoprotein, tripalmitoyl pentapeptide, constitutes a potent immune adjuvant. J. Immunol. **137:**2676–2681.
26. **Livermore, B. P., and R. C. Johnson.** 1974. Lipids of the *Spirochaetales*: comparison of the lipids of several members of the genera *Spirochaeta*, *Treponema*, and *Leptospira*. J. Bacteriol. **120:**1268–1273.
27. **Livermore, B. P., and R. C. Johnson.** 1978. Lipid metabolism of *Borrelia hermsii*. Infect. Immun. **20:**215–220.
28. **Ma, Y., and J. J. Weis.** 1993. *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic and cytokine-stimulatory properties. Infect. Immun. **61:**3843–3853.
29. **Metzger, R., G. Jung, W. G. Bessler, P. Hoffmann, M. Strecker, A. Lieberknecht, and U. Schmidt.** 1991. Lipopeptides containing 2-(palmitoylamino)-6,7-bis(palmitoxy)heptanoic acid: synthesis, stereospecific stimulation of B lymphocytes and macrophages, and adjuvanticity in vivo and in vitro. J. Med. Chem. **34:**1969–1974.
30. **Mizuno, T.** 1979. A novel peptidoglycan-associated lipoprotein found in the cell envelope of *Pseudomonas aeruginosa* and *Escherichia coli*. J. Biochem. **86:**991–1000.
31. **Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour.** 1992. Low-passage-associated proteins of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect. Immun. **60:**4662–4672.
32. **Plückthun, A., and E. A. Dennis.** 1982. Acyl and phosphoryl migration in lysophospholipids: importance in phospholipid synthesis and phospholipase specificity. Biochemistry **21:**1743–1750.
33. **Purcell, B. K., M. A. Swancutt, and J. D. Radolf.** 1990. Lipid modification of the 15 kilodalton major membrane immunogen of *Treponema pallidum*. Mol. Microbiol. **4:**1371–1379.
34. **Radolf, J. D., J. S. Brusca, M. E. Brandt, and M. V. Norgard.** 1992. Spirochete molecular architecture and Lyme disease pathogenesis, p. 119–134. *In* S. E. Schutzer. (ed.), Lyme disease: molecular and immunologic approaches. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard.** 1988. Identification and localization of integral membrane proteins of *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. **56:**490–498.
36. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis: analysis using a CAT reporter construct. J. Immunol. **147:**1968–1974.

37. **Reitermann, A., J. Metzger, K.-H. Wiesmüller, G. Jung, and W. G. Bessler.** 1989. Lipopeptide derivatives of bacterial lipoprotein constitute potent immune adjuvants combined with or covalently coupled to antigen or hapten. Biol. Chem. Hoppe-Seyler **370:**343–352.
38. **Riley, B. S., N. Oppenheimer-Marks, E. J. Hansen, J. D. Radolf, and M. V. Norgard.** 1992. Virulent *Treponema pallidum* activates human vascular endothelial cells. J. Infect. Dis. **165:**484–493.
39. **Roberts, M. F., R. A. Deems, and E. A. Dennis.** 1977. Spectral perturbations of the histidine and tryptophan in cobra venom phospholipase A_2 upon metal ion and mixed micelle binding. J. Biol. Chem. **252:**6011–6017.
40. **Robertson, S. M., J. R. Kettman, J. N. Miller, and M. V. Norgard.** 1982. Murine monoclonal antibodies specific for virulent *Treponema pallidum* (Nichols). Infect. Immun. **36:**1076–1085.
41. **Rock, C. O.** 1984. Turnover of fatty acids in the 1-position of phosphatidylethanolamine in *Escherichia coli*. J. Biol. Chem. **259:** 6188–6194.
42. **Rock, C. O., and S. Jackowski.** 1985. Pathways for the incorporation of exogenous fatty acids into phosphatidylethanolamine in *Escherichia coli*. J. Biol. Chem. **260:**12720–12724.
