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# Coprecipitation of Lipopolysaccharide and the 39,000-Molecular-Weight Major Outer Membrane Protein of *Haemophilus influenzae* Type b by Lipopolysaccharide-Directed Monoclonal Antibody

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**The major outer membrane protein of *Haemophilus influenzae* type b (Hib) with an apparent molecular weight of 39,000 (39K) was purified from three different Hib strains and was shown to be free from detectable contamination with other proteins. However, these purified 39K protein preparations were found to contain Hib lipopolysaccharide (LPS). Immunization of rats with these 39K protein preparations resulted in the production of antisera containing both 39K protein-directed and LPS-directed antibodies, as determined by Western blot analysis. The reactivity pattern of the LPS-directed serum antibodies with different Hib strains was identical to the reactivity of these Hib strains with a set of monoclonal antibodies (mabs) previously shown to immunoprecipitate the 39K protein in a radioimmunoprecipitation (RIP) system. Examination of the antigenic specificities of the 39K protein-immunoprecipitating mabs by using Western blot analysis showed that these mabs were actually directed against Hib LPS. RIP analysis of $^{125}$I-labeled Hib cells and $^{32}$P-labeled Hib cells revealed that the 39K protein and LPS existed as a complex in a RIP system, which resulted in the coprecipitation of both antigens by LPS-directed mabs. The interaction between LPS and the 39K protein was highly selective for this protein and did not involve other outer membrane proteins. The LPS/39K protein complex could be reconstituted by mixing purified LPS and purified 39K protein; it could also be reconstituted with 39K protein from one Hib strain and LPS from another Hib strain. These findings have necessitated the reinterpretation of previous studies involving the 39K protein-immunoprecipitating mabs. Of primary importance is the fact that the demonstrated immunoprotective ability of a 39K protein-immunoprecipitating mab (E. J. Hansen, S. M. Robertson, P. A. Gulig, C. F. Frisch, and E. J. Haanes, Lancet i:366–368, 1982) must now be regarded as evidence that antibody directed against Hib LPS can be protective against experimental Hib disease.**

Although it has been well established that antibodies directed against the capsular polysaccharide of *Haemophilus influenzae* type b (Hib) are protective against disease caused by this pathogen (3, 19, 31), it has also been shown that antibodies directed against noncapsular Hib surface antigens are protective against experimental Hib disease (13, 18, 24). The principal somatic antigens of the outer membrane of Hib are lipopolysaccharide (LPS) and proteins. We developed a radioimmunoprecipitation (RIP) procedure to identify Hib outer membrane proteins which are both immunogenic and accessible to antibody on the Hib cell surface (6). This whole cell-RIP system was used to study the immune responses of both human infants (5) and laboratory animals (6) after systemic Hib disease. In addition, this whole cell-RIP system was used to identify monoclonal antibodies (mabs) which immunoprecipitated Hib outer membrane proteins. A mab which immunoprecipitates a major outer membrane protein with an apparent molecular weight of 39,000 (39K) was identified in this manner (20) and has been shown to be protective against experimental Hib disease (8). In addition, a set of four 39K protein-immunoprecipitating mabs was used to divide Hib strains into several different antigenic groups (4).

We report here that four of the previously described mabs which immunoprecipitate the 39K protein are actually directed against Hib LPS. The ability of these LPS-directed mabs to immunoprecipitate the 39K protein in the whole cell-RIP system is based on a noncovalent and highly selective interaction between Hib LPS and the 39K protein. This finding, together with the demonstrated protective ability of one of these LPS-directed mabs against experimental Hib disease (8), has considerable significance with regard to the possible use of Hib LPS as a source of vaccine components.

## MATERIALS AND METHODS

**Bacterial strains and culture media.** The four Hib strains representative of antigenic groups 1 (DL26), 2 (DL42), 3 (DL41), and 4 (NO100) have been described previously (4). Information relevant to the present study is provided in Table 1. The bacterial culture media (brain heart infusion broth supplemented with Levinthal base) and conditions used for bacterial culture were described previously (7).

