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Analyses of Gonococcal Lipopolysaccharide in Whole-Cell Lysates by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis: Stable Association of Lipopolysaccharide with the Major Outer Membrane Protein (Protein I) of *Neisseria gonorrhoeae*

PENNY J. HITCHCOCK

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

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The lipopolysaccharide (LPS) of *Neisseria gonorrhoeae* whole-cell lysates and proteinase K-digested lysates was examined and compared with purified homologous LPS by a method which preferentially stains LPS in polyacrylamide gels. The silver-stained profile of gonococcal LPS in the proteinase K-digested lysate was similar to that of homologous purified LPS; however, the LPS profile in whole-cell lysates was much smaller than that of digested lysates or purified LPS. Conditions of solubilization did not affect these differences. Since it is known that LPS migrates in a unique fashion in second-dimension electrophoresis, the location of LPS in the whole-cell lysates was probed by second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a variety of stains and radiolabels. Results from these experiments indicated a stable and reproducible association of LPS with proteins ranging between 23,000 to 36,000 in M_r, in particular major outer membrane protein I. In addition to staining with the silver method, which preferentially stains LPS, the putative LPS was resistant to digestion by proteinase K, did not stain with Coomassie brilliant blue, and was not labeled extrinsically with ^{125}I (Iodogen method) or intrinsically with [^{35}S]methionine. Analysis of two-dimensional gels by immunoblotting with rabbit antisera prepared from protein I bands removed from a polyacrylamide gel revealed the presence of antigens in the same area of the gel (below proteins that were 23,000 to 36,000 in M_r). Antibodies to constituents which migrated below the diagonal were essentially removed by adsorption of antisera with purified LPS, as were antibodies to homologous LPS and LPS in proteinase K-digested whole-cell lysates. Immunoblotting with a monoclonal antibody specific for LPS demonstrated reactivity of the antibody with LPS and with the protein I band. On the basis of these data, we conclude that protein I and perhaps other proteins in the whole-cell lysate are stably associated with LPS; this complex is resistant to dissociation in sodium dodecyl sulfate at high temperature (~100°C) but does, for unknown reasons, dissociate with electrophoresis in the second dimension. The association of LPS with protein antigens in sodium dodecyl sulfate-polyacrylamide gels adds another dimension of complexity to analysis of these antigens by immunoelectroblotting. Furthermore, the tight association of LPS with the major outer membrane protein I may alter the nature of the immune response generated by "purified" protein I vaccine antigens. The possible role of protein-LPS complexes in the pathogenesis of gonorrhea is discussed.

The outer membrane of gram-negative bacteria is composed of a mosaic of lipopolysaccharides (LPSs), proteins, and phospholipids. The outer membrane constituents associate so tightly (probably without the benefit of covalent bonds) that they form an impervious barrier capable of excluding many noxious substances (for a recent review, see reference 24). Even after organic solvent extraction, detergent lysis, heat treatment, ultracentrifugation, or chromatographic separation, LPSs and outer membrane proteins are often still associated (3, 15, 36, 37, 45, 46, 59, 87, 89). Copurification or contamination complicates biochemical characterization of individual membrane components and analyses of the antigenic character of each of the surface constituents.

The functional association of LPSs with major outer membrane proteins has been well characterized in *Salmonella* sp., *Escherichia coli*, and *Pseudomonas aeruginosa*, in which both LPSs and proteins are required for reconstitution of the outer membrane (50, 90, 91), for porin function (48, 49, 57, 58, 93), for colicin receptor function (6, 30, 33), for phage receptor function (OmpF, OmpC, and OmpA) (9, 21, 47, 93), and for conjugation receptor function (OmpA) (11, 61). Heat modifiability (of OmpA) is also thought to reflect association of this family of proteins with LPSs (53, 62). The topic of protein-LPS interactions has been reviewed recently (39; P. J. Hitchcock and D. C. Morrison, *in* E. T. Rietschel (ed.), *Handbook of Endotoxins*, vol. 1, in press).

The relationship between LPSs and the major outer membrane proteins of the gonococci, however, has not been investigated. The outer membrane of *Neisseria gonorrhoeae* is also composed of a mosaic of LPSs, proteins, and phospholipids (27, 56). The individual proteins of the outer membrane have been carefully studied. It is known that gonococci possess several major outer membrane proteins, designated protein I (P.I), P.II, and P.III (76); these have been well characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (18, 20, 27, 36, 68, 69, 74, 84). The three serotypes of P.I, the porin protein (92), ranging from 34,000 in M_r (34K) to 36 K, have also been characterized by peptide mapping, radioimmunoprecipitation, immunoblotting, and amino acid analysis (19, 29, 70, 73, 77). P.I is the dominant band of the whole-cell lysate in the Coomassie brilliant blue-stained polyacrylamide gels and in gel profiles of ^{125}I-surface-labeled (Iodogen) whole cells (17–19, 27, 70). P.IIs constitute a group of heat-modifiable proteins ranging from 26K to 28K (20, 35, 68, 69, 74, 84).

P.IIs are present in the outer membrane in positive correlation with the acquisition of colonial opacity; however, phenotypically transparent P.IIs bearing gonococci have recently been described (74). Structural and immunological analyses have also been carried out on P.IIs (72, 74, 75). PIII is thought to be common to the outer membrane of all gonococci, irrespective of strain or colony phenotype (51, 77). This 33K protein is readily modified by 2-mercaptoethanol and appears to be closely associated with P.I (28, 42, 51, 77).

The LPS of *N. gonorrhoeae*, also located in the outer membrane, probably constitutes up to 50% of the surface (by way of analogy with *E. coli* and *Salmonella* sp.). The LPSs of numerous strains of gonococci have been analyzed biochemically (16, 52, 56, 60, 67). Recently, Guymon et al. published photographs of silver-stained polyacrylamide gels containing gonococcal LPS (16). Gel profiles are consistent with biochemical analysis: gonococcal LPS lacks O antigen side chains, and the oligosaccharide core is likely to be similar in size to the Rc chemotype of *Salmonella* sp. (60).

