Absence of Lipopolysaccharide in the Lyme Disease Spirochete, *Borrelia burgdorferi*

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We were unable to demonstrate the presence of the classic enterobacterium-type lipopolysaccharide in the cells of the Lyme disease spirochete, *Borrelia burgdorferi* B31. This finding was primarily based on chemical analysis and the absence of free lipid A upon mild acid hydrolysis of the appropriate cell extracts. These results do not preclude the possible existence of an unusual lipopolysaccharide-like compound(s) in *B. burgdorferi*.

Beck et al. (4) recently reported the presence of lipopolysaccharide (LPS) in the Lyme disease spirochete (*Borrelia burgdorferi*). We wanted to confirm their results and then examine the nature of the lipid A moiety of LPS from this source. We extracted the cells of *B. burgdorferi* B31 by two well-known methods used in the isolation of LPS from gram-negative bacteria. The appropriate fractions obtained by these two methods were hydrolyzed under mild acid conditions and examined by thin-layer chromatography (TLC) for the presence of free lipid A. Chemical analyses for the presence of 2-keto-3-deoxyoctonate, glucosamine, and hydroxy fatty acids were performed on the extracts. We found no evidence for the presence of lipid A in any of the fractions tested. Thus, we must conclude that the classic LPS associated with gram-negative bacteria is absent in cells of *B. burgdorferi*.

Cells of *B. burgdorferi* B31 (ATCC 35210) were grown in BSK II medium (1), washed three times with 50 mM Tris (pH 7.4)–150 mM NaCl–5 mM $MgCl_2$ (2), and lyophilized. In one experiment, the extraction method of Galanos et al. (7) with modifications was used (12, 14). The results of this extraction are summarized in Table 1. Almost all of the extractable material was found in the 90% aqueous ethanol fraction, whereas very little material appeared in the phenol-water-chloroform-petroleum ether fraction, which should contain the rough-type LPS (R-LPS). This latter fraction was hydrolyzed in 0.1 N HCl as previously described for the preparation of monophosphoryl lipid A (14), and 30 μg of the organic extract was analyzed by TLC for the presence of lipid A (12, 14). A mixture of hexa-, penta-, and tetraacyl monophosphoryl lipid A (from LPS of *Salmonella typhimurium*) was used as a chromatographic standard (13). There was no evidence of the presence of free lipid A in this sample, indicating the absence of R-LPS in the original extract (phenol-water-chloroform-petroleum ether).

Previous studies of lipids X and Y (monosaccharide precursors of LPS) showed that much of X and Y was extracted into the aqueous ethanol fraction (15, 16). Thus, it is conceivable that the 113.2 mg of the 90% ethanol extract (Table 1) might contain the R-LPS. This material was initially fractionated by solvent precipitation to yield 79.9 mg of an acetone-insoluble glycolipid-phospholipid fraction. This

fraction was further purified by preparative TLC on Silica Gel G (Merck & Co., Inc.) by a solvent system of chloroform-methanol-water (65:25:4). Five major fractions (designated A to E) were obtained. Each of these fractions was acid hydrolyzed and analyzed by TLC for the presence of free lipid A. The results of the analysis of fractions A to D were negative. Fraction E, the slowest-moving TLC band, yielded a hydrolysis product that comigrated near the tetraacyl monophosphoryl lipid A standard. However, this fraction was devoid of 2-keto-3-deoxyoctonate, glucosamine, and hydroxy fatty acid and resulted in no reaction in the *Limulus* amebocyte lysate test. It did not stimulate prostaglandin E_2 (PGE_2) by monolayer cultures of rabbit synoviocytes. LPS from several gram-negative organisms stimulate PGE_2 (20), and we have confirmed this finding in unpublished studies (R. J. Rothenberg and K. Takayama). Moreover, fraction E had a higher R_f value than the standard value for monophosphoryl lipid A. Normally, the R-LPS is a slower-moving band on TLC, which upon hydrolysis yields the faster-moving lipid A bands. All of these results together appear to indicate that the hydrolysis of fraction E does not yield lipid A and that this fraction does not contain R-LPS.