**Purification of the 39K protein.** The purification procedure of Munson et al. (18), which is based on the differential solubility of the 39K protein in various detergents, was used to purify the 39K proteins of Hib DL26, DL42, and DL41.

**Purification of LPS.** Hib LPS was purified by two different procedures. One preparation of LPS from Hib DL26 was purified by R. S. Munford by the phenol-chloroform-petroleum ether method of Galanos et al. (1). The second set of LPS preparations was purified from Hib DL26 and Hib DL42 by the hot phenol-water method of Johnson and Perry (11). Comparison by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the DL26

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TABLE 1. Characteristics of Hib strains and mabs used in this study

| Mab | Reactivity in: | | Reactivity with Hib strain (antigenic group)[c] | | | |
|---|---|---|---|---|---|---|
| | RIP analysis[a] | WB analysis[b] | DL26 (1) | DL42 (2) | DL41 (3) | NO100 (4) |
| 12D9/1 | 39K protein | LPS | + | - | + | - |
| 6A2/1 | 39K protein | LPS | + | - | + | - |
| 4C4/2 | 39K protein | LPS | - | + | + | - |
| 5G8/2 | 39K protein | LPS | - | + | + | - |
| 7B2 | 27K protein | 27K protein | + | + | + | ND[d] |
| 6G12 | 98K protein | 98K protein | - | + | + | ND |

[a] $^{125}$I-labeled antigen immunoprecipitated in the whole-cell- or PS-RIP system.
[b] Antigen which bound the mab in Western blot (WB) analysis.
[c] Antigenic groups are defined by the reactivity of these Hib strains with the 39K protein-immunoprecipitating mabs 12D9/1 and 4C4/2 as previously described (4).
[d] ND, Not determined.

LPS preparation derived by using the phenol-chloroform-petroleum ether method with that prepared by the hot phenol-water method showed that the LPS samples were identical in their purity and composition (see below). Purified smooth and rough LPS standards from *Salmonella typhimurium* were also provided by R. S. Munford (17).

**SDS-PAGE, staining procedures, and autoradiography.** Two different SDS-PAGE systems were used in this study. For optimal resolution of LPS, 10 to 20% polyacrylamide gradient SDS gels were used. The separating gel consisted of a linear gradient of 10% (wt/vol) polyacrylamide, 0.9% (wt/vol) bisacrylamide, and 0% glycerol increasing to 20% (wt/vol) polyacrylamide, 1.8% (wt/vol) bisacrylamide, and 50% (vol/vol) glycerol. This separating gel also contained 0.375 M Tris hydrochloride (pH 8.8) and 0.1% (wt/vol) SDS. The stacking gel contained 4% (wt/vol) polyacrylamide as previously described (7). The running buffer consisted of 0.025 M Tris hydrochloride (pH 8.3), 0.192 M glycine, and 0.1% (wt/vol) SDS. The sample buffer consisted of 0.0625 M Tris hydrochloride (pH 6.8), 0.1% (wt/vol) SDS, 10% (vol/vol) glycerol, and a trace of pyronin Y. The SDS-PAGE system used for the analysis of protein samples has been described previously (7). Coomassie brilliant blue staining was used for the visualization of proteins in gels (7). The silver stain of Tsai and Frasch (28) was used for the staining of LPS. Autoradiography of radiolabeled antigens resolved by SDS-PAGE has been described before (7).

**Immunization with 39K protein preparations.** Adult Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, Tex.) were given a primary immunization by intraperitoneal injection of 100 μg of purified 39K protein from Hib DL26, DL42, or DL41 mixed with complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). Four weeks later, the rats were given a second intraperitoneal injection with 100 μg of the same purified 39K protein in incomplete Freund adjuvant (Difco). Two further intraperitoneal injections of 100 and 25 μg of this 39K protein in pH 7.2 phosphate-buffered saline (PBS) were administered at 2-week intervals thereafter. The rats were bled by cardiac puncture 2 weeks after the final injection, and sera were prepared from the blood by standard methods. Control rats were immunized with the appropriate adjuvant mixed with PBS.