Since the outer membrane proteins of *N. gonorrhoeae* are thought to be possible candidates for a gonococcal vaccine, we decided to explore the relationship between the gonococcal outer membrane proteins and LPS. In this report we present a study of the association between LPS and P.I. Gonococcal LPS, whole-cell lysates, and proteinase K-digested lysates were separated by one- and two-dimensional SDS-PAGE and analyzed by using a variety of stains, radiolabels, and antibodies. From the data presented, we conclude that LPS and proteins, in particular the major outer membrane P.I, seem to remain tightly associated even after being subjected to harsh dissociating conditions, i.e., the presence of SDS at high temperatures.

MATERIALS AND METHODS

Bacteria and culture conditions. *N. gonorrhoeae* JS1 was obtained from John Swanson (Laboratory of Microbial Structure and Function, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.), and *N. gonorrhoeae* FA1090 was obtained from Janne Cannon (School of Medicine, University of North Carolina at Chapel Hill). Organisms were grown at 36°C for 18 h in a moist atmosphere containing 5% CO_2 on gonococcal clear typing solid medium, originally described by James and Swanson (25) and modified by Swanson (73); Bacto-Agar (Difco Laboratories, Detroit, Mich.) was substituted for Noble agar. For extraction of LPS, gonococci were grown in defined liquid medium as described by Wong et al. (88). Nonpiliated, transparent organisms (P^- O^-) were used in all studies (71).

Radiolabeling of gonococci. For intrinsic labeling with radioactive sulfur (^{35}S), organisms were grown in 75 ml of Wong-Shockley-Johnston defined liquid medium, without methionine, in a shaking water bath at 37°C until mid-log phase (for approximately 3 h; measured by increased turbidity in a Klett-Summerson colorimeter with a blue filter). [^{35}S]methionine (100 μCi; Amersham Corp., Arlington Heights, Ill.) was added to the culture, and incubation was continued at 37°C for 40 min. Radiolabeled organisms were washed twice and solubilized as described below. Incorporation of radiolabel (^{35}S) was measured by trichloroacetic acid precipitation, and the amount of precipitated radioactive material was measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Organisms were extrinsically labeled with radioactive iodine (^{125}I) as described previously by Swanson (70).

Autoradiography of radioactive gels was carried out with Kodak A-R film (Eastman Kodak Co., Rochester, N.Y.).

Whole-cell lysates and proteinase K digestion. Organisms grown on solid medium were harvested with a sterile dacron swab and suspended in 10 ml of cold Dulbecco phosphate-buffered saline (12) to a turbidity of 100 Klett units (such a suspension commonly contains 5×10^8 to 6×10^8 organisms per ml, as measured by enumeration of CFU). A 1.5-ml amount of this suspension was microfuged for 1.5 min. The pellets were solubilized in 50 μl of lysing buffer containing 2% SDS (BDH [British Drug Houses], Poole, England), 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8), and bromphenol blue. Lysates were heated at 100°C for at least 10 min. Preparation of proteinase K-digested lysates has been described in an earlier publication (23). Briefly, after heating, whole-cell lysates were incubated at 60°C for 60 min with 25 μg of proteinase K solubilized in lysing buffer. (It should be noted that enzyme in lysing buffer is stored at −20°C before use; it should not be heated or allowed to incubate at room temperature before the addition of substrate.)

LPSs. LPS from *Salmonella typhimurium* LT_2, smooth chemotype (S), and G30/C21, deep rough chemotype (Re), were purified by the procedure of either Westphal and Jann (86) or Galanos et al. (13) and were the gift of Charles McLaughlin (Laboratory of Microbial Structure and Function). LPS from *N. gonorrhoeae* strains JS1 and FA1090 was purified by the Westphal procedure as modified by Perry et al. (52). All LPS preparations contained <1% protein as determined by the method of Lowry et al. (38).

SDS-PAGE. Preparations were subjected to SDS-PAGE by the Laemmli buffer system (34). The 4% stacking gel and the 12.5% separating gel did not contain SDS (M. Wycoff, R. Rudbard, and A. Chrambach, Fed. Proc. **35**:1383, 1976). Electrophoresis was carried out at a constant current of 35 mA with Tris-glycine (pH 8.3) plus 0.1% SDS buffer for approximately 2.5 h. Low-molecular-weight markers (Bio-Rad Laboratories, Richmond, Calif.) were used in several gels; the protein standards included phosphorylase (94K), bovine serum albumin (68K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (21K), and lysozyme (14.3K). Several gels were stained with 0.2% (wt/vol) Coomassie brilliant blue R250 (Fisher Scientific Co., Fairlawn, N.J.) in 25% (vol/vol) isopropanol–7% (vol/vol) acetic acid. For second-dimension electrophoresis (described previously [22]), the lane (5 by 70 mm) containing whole-cell lysate or LPS was excised from the first-dimension gel, placed on the second stacking gel, and electrophoresed into the gel. In some cases, the excised lane was placed in a sealed plastic bag with lysing buffer with or without 1 mg of proteinase K and heated at 60°C for 60 min. After incubation or proteolysis or both, the excised lane was placed on the second stacking gel and electrophoresed.

Silver stain. The silver stain methods used have been described in detail elsewhere (23). Both methods, that which preferentially stains LPS (referred to as LPS only) and that which stains both proteins and LPS, include a peroxidation step, extrapolated from the observations made by Tsai and Frasch (81).

Electroblotting. Electroblotting was performed with the buffer system of Towbin et al. (79) and Renart et al. (54), as modified by Barbour et al. (1). Electrophoretic transfer conditions were 15 V, <100 mA for 12 h at 18°C. After incubation with antisera, ^{125}I-radiolabeled protein A was used to identify antibody (immunoglobulin G) antigen complexes (also described by Barbour et al. [1]).

Antisera. Hyperimmune rabbit antiserum (αHRI) was elicited to P.I bands excised from polyacrylamide gels by the method of Caldwell and Schachter (5) and was the gift of Leonard Mayer (Laboratory of Microbial Structure and Function).