43. **Schiller, N. L., and C. D. Cox.** 1977. Catabolism of glucose and fatty acids by virulent *Treponema pallidum*. Infect. Immun. **16:**60–68.
44. **Schouls, L. M., R. Mout, J. Dekker, and J. D. A. Van Embden.** 1989. Characterization of lipid-modified immunogenic proteins of *Treponema pallidum* expressed in *Escherichia coli*. Microb. Pathog. **7:**175–188.
45. **Schwan, T. G., W. Burgdorfer, M. E. Schrumpf, and R. H. Karstens.** 1988. The urinary bladder, a consistent source of *Borrelia burgdorferi* in experimentally infected white-footed mice (*Peromyscus leucopus*). J. Clin. Microbiol. **26:**893–895.
46. **Siakotos, A. N., and G. Rouser.** 1965. Analytical separation of nonlipid water soluble substances and gangliosides from other lipids by dextran gel column chromatography. J. Am. Oil Chem. Soc. **42:**913–919.
47. **Sjöstedt, A., A. Tärnvik, and G. Sandström.** 1991. The T-cell-stimulating 17-kilodalton protein of *Francisella tularensis* LVS is a lipoprotein. Infect. Immun. **59:**3163–3168.
48. **Stamm, L. V., and P. J. Bassford, Jr.** 1985. Cellular and extracellular protein antigens of *Treponema pallidum* synthesized during in vitro incubation of freshly extracted organisms. Infect. Immun. **47:**799–807.
49. **Swancutt, M. A., J. D. Radolf, and M. V. Norgard.** 1990. The 34-kilodalton membrane immunogen of *Treponema pallidum* is a lipoprotein. Infect. Immun. **58:**384–392.
50. **Swancutt, M. A., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1989. Molecular characterization of the pathogen-specific 34-kilodalton membrane immunogen of *Treponema pallidum*. Infect. Immun. **57:**3314–3323.
51. **Tramont, E. C.** 1990. Treponema pallidum (syphilis), p. 1794–1808. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases. Churchill Livingtone, Inc., New York.
52. **Van Golde, L. M. G., and L. L. M. Van Deenen.** 1967. Molecular species of extracellular phosphatidylethanolamine from *Escherichia coli*. Chem. Phys. Lipids **1:**157–164.
53. **Weigel, L. M., M. E. Brandt, and M. V. Norgard.** 1992. Analysis of the N-terminal region of the 47-kilodalton integral membrane lipoprotein of *Treponema pallidum*. Infect. Immun. **60:**1568–1576.
54. **Weyer, K. A., W. Schäfer, F. Lottspeich, and H. Michel.** 1987. The cytochrome subunit of the photosynthetic reaction center from *Rhodopseudomonas viridis* is a lipoprotein. Biochemistry **26:**2909–2914.
55. **Wheeler, C. M., J. C. G. Monoco, J. L. Benach, M. G. Golightly, G. S. Habicht, and A. C. Steere.** 1993. Nonprotein antigens of *Borrelia burgdorferi*. J. Infect. Dis. **167:**665–674.
56. **Wiesmüller, K.-H., G. Jung, and G. Hess.** 1989. Novel low-molecular weight synthetic vaccine against foot-and-mouth disease containing a potent B-cell and macrophage activator. Vaccine **7:**29–33.
57. **Wilske, B., V. Preac-Mursic, S. Jauris, A. Hofmann, I. Pradel, E. Soutschek, E. Schwab, G. Will, and G. Wanner.** 1993. Immunological and molecular polymorphisms of OspC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. Infect. Immun. **61:**2182–2191.
58. **Zlotnick, G. W., V. T. Sanfilippo, J. A. Mattler, D. H. Kirkley, R. A. Boykins, and R. C. Seid, Jr.** 1988. Purification and characterization of a peptidoglycan-associated lipoprotein from *Haemophilus influenzae*. J. Biol. Chem. **263:**9790–9794.