**Immunization with purified Hib LPS.** Adult rats were given a primary immunization by intraperitoneal injection of 20 μg of purified DL26 LPS suspended in incomplete Freund adjuvant. At 2, 3, and 4 weeks postimmunization, the animals received additional intraperitoneal injections of 20 μg of LPS suspended in PBS. Ten days after the last injection of LPS, blood was drawn for serum preparation.

**Monoclonal antibodies.** Mabs 12D9, 4C4, 6A2, and 5G8, which are used in the antigenic grouping system for Hib, have been previously described (4). For purposes of clarity, the antigenic group of the homologous Hib strain (against which each LPS-specific mab was raised) is affixed to the designation of the mab throughout this report (e.g., mab 12D9 is designated 12D9/1 because it was raised against group 1 Hib DL26). Mab 6G12, which is directed against the 98K outer membrane protein of Hib, is described elsewhere (13). Mab 7B2 is directed aginst the 27K outer membrane protein of Hib (P. L. Holmans, T. A. Loftus, and E. J. Hansen, manuscript in preparation). The relevant characteristics of these mabs are summarized in Table 1. Culture supernatant fluid from the plasmacytoma SP2/0-Ag14 was used as a negative antibody control in some experiments.

**RIP analyses.** The presolubilized (PS)-RIP system for use with solubilized, $^{125}$I-labeled Hib cells has been described elsewhere (5, 6). For use in the RIP analysis of LPS, Hib cells were intrinsically radiolabeled with [$^{32}$P]phosphoric acid by the method of Hitchcock and Brown (9). The culture medium for the latter experiment was depleted of inorganic phosphate by raising the pH to 10 with NaOH, adding 3.5 M $MgCl_2$ until precipitation ceased, and removing the precipitates by centrifugation. The media were adjusted to pH 7.3 with HCl and filter sterilized before use. Hib strains were grown overnight in 15 ml of phosphate-depleted medium containing 1.3 mCi of [$^{32}$P]phosphoric acid (ICN Pharmaceuticals Inc., Irvine, Calif.; specific activity, 285 Ci/mg). Cells were harvested, washed three times with PBS, solubilized, and then used as antigen in the PS-RIP system.

**Western blot analyses.** Electrophoretic transfer of antigens from SDS-PAGE gels to nitrocellulose membranes was performed as described by Towbin et al. (27). The basic

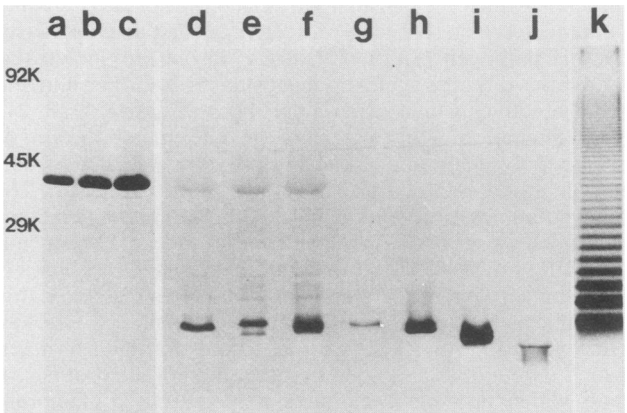


FIG. 1. Protein and LPS content of purified 39K protein preparations. Purified 39K protein preparations and LPS were resolved by SDS-PAGE in 10 to 20% polyacrylamide gradient gels and stained with either Coomassie brilliant blue (lanes a to c) or silver stain (lanes d to k). Lanes a to c: 20 μg of purified 39K protein from group 1 Hib, group 2 Hib, and group 3 Hib, respectively. Lanes d to f: 10 μg of purified 39K protein from group 1 Hib, group 2 Hib, and group 3 Hib, respectively. Lane g: 1.0 μg of LPS from group 1 Hib purified by the method of Galanos et al. (1). Lanes h to j: purified LPS of Ra (molecular weight, 4,200), Rc (molecular weight, 3,100), and Re (molecular weight, 2,600) rough mutants, respectively, of *S. typhimurium*. Lane k: purified LPS from a smooth strain of *S. typhimurium*. Molecular weight markers appear on the left.