By the method of Wallace et al. (82), antiserum (αHRI) was adsorbed with LPS of strain JS1 (P^- O^-) by incubating 250 μl of antiserum with 250 μl of glass distilled water containing 1 mg of LPS (control antiserum was incubated with glass distilled water) at 37°C for 1 h. The mixture was centrifuged in a Microfuge B for 5 min. The adsorption was repeated a total of three times. Adsorbed αHRI and control were diluted to a final concentration of 1:200.

Monoclonal antibody (McAb) H10d was the gift of Janne Cannon. This McAb has been described in detail elsewhere (W. J. Black, I. Nachamkin, and J. Cannon, submitted for publication); it was elicited to gonococcal strain FA1090 and reacts with the purified LPS of this and other gonococcal strains; the degree of cross-reactivity with heterologous gonococcal LPSs varies from strain to strain. Adsorption of McAb H10d was done under conditions similar to those described above; however, homologous LPS was used.

The amount of antigen, concentration of antisera, autoradiographic exposure parameters, and photographic exposure and printing factors were identical for all immunoblotting experiments.

RESULTS

Comparison of gonococcal LPS in silver-stained polyacrylamide gels. The polyacrylamide gel profiles of LPS in whole-cell lysates and in proteinase K-digested lysates were compared with that of homologous purified LPS. Figure 1A shows paired samples of whole-cell lysates and proteinase K-digested lysates in a 12.5% polyacrylamide gel stained with the LPS-only method (10 μl of whole-cell lysate contains 30 μg of protein by the assay of Lowry et al. [38]). In each pair, the LPS stained in the whole-cell lysate was smaller, stained less intensely, and occupied a more circumscribed space compared with the LPS of the proteinase K-digested lysate. The pattern of proteinase K-digested lysate (30 μl) was very similar to that of 20 μg of purified homologous LPS. (Several RNase-sensitive bands were present in the proteinase K-digested lysate which were not affected by proteolysis; these bands did not stain with silver or Coomassie brilliant blue, nor did they transfer by electroblotting to nitrocellulose paper under conditions employed in this study [data not shown].) In the gonococcal LPS profile, diffuse grey staining material was present in the space between the slow single band and the discrete doublet (the doublet had similar electrophoretic mobility in both proteinase K-digested lysate and purified LPS). Compared with the salmonella LPS controls, the purified gonococcal LPS lacked the doublet ladder seen in the S LPS and was less electrophoretically mobile than the Re LPS. The same gel was secondarily stained with Coomassie brilliant blue (Fig. 1B). P.I (36K) was the dominant Coomassie brilliant blue-staining band. In the whole-cell lysate, Coomassie brilliant blue bands (Fig. 1B, Coomassie brilliant blue; arrows) occupied the previously unstained area (Fig. 1A, LPS only stain; arrow) adjacent to the LPS band.

Since it seemed likely that either the LPS of the whole-cell lysate was migrating near the dye front and not staining or that the LPS was migrating elsewhere in the gel and not staining because of association with other (protein) constituents, experiments were designed to encourage dissociation of the LPS from proteins in the undigested whole-cell lysate. However, solubilization in 2 or 4% SDS lysing buffer for up to 40 min at 100°C did not alter the LPS profile of the whole-cell lysate or proteinase K-digested lysate (data not shown).

Second-dimension SDS-PAGE analysis of whole-cell lysates. Two-dimensional electrophoresis should produce a series of bands which form a diagonal when the sample is unaltered by the intervening experimental condition, e.g., increased temperature. If the sample is affected by the variation in experimental conditions, bands of increased or decreased apparent molecular weight (for proteins) or with faster or slower migration patterns (for LPS) will appear above or below the diagonal. Since it had been demonstrated in previous studies (22) that LPS is somehow altered during

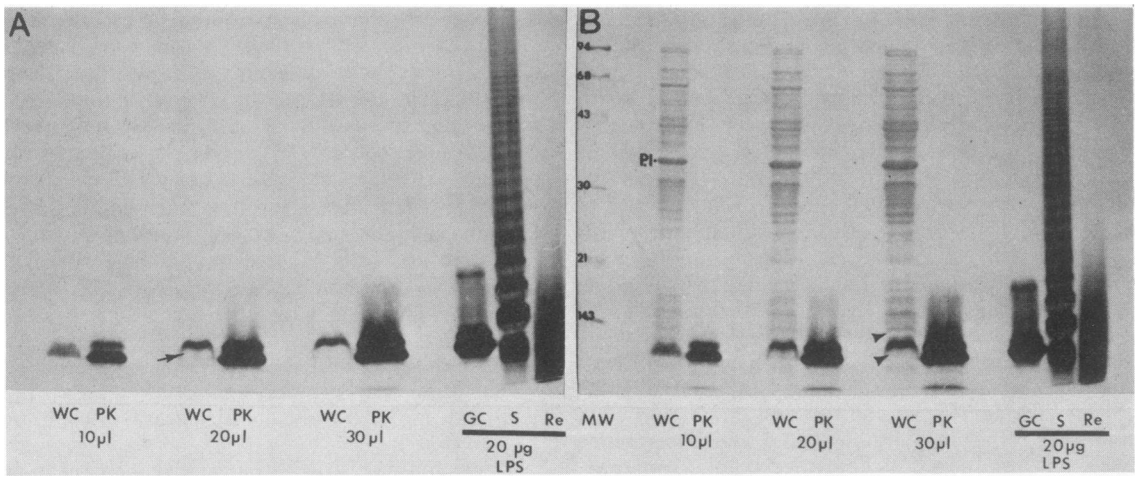

FIG. 1. (A) Paired samples of whole-cell lysates (WC) and proteinase K-digested lysates (PK) in an LPS-only silver-stained 12.5% polyacrylamide gel. In each pair, the LPS in the whole-cell lysate was smaller, stained less intensely, and occupied a more circumscribed area than the proteinase K-digested lysate. Purified LPSs from *N. gonorrhoeae* and *S. typhimurium* LT_2 smooth (S) and deep rough (Re) chemotypes are included. (B) The same gel was secondarily stained with Coomassie brilliant blue. P.I was the dominant staining band. Molecular weight protein standards (MW) were stained by Coomassie brilliant blue. In the whole-cell lysate, Coomassie brilliant blue bands (arrows) occupied the previously unstained area (see panel A, LPS-only stain, arrow) adjacent to the LPS.