In another experiment, the extraction method of Westphal and Jann (18) was used. Table 1 shows that a considerable amount of material was extracted with the aqueous hot phenol (166.6 mg, 48%), the distribution of which in the three subfractions is indicated (Table 1). The smooth-type LPS (S-LPS) is normally found in the aqueous layer of the phenol extract, but in certain bacteria (i.e., *Brucella abortus* and *Rhodopseudomonas palustris*), the LPS can partition into the phenol layer (9, 17). All three fractions from the aqueous phenol extract were hydrolyzed under mild acid conditions and analyzed by TLC for the presence of lipid A as previously described (Fig. 1). The acid-hydrolyzed aqueous layer (lane A) was completely devoid of lipid A. The acid-hydrolyzed water-soluble phenol layer (lane C) contained a very light band which had an R_f value similar to that of the tetraacyl monophosphoryl lipid A. The acid-hydrolyzed water-insoluble phenol layer (lane D) contained more of this slower-moving band. However, all three hydrolyzed samples were devoid of bands migrating at the R_fs of the hexa- and pentaacyl monophosphoryl lipid A (the expected products of hydrolysis of LPS). Lane E of Fig. 1 shows that the unhydrolyzed water-insoluble phenol layer contained a com-

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TABLE 1. Extraction of cells of *B. burgdorferi* by the methods of both Galanos et al. (7) with modifications (12, 14) and Westphal and Jann (18)

Cell or extract	Dry wt (mg)	% Yield
Method of Galanos et al.		
Cells	460	100
Extracts		
90% Ethanol extract	113.2	24.6
Acetone and diethyl ether	0.0	0.0
PWCP[a]	0.3	0.07
Method of Westphal and Jann		
Cells	343	100
Aqueous phenol extracts[b]		
Aqueous layer	30.6	8.9
Phenol layer		
Water soluble	16	4.7
Water insoluble	120	35.0

[a] PWCP, Phenol-water-chloroform-petroleum ether; only the acetone-diethyl ether-insoluble fraction was recovered. This fraction would normally represent the R-LPS.

[b] The S-LPS would normally be present in the aqueous layer, but Beck et al. (4) reported it to be present in the phenol layer.

ponent with an R_f identical to that of fraction E of the 90% ethanol extract previously discussed. The hydrolysis of this fraction yielded a product that was identical to the slower-moving TLC band (the lipid A-like material) of the two hydrolyzed phenol layers. However, it was already shown that fraction E does not contain lipid A or R-LPS. All three of the aqueous phenol-extracted fractions were devoid of 2-keto-3-deoxyoctonate. These results suggested that the S-LPS was also absent in cells of *B. burgdorferi*.

The aqueous layer of the phenol extract did result in a positive response in the *Limulus* amebocyte lysate test at a concentration of 0.02 ng/ml, a response similar to the limit of detection of the reference standard. The residue was positive only at concentrations of 200 ng/ml, and the water-insoluble material of the phenol extract had no reaction in the lysate test at this concentration. This finding indicates that some type of water-soluble material can cause a positive lysate test reaction but that it is not LPS.

Extracts of the Lyme disease spirochete were incubated with a monolayer culture of rabbit synoviocytes which were previously incubated with [^{3}H]arachidonic acid to label phospholipids. The release of [^{3}H]PGE$_2$ was monitored as a measure of cellular activation by LPS. Control cultures released 2,034 ± 133 cpm of [^{3}H]PGE$_2$ (n = 4). *Salmonella minnesota* (100 μg/ml) resulted in 11,039 ± 518 cpm released (n = 4). In other studies by radioimmunoassay techniques, this finding corresponds to a doubling of basal PGE$_2$ levels in the medium. The lyophilized material from the aqueous layer at 100 μg/ml resulted in only 2,144 ± 27 cpm (n = 3), and the water-insoluble material caused a release of 2,513 ± 263 cpm (n = 4). Comparison of the control samples, the aqueous layer, and the water-insoluble layer by analysis of variance yielded no significant difference in the groups (P = 0.213). Similarly, no increase in the synthesis of [^{3}H]PGE$_2$ was observed when the fractions B through E of the material which would extract with lipid A were tested (data not shown). Therefore, no biological activity of these extracts was detected, in contrast to the marked response to *S. minnesota* LPS.