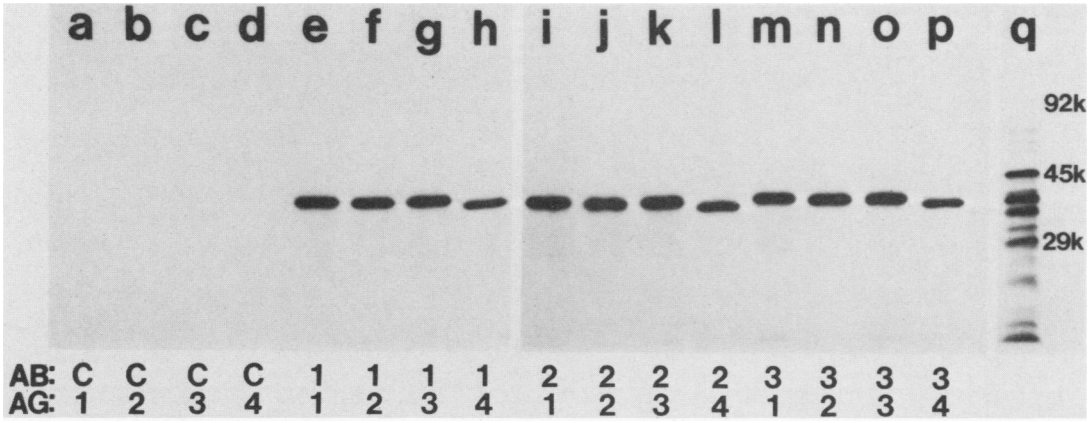


FIG. 2. RIP analysis of anti-39K protein sera. Each of the anti-39K protein sera was reacted with solubilized, radioiodinated Hib cells from antigenic groups 1 to 4 and then processed in the RIP system. Radiolabeled proteins in the immune precipitates were resolved by SDS-PAGE in 10% polyacrylamide gels and identified by autoradiography. AB, antigenic group classification of the Hib strain against which the anti-39K protein serum was raised; AG, antigenic group classification of the Hib strain used as antigen in RIP analysis; C, control serum. Lane q contains a sample of radioiodinated and solubilized cells of Hib DL42, provided as a reference for radiolabeled outer membrane proteins.

procedure for the probing of antigens on nitrocellulose membranes was (i) presoak in buffer for 1 h; (ii) incubation in primary antibody for at least 4 h at room temperature; (iii) three washes (20 min each) in washing buffer; (iv) incubation in a 1:1,000 dilution of secondary antibody (horseradish peroxidase-coupled goat anti-mouse immunoglobulin G [Cappel Laboratories, Cochranville, Pa.]) overnight at 4°C; (v) washes as in step iii; (vi) incubation in a 1:1,000 dilution of tertiary antibody (horseradish peroxidase-coupled rabbit anti-goat immunoglobulin [Cappel]) for 2 h at room temperature; (vii) washes as in step iii; and (viii) color development by incubation of the membranes in 0.05 M Tris hydrochloride (pH 7.4) containing 0.20 M NaCl, 0.5 mg of 4-chloro 1-naphthol per ml, 3.3% (vol/vol) methanol, and 0.01% (vol/vol) hydrogen peroxide. Color development was stopped by rinsing the membranes in water.

Two derivatives of this basic procedure were used to detect LPS-specific and protein-specific antibodies. For optimal detection of antibodies directed against LPS, presoaking (blocking) of nitrocellulose membranes containing transferred antigens was performed in PBS containing 2.5% (wt/vol) bovine serum albumin, all antibodies were diluted in PBS-bovine serum albumin, and the washing buffer consisted of PBS. For optimal detection of antibodies directed against protein antigens, buffers for presoaking, for antibody dilution, and for washing consisted of PBS containing 0.01% polyoxyethylene sorbitan monolaurate (Tween 20).

**Lithium chloride-based extraction of outer membrane vesicles.** The LiCl-based extraction of outer membrane vesicles from Hib cells has been described elsewhere (5, 16).