electrophoresis and subsequently migrates aberrantly (and quite characteristically) in second-dimension SDS-PAGE, this technique was used to probe the location of LPS in the whole-cell lysates. Strain JS1 P^- O^- whole-cell lysates were electrophoresed in two dimensions as described in Materials and Methods. The gels were stained with Coomassie brilliant blue and silver stain for proteins and LPS (Fig. 2A and B, respectively). A first-dimension control (which was stained simultaneously) is juxtaposed to the second-dimension gel. In the Coomassie brilliant blue-stained gel (Fig. 2A), P.I was the dominant band. Some smearing of larger proteins occurred, the extent of which was affected by the width of the first-dimension gel strip (data not shown). Some smearing of P.III (first band below P.I in Fig. 2A) occurred (arrow); heating (100°C) of the gel strip with lysing buffer containing 2-mercaptoethanol for 10 min before second-dimension electrophoresis eliminated this smearing (data not shown). The silver method for proteins and LPS was used to stain the gels shown in Fig. 2B. The P.I band stained orange-amber with a darker brown bottom edge, and P.III (33K) was a dark brown band, the first prominent band below P.I in Fig. 2B. In the silver-stained gel, additional material which migrated aberrantly in the second dimension was stained. This additional material was seen as several grey spots which had greater electrophoretic mobility in second-dimension electrophoresis than in first-dimension electrophoresis. These spots all migrated at ca. 23K (assessing mobility based on protein molecular weight markers) and lined up between the two arrows marking the tail. It has been demonstrated that the behavior of LPS in second-dimension gels is quite characteristic; specifically, the LPS which migrates above the diagonal does not align with the LPS on the diagonal (22).

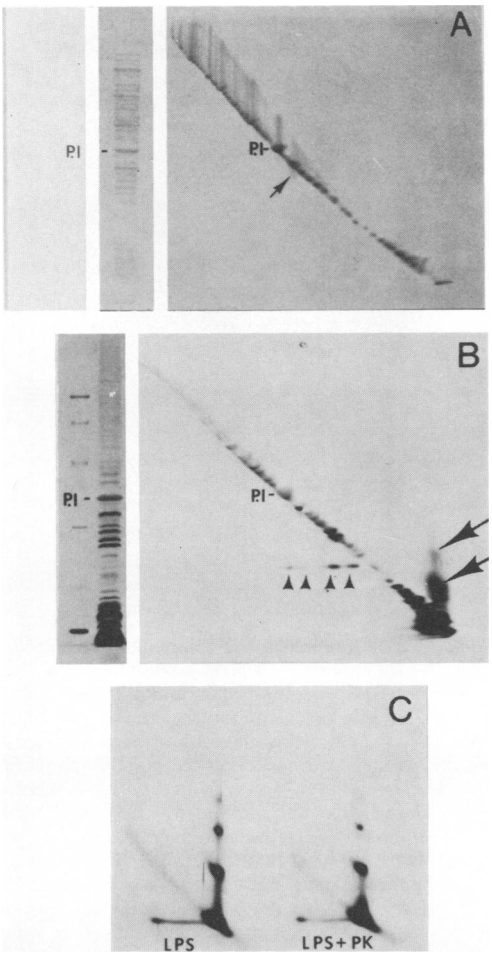

FIG. 2. Whole-cell lysates (WC) were electrophoresed in second-dimension 12.5% polyacrylamide gels. Coomassie brilliant blue and the silver stain for LPS and proteins, respectively, were used to stain the gels. A first-dimension control which was stained simultaneously is juxtaposed to the second-dimension gels. In the Coomassie brilliant blue-stained gel (A), P.I was the dominant band; some smearing of larger proteins occurred, the extent of which was affected by the width of the first-dimension gel strip. Some smearing of P.III occurred (arrow). The silver stain was used to stain the gels shown in panel B. P.I stained orange-amber, with a darker-brown bottom edge; P.III was a dark-brown band. Below the diagonal are several grey spots (arrowheads) which migrated at ca. 23K (assessing mobility based on protein molecular weight markers). Associated with the fastest-migrating material of the whole-cell lysate was an irregular, orange-brown and black tail (arrows). (C) The silver-stained gel contains purified LPS which was subjected to second-dimension electrophoresis with or without proteinase K (PK) digestion before first-dimension electrophoresis. Like the tail in the whole-cell lysate (B), LPS migrated above and below the diagonal; proteinase K digestion of the LPS did not affect its migration characteristics.

Associated with the fastest-migrating material of the whole-cell lysate was an irregular orange-brown and black "tail" (Fig. 2B, arrows), most of which was above the diagonal (less electrophoretically mobile in the second dimension); however, some material was seen below the diagonal. Since this material migrated near the dye front, as does gonococcal LPS, homologous purified LPS was subjected to second-dimension electrophoresis. A photograph of the silver-stained gel is shown in Fig. 2C. Like the tail in the whole-cell lysate, LPS migrated above and below the diagonal. The LPS above the diagonal was composed of discrete, silver-stained spots; antigenic analysis of purified LPS (by immunoelectroblotting) revealed a diffuse tail of LPS, non-discrete spots, migrating above the diagonal (data not shown). Proteinase K digestion of the LPS did not affect its migration characteristics.

Results from the second-dimension-stained gels (Fig. 2), specifically, the lack of staining by Coomassie brilliant blue, suggested that material which migrated aberrantly might not be proteinacious. To further investigate this possibility, proteins of strain JS1 (P^- O^-) were intrinsically labeled with [^{35}S]methionine and extrinsically labeled with ^{125}I, as described above. Figure 3A shows autoradiograms of one- and two-dimension polyacrylamide gels containing ^{35}S-labeled whole-cell lysates. The locations of P.I and P.III were determined by superimposing the dried Coomassie brilliant blue-stained gel on the autoradiogram. P.I did not label with [^{35}S]methionine, whereas P.III labeled very well. Figure 3B shows autoradiograms of surface-labeled gonococci (Iodogen method). P.I labeled well, as did P.III, with this method. The arrow corresponds to the location of P.III in its unmodified (without the influence of 2-mercaptoethanol) form (see Fig. 2A). Neither isotope was present in the aberrantly migrating material demonstrated in the silver-stained gel (Fig. 2B).