Several past studies on *Borrelia* and *Treponema* spp. suggested the possible involvement of endotoxinlike activity

associated with those organisms (3, 5, 10, 11, 19). However, no precise chemical characterization was performed in these studies. Fumarola et al. (6) showed that the spirochete from the *Ixodes ricinus* ticks gave a positive *Limulus* lysate reaction at a concentration of 10^5 cells per ml. Beck et al. (4) recently reported the isolation of an LPS from *B. burgdorferi*. Using a more precise definition of LPS, we were not able to confirm this finding. We did not perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis on these samples because it is, at most, a supplemental analytical technique. It alone does not add to the knowledge of the presence or absence of LPS. The interesting stained bands that appear still have to be carefully characterized. However, this characterization can be done much more easily at the lipid A level by TLC.

We made a basic assumption in this study that when a true LPS is treated under mild acid conditions, free lipid A must be liberated which should contain one or more of the following: an amino sugar (either glucosamine or diamino sugar), normal or hydroxy fatty acids, and phosphate. We were unable to generate free lipid A from the two extracts of *B. burgdorferi*, which were prepared by methods known to yield either S-LPS or R-LPS from enterobacterial cells. This finding strongly suggests that the classic enterobacterium-type LPS is absent in cells of *B. burgdorferi*. It is possible that an LPS-like or a non-LPS compound might be present in these cells as it appears to be in *Bacteroides gingivalis* (8). It
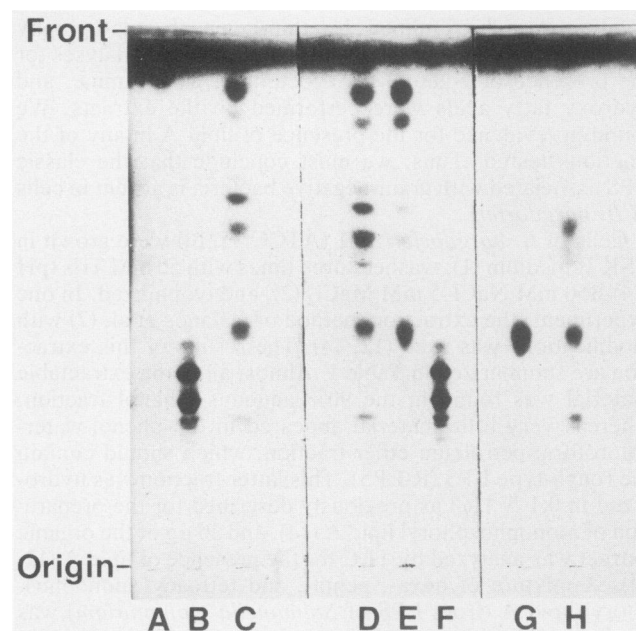

FIG. 1. Analytical TLC of acid-hydrolyzed aqueous phenol extracts of *B. burgdorferi*. The samples were hydrolyzed in 0.1 N HCl at 100°C for 30 min. The samples were spotted on a Silica Gel H (250-μm) plate and developed in chloroform-methanol-water-concentrated ammonium hydroxide (50:25:4:2). The plate was sprayed with sulfuric acid-dichromate reagent and charred to visualize the spots. Lanes: A, acid-hydrolyzed aqueous layer (100 μg); B and F, standard monophosphoryl lipid A mixture (40 μg); C, acid-hydrolyzed water-insoluble phenol layer (150 μg); E, unhydrolyzed water-insoluble phenol layer (chloroform-methanol extract) (150 μg); G, fraction E of 90% ethanol extract (50 μg); H, acid-hydrolyzed fraction E (50 μg). In lanes B and F, major bands in order of decreasing mobility are the standards, i.e., hexa-, penta-, and tetraacyl monophosphoryl lipid A.

could elicit some of the LPS-like biological activities, as shown by Beck et al. (4).

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