## RESULTS

**SDS-PAGE analysis of purified 39K protein preparations.** The 39K proteins from Hib strains of antigenic groups 1, 2, and 3 (4) were purified by the procedure of Munson et al. (18). When resolved by SDS-PAGE and stained with Coomassie brilliant blue, these purified 39K protein preparations were found to be essentially free of contamination with other proteins (Fig. 1, lanes a to c). However, upon analysis of these SDS-PAGE-resolved 39K protein preparations with the silver stain of Tsai and Frasch (28), which stains primarily carbohydrates, it was apparent that they possessed LPS contamination (Fig. 1, lanes d to f). Munson et al. (18) similarly found that their purified 39K protein preparations were contaminated with LPS. To verify that these low-molecular-weight silver stain-positive bands were Hib LPS, purified LPS from group 1 Hib was included as a standard (Fig. 1, lane g). The Hib LPS had an electrophoretic mobility similar to that of the LPS of an Ra mutant of *S. typhimurium*; this latter LPS molecule contains a complete core polysaccharide but lacks O-antigen units (Fig. 1, lane h).

**RIP analysis of anti-39K protein sera.** The three purified 39K protein preparations were used to immunize adult rats, and the resultant anti-39K protein sera were examined for 39K protein-specific antibody activity by using solubilized $^{125}$I-labeled Hib cells from antigenic groups 1 through 4 in the PS-RIP procedure. All three anti-39K protein sera immunoprecipitated the radioiodinated 39K protein from all four antigenic groups (Fig. 2, lanes e to p). It should be noted that the group 4 Hib (Fig. 2, lanes h, l, and p) has a 38K protein instead of a 39K protein (4). Because this difference is irrelevant to the present study, this 38K protein will be referred to as a 39K protein in this report. Control rat serum did not contain any detectable anti-39K protein activity (Fig. 2, lanes a to d).

**Western blot analysis of the anti-39K protein sera.** Because the purified 39K protein preparations were contaminated with LPS and because LPS is not labeled with $^{125}$I by the lactoperoxidase-catalyzed radioiodination procedure, we examined these antisera for LPS-directed antibody activity by using Western blot analysis. Antigens contained in LiCl-extracted outer membrane vesicles of strains from antigenic groups 1 through 4 were resolved by SDS-PAGE in 10 to 20% polyacrylamide gradient gels, electrophoretically transferred to nitrocellulose paper, and probed with the different anti-39K protein sera. Both the 39K protein and LPS were recognized by each of the homologous antisera in this experiment (Fig. 3, lanes e, j, and o). Therefore, the anti-39K protein sera did contain LPS-directed antibodies raised against the LPS present in these 39K protein preparations.

The antigenic cross-reactivity of LPS among Hib strains from the four antigenic groups was also examined by using these anti-39K protein sera in Western blot analysis. As expected from the immunoprecipitation results depicted in Fig. 2, each anti-39K protein serum reacted with the 39K protein of every strain tested (Fig. 3, lanes e to p). However, the reactivity of these anti-39K protein sera with the LPS of