To further exclude the possibility of a proteinacious component, the effect of proteolysis on the material migrating above and below the second-dimension diagonal was assessed by incubating the first-dimension gel strip in SDS lysing buffer containing 1 mg of proteinase K, as described above, before second-dimension electrophoresis. The second dimension gel (Fig. 4A) was silver stained by the LPS-

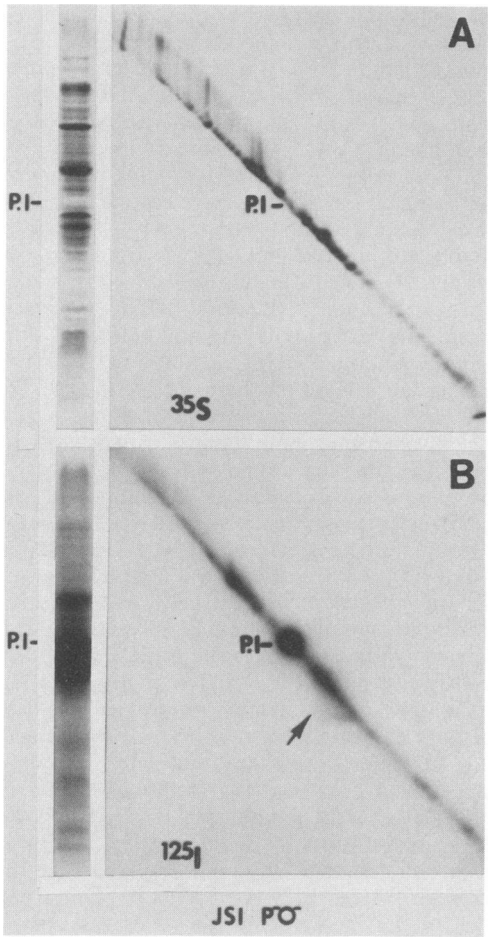

FIG. 3. Proteins of strain JS1 ($P^- O^-$) were labeled intrinsically with [^{35}S]methionine and extrinsically with ^{125}I. (A) Autoradiograms of first- and second-dimension polyacrylamide gels containing ^{35}S-labeled whole-cell lysates. The locations of P.I and P.III were determined by superimposing the dried, stained gel on the autoradiogram. P.I did not label with [^{35}S]methionine; however, P.III labeled well. (B) Autoradiograms of surface-labeled gonococci. P.I labeled well, as did P.III, with this method. The arrow corresponds to the location of P.III in its unmodified (without the influence of 2-mercaptoethanol) form (see panel A). Neither isotope was present in the aberrantly migrating material demonstrated in the silver stained gel (B).

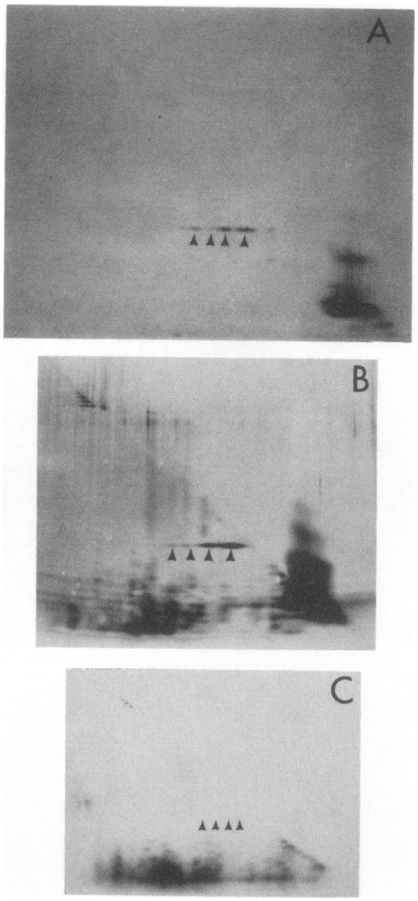

FIG. 4. (A) Second-dimension gel stained by the LPS-only silver method. The first-dimension gel strip containing whole-cell lysate was incubated in SDS-lysing buffer containing 1 mg of proteinase K before second-dimension electrophoresis. The aberrantly migrating material above and below the diagonal (arrows) was resistant to proteolysis and stained with the silver method, which preferentially stains LPS. The experiment was repeated with ^{35}S-labeled organisms. (B) Gel stained with the method which stains both LPS and protein. In addition to material seen in panel A (arrows), numerous spots of low M_r were seen below the diagonal. (C) Autoradiogram of this gel demonstrating that these spots were radioemitting and suggesting that they are products of proteolysis. Conspicuously absent from the autoradiogram are radioemitting spots which correspond to the tail and grey spots (arrows) in panels A and B.

only method. The material above and below the diagonal was resistant to proteolysis and stained with the silver method, which preferentially stains LPS. This experiment was repeated with ^{35}S-labeled organisms (Fig. 4B and C); however, the gel shown in Fig. 4B was stained with the silver method for proteins and LPS. The tail and the grey spots (arrows) at ca. 23K stained with silver; however, numerous smaller spots were also seen below the diagonal (cf. Fig. 4A). An autoradiogram of this gel (Fig. 4C) demonstrated that the smaller spots were radioemitting, suggesting that they are products of proteolysis. Conspicuously absent from the autoradiogram were radioemitting areas which correspond to the tail and to the grey spots (arrows), even though P.III labeled well with ^{35}S and was directly above one of moieties migrating at 23K.