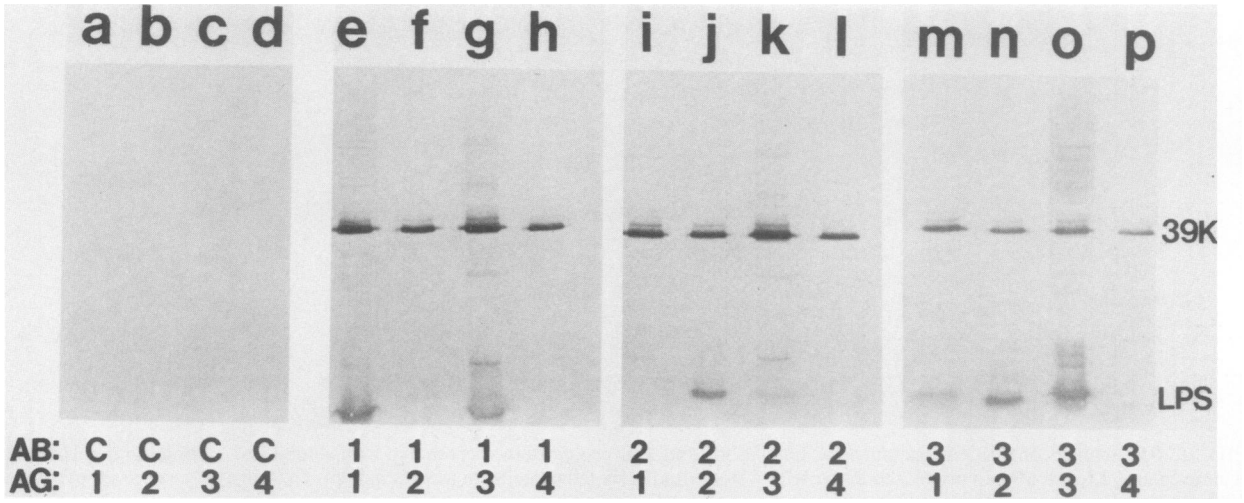


FIG. 3. Western blot analysis of anti-39K protein sera. LiCl-extracted outer membrane vesicles of Hib strains from antigenic groups 1 to 4 were resolved by SDS-PAGE in 10 to 20% polyacrylamide gradient gels, electrophoretically transferred to nitrocellulose membrane, and probed with anti-39K protein sera. The antibody and antigen combinations (lanes a to p) are identical to those described in the legend to Fig. 2. AB, antigenic group classification of the Hib strain against which the anti-39K protein serum was raised; AG, antigenic group classification of the Hib strains used as antigen in Western blot analysis; C, control serum. The positions of the 39K protein and LPS in this Western blot are indicated by the markers on the right side of the figure.

homologous and heterologous Hib strains yielded a distinctive pattern. That is, anti-group 1 39K protein serum reacted with LPS from groups 1 and 3 (Fig. 3, lanes e and g), anti-group 2 39K protein serum reacted with LPS from groups 2 and 3 (Fig. 3, lanes j and k), and anti-group 3 39K protein serum reacted with LPS from groups 1, 2, and 3 (Fig. 3, lanes m, n, and o). In addition, the anti-group 3 39K protein serum reacted slightly with the LPS of the group 4 strain (Fig. 3, lane p). With the exception of this latter result, this pattern of reactivity with LPS obtained with these antisera was identical to the pattern previously observed with the set of mabs which immunoprecipitate the 39K protein (4; Table 1). That is, group 1-directed mabs react with only group 1 and 3 Hib, whereas group 2-directed mabs react with only group 2 and 3 Hib.

**Western blot analysis of the 39K protein-immunoprecipitating mabs.** The fact that the cross-reactivity of the LPS-directed antibody in the anti-39K protein sera with Hib strains from antigenic groups 1, 2, and 3 (Fig. 3) matched that of the 39K protein-immunoprecipitating mabs (Table 1) led us to reexamine by Western blot analysis the antigenic specificity of these mabs, which had originally been characterized by RIP analysis (20). Anti-group 1 39K protein serum was included in this experiment as a positive control for antibodies directed against the 39K protein and LPS (Fig. 4, lane a). These 39K protein-immunoprecipitating mabs were found to bind to LPS but not to the 39K protein of the homologous Hib strains (Fig. 4). This result is illustrated for mab 12D9/1 with purified group 1 LPS, purified group 1 39K protein, and group 1 outer membrane vesicles as antigen (Fig. 4, lanes b to d, respectively). The LPS-directed specificities of three other 39K protein-immunoprecipitating mabs are shown by the reactivity of these mabs (6A2/1, 4C4/2, and 5G8/2) with LPS from their homologous Hib strains (Fig. 4, lanes e to g).