Analysis of second-dimension SDS-PAGE by immunoelectroblotting. The whole-cell lysate of strain JS1 ($P^- O^-$) was electrophoresed in a second-dimension polyacrylamide gel and electrophoretically transferred to nitrocellulose paper by the method of Towbin et al. (79), modified as described above. The blots were incubated with αHRI raised against a P.I band removed from a polyacrylamide gel. The antiserum was adsorbed as described. In Fig. 5A and B, the autoradiograms of blots with unadsorbed αHRI and αHRI absorbed with LPS are shown. In addition to P.I, numerous constituents in the whole-cell lysate were recognized by αHRI (Fig. 5A). In the first dimension, whole-cell lysate, a band (~40K) slightly higher in M_r than P.I, as well as numerous bands of lower M_r, were recognized. In the second dimension, whole-cell lysate, the LPS tail, material directly below P.I, and material which migrated at ca. 23K (below proteins of 36K to 23K coincident with the silver-staining grey spots [Fig. 2B, etc.]) were recognized by αHRI. The blot shown in Fig. 5B was incubated with αHRI adsorbed with LPS (3×). When

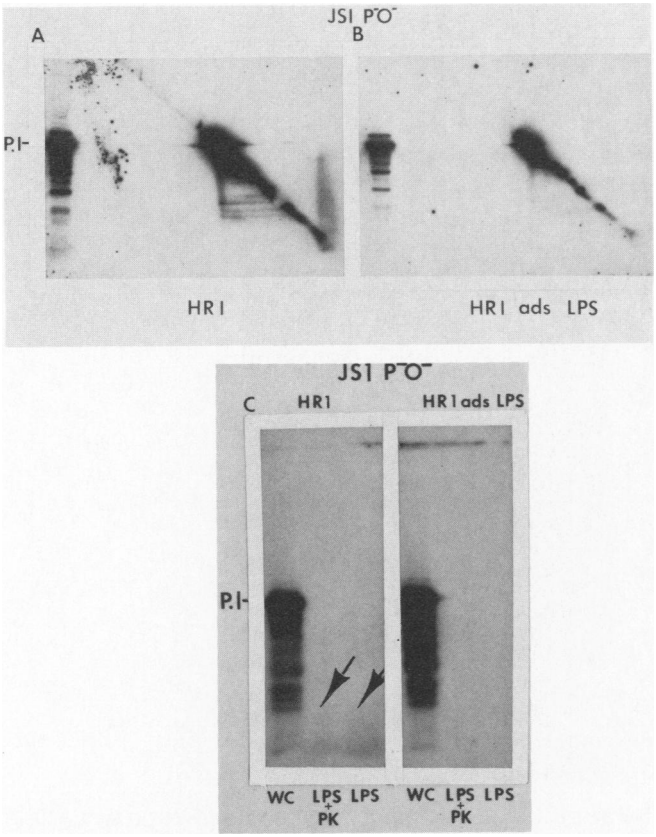

FIG. 5. Autoradiograms of blots with αHRI (HRI) and αHRI adsorbed with LPS (HRI ads LPS). In addition to P.I, numerous constituents in the whole-cell lysate (WC) were recognized by the αHRI. In the first-dimension whole-cell lysate (A), a band (~40K) slightly higher in M_r than P.I, as well as numerous bands of lower M_r were recognized. In the second-dimension whole-cell lysate (A), the LPS tail, material directly below P.I, and material which migrated at ca. 23K (below proteins of 36K to 23K) and coincident with the silver-staining grey spots were recognized by αHRI. (B) Blot incubated with αHRI adsorbed with homologous LPS (3×). Adsorption removed antibodies directed towards the LPS tail and most of the material below the diagonal. (C), Whole-cell lysate, homologous purified LPS, and LPS treated with proteinase K (LPS + PK) were subjected to SDS-PAGE and immunoblotting. Blots were reacted with αHRI and αHRI adsorbed with LPS. Antibodies which reacted with LPS (arrows) were removed by adsorption with homologous purified LPS.

the antibodies were adsorbed with LPS, the specificity of the antiserum changed; in the second dimension, whole-cell lysate antibodies directed towards the LPS tail and the 23K material below the diagonal were removed. In both blots, immunoglobulins were not associated with bands above PI except as noted (one band of ca. 40K).

Strain JS1 (P^- O^-) whole-cell lysate, homologous purified LPS, and LPS treated with proteinase K were subjected to SDS-PAGE and immunoblotting (Fig. 5C). Blots were reacted with αHRI adsorbed with LPS and αHRI control. Antibodies which reacted with LPS and proteinase K-treated LPS (arrows) were removed by adsorption with homologous purified LPS.

The nitrocellulose paper was also incubated with periodic acid or distilled water before being processed with buffer and αHRI. (Periodic acid modifies carbohydrate structure by converting vicinyl hyroxyl and ketone groups to aldehyde groups.) Periodate oxidation reduced the antigenicity of the material below the diagonal, the LPS tail of the whole-cell lysate, purified LPS, and LPS treated with proteinase K (data not shown).

Samples of ^{125}I-surface-labeled whole-cell lysates, unlabeled whole-cell lysates, and purified LPS from strains FA1090 and JS1 were electrophoresed in a polyacrylamide gel and transferred to nitrocellulose paper (Fig. 6A). The blot was then incubated with McAb H10d. In addition to the LPS, the McAb reacted with a band having an electrophoretic mobility identical to that of P.I (arrows). The McAb reacted strongly with homologous LPS purified or in the whole-cell lysate; the heterologous reaction was comparatively weak, and the McAb recognized only the purified LPS. Note that reactivity of McAb H10d with homologous and heterologous P.Is and LPSs are similar. Protein A controls did not bind to either constituent (data not shown). Samples from the same preparations were electrophoresed and transferred to nitrocellulose paper (Fig. 6B). The blot was incubated with McAb H10d which had been adsorbed with homologous LPS by the method of Wallace et al. (82). Adsorbtion removed antibodies which bound LPS and PI.

DISCUSSION

Results from these studies support several conclusions. First, LPS appears to be stably associated with proteins, in particular outer membrane P.I (and others ranging from 36K to 23K) in whole-cell lysates displayed by SDS-PAGE. The evidence for this association is derived from analyses of silver-stained grey spots which migrate aberrantly in second-dimension electrophoresis. These spots have the following characteristics: (i) refractory to staining by Coomassie brilliant blue; (ii) not surface labeled by ^{125}I (Iodogen method); (iii) not intrinsically labeled by [^{35}S]methionine; (iv) resistant to proteinase K digestion (in the presence of SDS); (v) stainable by LPS-only silver method; and (vi) antigenically cross-reactive with gonococcal LPS. In addition, McAb directed towards gonococcal LPS also reacted with P.I. Second, like the salmonella LPSs (23), SDS-PAGE of proteinase K-digested gonococcal whole-cell lysates results in a stainable gel profile that is very similar to that of homologous purified LPS. Third, gonococcal LPS moves aberrantly in second-dimension electrophoresis in a fashion similar to that of salmonella LPSs (22).

In previous studies with salmonella chemotypes (23), it has been shown that the LPS of whole-cell lysate stains to a lesser extent than does compared LPS of proteinase K-digested whole-cell lysate when stained by the LPS only method. The LPS which is stained by silver corresponds to an area which is refractory to staining by Coomassie brilliant blue. In this study, silver staining of gonococcal whole-cell LPS was significantly less than that of proteinase K-digested LPS (Fig. 1). Examination of autoradiograms of gels containing ^{125}I- and ^{35}S-labeled gonococcal whole-cell lysates (Fig. 3) revealed the presence of numerous labeled (protein) bands in the area adjacent to the silver-staining LPS (LPS-only method). Furthermore, Coomassie brilliant blue bands were located next to the silver-stained LPS (Fig. 1). Thus it was concluded that, like the Salmonella chemotypes, gonococcal LPS which migrates adjacent to proteins stains differently by the method which preferentially silver stains LPS, because of either altered migration due to compression of the LPS by proteins or protein interference with staining. However, by use of second-dimension electrophoretic analyses and by taking advantage of the fact that LPS has unusual migration characteristics in second-dimension SDS-PAGE, it was pos-

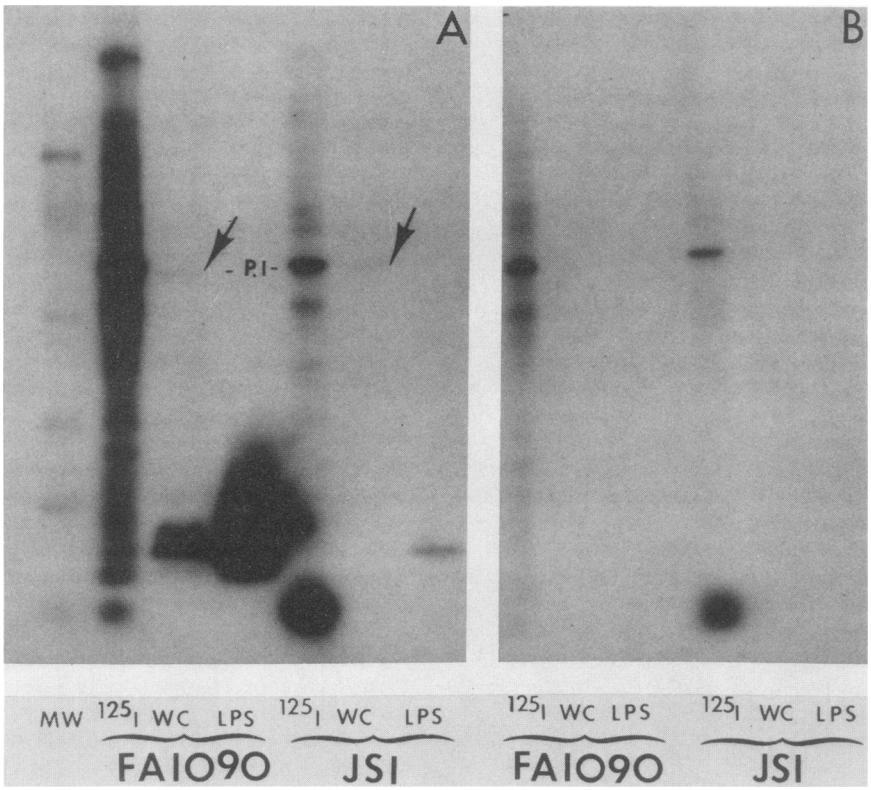

FIG. 6. Autoradiograms of blots with McAb H10d (A) and H10d adsorbed with homologous LPS (B). (A) McAb bound well to the FA1090 LPS of the whole-cell lysate (WC) and the purified LPS; in addition, a band corresponding to P.I was recognized by the McAb (arrows). The McAb cross-reacted to a lesser degree with the purified LPS of strain JS1 and P.I of this strain. (B) Adsorbed McAb H10d did not recognize the LPS or the P.Is of either strain. ^{125}I-labeled whole-cell lysates (^{125}I) are included, as well as ^{125}I-labeled molecular weight markers (MW) as controls.

sible to probe the location of additional LPS molecules in the whole-cell lysate. In regard to the [^{35}S]methionine labeling data, it should be noted that Blake and Gotschlich (2) have published the P.I amino acid composition of two strains of gonococci, MS11 and R10, both of which have four methionines. Their data are in contrast with the fact that P.I did not label with [^{35}S]methionine in these experiments. Whether the discrepancy is due to differences in gonococcal strains or methods of culture is unclear.

In previous studies with salmonella chemotypes (22), it has been demonstrated that LPS migrates aberrantly in second-dimension electrophoresis. Numerous LPS species migrate both slower and faster than the parent material found on the diagonal. The exact nature of these LPS molecules and the reason for their aberrant migration characteristics are unknown; however, this finding undoubtedly reflects physical or chemical alteration of the LPS during electrophoresis and is the subject of further investigation in this laboratory. Preliminary evidence from other studies of encapsulated bacteria has indicated that carbohydrate moieties may be essential for this migration characteristic. Regardless of the mechanism, this migration characteristic of LPS seems to be useful to identify and characterize LPS of unknown phenotype in a whole-cell lysate. Examination of gonococcal whole-cell lysates with second-dimension SDS-PAGE revealed the presence of aberrantly migrating material. The electrophoretic mobility of this 23K species overlapped that of purified LPS (Fig. 2C). The silver-stained second-dimension profile of purified LPS was similiar to the material seen below the diagonal in that both comprised discrete spots; however, the spots aligned between the silver-stained spots of the tail. In this respect, gonococcal LPS behaved identically with salmonella LPSs in second-dimension electrophoresis (22). The sensitivity of the silver stain is considerably less than the electroblot-immunological assay; in Fig. 5, the LPS tail and the material which migrated below the diagonal are demonstrated much more extensively in the immunoelectroblot than in the silver-stained gel (Fig. 2B). The discrete staining spots appeared as a continuous smear below the 36K to 23K proteins when detected by antiserum and ^{125}I-labeled protein A.