**ELISA-based reactivity of the LPS-directed mabs.** The mabs which immunoprecipitated the 39K protein in RIP analysis and which bound to LPS in Western blot analysis were further analyzed for their strain specificity in an enzyme-linked immunosorbent assay (ELISA) system. Outer membrane vesicles of Hib strains from antigenic groups 1, 2, 3, and 4 were used as antigen in an ELISA with mabs 12D9/1, 6A2/1, 4C4/2, and 5G8/2. The results (Table 2) indicate that these four mabs exhibited the same pattern of reactivity with these Hib strains in an ELISA system as they did in both RIP and Western blot analyses. Therefore, the differences detected among the LPS antigenic determinants of these strains were not the result of possible configura-

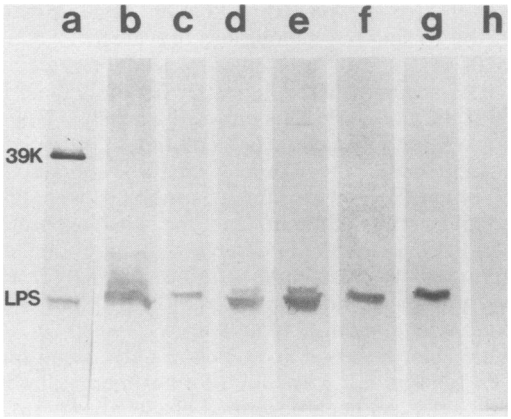


FIG. 4. Western blot analysis of 39K protein-immunoprecipitating mabs. Various outer membrane antigens were resolved by SDS-PAGE in 10 to 20% polyacrylamide gradient gels, electrophoretically transferred to nitrocellulose membrane, and probed with mabs which immunoprecipitate the 39K protein (4; Table 1). Antigen-antibody combinations: a, group 1 outer membrane vesicles–anti-group 1 39K serum (positive control); b, purified group 1 LPS–mab 12D9/1; c, purified group 1 39K protein–mab 12D9/1; d, group 1 outer membrane vesicles–mab 12D9/1; e, group 1 outer membrane vesicles–mab 6A2/1; f, group 2 outer membrane vesicles–mab 4C4/2; and g, group 2 outer membrane vesicles–mab 5G8/2. h, Group 2 outer membrane vesicles–irrelevant mab (negative control). The positions of the 39K protein and LPS in this Western blot are indicated by the markers on the left side of the figure.

TABLE 2. Reactivity of LPS-directed mabs with Hib strains in an ELISA system[a]

| Mab | Reactivity ($OD_{405}$) with Hib antigenic group[b] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 12D9/1 | 1.352 | 0.204 | 1.208 | 0.207 |
| 6A2/1 | 1.871 | 0.170 | 1.751 | 0.186 |
| 4C4/1 | 0.191 | 1.021 | 1.023 | 0.251 |
| 5G8/1 | 0.210 | 0.946 | 0.871 | 0.182 |
| SP2/0-Ag14[c] | 0.200 | 0.191 | 0.236 | 0.164 |

[a] Outer membrane vesicles were used as antigen in an ELISA system as described in the text. The volume of mab (hybridoma culture supernatant fluid) used in each well was 100 μl.

[b] $OD_{405}$ (optical density at 405 nm) of reaction mixture after 30 min at room temperature.

[c] Negative control for background level of binding of the alkaline phosphatase-coupled goat anti-mouse immunoglobulin probe.

tional restraints imposed on the LPS antigens by the nitrocellulose solid phase and the SDS treatment involved in Western blot analysis.

**LPS and the 39K protein exist as a complex in RIP analysis.** The immunoprecipitation of the 39K protein by LPS-directed mabs involved the following considerations. First, the 39K protein and LPS must exist as a complex in the detergent system used for RIP analysis, resulting in the coprecipitation of these two antigens by LPS-directed antibody. Second, this interaction between the 39K protein and LPS must be highly selective for the 39K protein because no other radioiodinated outer membrane proteins were immunoprecipitated by these LPS-directed mabs.

To examine the selectivity of this interaction, both the LPS-directed mabs and additional mabs which immunoprecipitate other outer membrane proteins were compared for their abilities to immunoprecipitate LPS in RIP analysis. Mab 7B2, which immunoprecipitates a 27K protein, and mab 6G12, which immunoprecipitates a 98K protein, in RIP analysis (Fig. 5, lanes g and h, respectively) bound to their respective protein antigens in Western blot analysis (Fig. 6, lanes c and d). Therefore, these two mabs are directed against antigenic determinants on these outer membrane proteins.