In addition to having staining characteristics, labeling affinities, and resistance to proteolysis consistent with those of LPS, the antigenic cross-reactivity of this aberrantly migrating material with homologous purified LPS was demonstrated by the results of adsorption experiments (Fig. 5). The fact that antisera elicited against a P.I band contained high titers of anti-LPS antibody is consistent with the hypothesis that LPS is associated with P.I in SDS-polyacrylamide gels. This hypothesis is further supported by cross-reaction of LPS McAb H10d specifically with P.I of the whole cell lysate.

All experimental parameters were carefully controlled in experiments in which αHRI and adsorbed αHRI were used; it is therefore significant that adsorption of αHRI with LPS measurably reduced but did not eliminate antibodies which recognized protein bands below P.I in the first-dimension gel. The reasons for this are unclear, but several possible

explanations are that (i) proteolysis of P.I (perhaps owing to endogenous proteases) results in fragments of lower M_r, (ii) there is antigenic cross-reactivity with smaller proteins, or (iii) adsorption of anti-LPS antibodies is incomplete. Cross-reactivity seems less likely, since almost none occurred with proteins migrating above P.I (except for the 40K band noted earlier). If it is the case that P.I fragments migrate at lower positions in the gel, it complicates analysis of proteins of lower M_r by SDS-PAGE either in terms of their antigenic character, e.g., immunoblotting, or their structure, e.g., peptide mapping (27, 38, 49, 70, 72, 73, 75). The third possibility, incomplete adsorption, cannot be ruled out, although reactivity of antiserum adsorbed 1× was similar to that of antiserum adsorbed 3×.

The reason that McAb H10d did not react with proteins below P.I is also unclear. It is possible that the LPS molecules which are associated with proteins represent unique species within a heterogeneous population of LPS molecules. Careful purification, separation, and analysis of LPS and LPS-protein complexes will be required to clarify such possibilities.

It is possible, although unlikely, that these aberrantly migrating moieties may be nucleic acid. This has been addressed in these studies by the use of nucleases which do not diminish staining or antigenicity of the aberrantly migrating material. Furthermore, controls of nucleic acid do not stain, nor do they transfer by the silver stain or immunoblotting methods employed in this study.

Finally, the possibility exists that these moieties are not LPS but are heretofore-undescribed constituents of the bacteria which copurify, stain, and immunologically cross-react with LPS. Again, purification is necessary to address these alternative explanations.

The precedents for the association of LPS and outer membrane proteins from both a structural and functional point of view are well documented in the literature dealing with *Salmonella* sp. and *E. coli* (6, 9, 11, 21, 30, 33, 47–50, 53, 57, 58, 61, 62, 90, 91, 93; for recent reviews, see reference 39 and Hitchcock and Morrison, in press). Reconstitution studies by Nakamura and Mizushima (50) have demonstrated that the association of LPS with outer membrane proteins is so specific that cytoplasmic membrane proteins cannot be substituted to form membranous vesicles with LPS. Numerous (more than 100) papers have been published on protein LPS interactions in the last 15 years. Although it is beyond the scope of this discussion to review this literature, it will suffice to say that from the "viewpoint" of the bacterium, the outer membrane is a functional unit, and regardless of whether the components are covalently bound, dissociation of proteins and LPS is not a simple matter.

It may be the case that LPS and outer membrane proteins are chemically associated in a unique way. Harlan Caldwell (Laboratory of Microbial Structure and Function) and I have recently determined that extraction of chlamydial LPS is facilitated by reduction and alkylation of the disulfide bonds of major outer membrane protein; otherwise, the LPS and the major outer membrane protein partition into the interface of the phenol-water mixture (4). Perhaps each species of bacteria has a particular kind of chemical interaction between LPS and major outer membrane protein; disruption of this chemical association might facilitate extraction of LPS.

This study presents evidence to suggest that strong LPS-protein interaction occurs in *N. gonorrhoeae*. Boiling of whole-cell lysates in an anionic detergent is not sufficient to interrupt this interaction. What might be, from a clinical standpoint, the significance of human exposure to protein-LPS complexes? To speculate on this, one must take several factors into consideration. First, in *N. gonorrhoeae* and *N. meningitidis*, the absence of lipoprotein (M. Inouye, unpublished data) is thought to account for blebbing of numerous outer membrane vesicles (comprised of proteins and LPS) in vitro and, most likely, in vivo as well (10, 40, 56). It is therefore probable that the infected human host is constantly exposed via these blebs to protein-LPS complexes. Second, a number of studies aimed at determining the presence and function of serum and secretory anti-gonococcal antibodies have been carried out. In most patients, both local and systemic gonorrheal infections elicit serum and secretory antibodies which are usually not protective against subsequent infection (7, 8, 32, 43, 44, 78, 80, 83).

The reasons for this lack of protection are unknown. The presence and role of blocking antibodies have been the subject of investigation by several investigators (41). The role of LPS in the induction of immunological tolerance must also be considered. Sjöberg and co-workers have demonstrated that multiple small injections of LPS into adult mice result in immunological tolerance to challenge with bacterial vaccines; in their studies, the tolerance persisted for 36 days (63, 65, 66). Furthermore, when gastrointestinal mucosa is exposed to foreign proteins in association with LPS, tolerance is induced (85). On the other hand, the presence of small amounts of LPS in protein antigens may markedly enhance the antibody response to both antigens (14, 26, 64). Also, carbohydrate antigens seem to modify the immunoglobulin subclass elicited against a given antigen (31, 55), and the efficacy of bactericidal and opsonic functions may vary among immunoglobulin subclasses.

In summary, the significance of human exposure to protein-LPS complexes via vaccines or natural infection depends upon the role of LPS antibodies in the prevention or the pathogenesis of gonorrhea. For better or for worse, the presence of LPS in P.I vaccine antigen is likely to influence its efficacy.

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