To label outer membrane proteins for RIP analysis, cells of Hib strains from antigenic groups 1, 2, and 3 were extrinsically radiolabeled with $^{125}I$. To radiolabel LPS and not outer membrane proteins, cells of the same three strains were also radiolabeled by growth in the presence of [$^{32}P$]phosphoric acid. These two different sets of radiolabeled Hib cells were then used in RIP analysis with the various mabs.

The results obtained in PS-RIP analysis with cells of group 3 Hib, which are recognized by all of the mabs, are depicted in Fig. 5. The LPS-directed mabs immunoprecipitated $^{125}I$-labeled 39K protein when used with the radioiodinated Hib cells (Fig. 5A, lanes c to f), whereas the other two protein-directed mabs immunoprecipitated their respective outer membrane proteins (Fig. 5A, lanes g and h). When the $^{32}P$-labeled cells were used as antigen, only the LPS-directed mabs immunoprecipitated radiolabeled LPS (Fig. 5B, lanes c to f). As expected, with cells of group 1 and group 2 Hib, mabs which immunoprecipitated the $^{125}I$-labeled 39K protein also immunoprecipitated $^{32}P$-labeled LPS (data not shown).

To further confirm that the interaction between the 39K protein and LPS is highly selective for the 39K protein, purified LPS from group 1 Hib was used to raise immune serum in rats. This anti-LPS serum was used in the PS-RIP

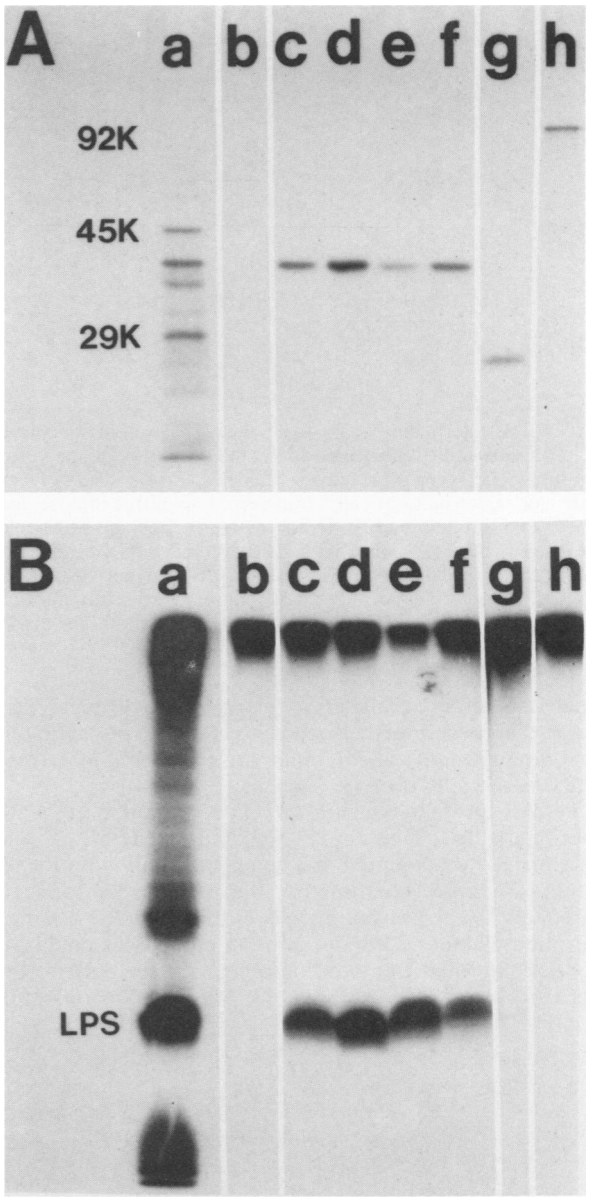


FIG. 5. RIP analysis of $^{125}I$- and $^{32}P$-labeled Hib cells. Cells of group 3 Hib were (A) extrinsically radiolabeled with $^{125}I$ or (B) intrinsically radiolabeled with [$^{32}P$]phosphoric acid, solubilized, and used as antigen in the PS-RIP system. Lane a of each figure is a standard which contains radiolabeled and solubilized cells of group 3 Hib which were used as antigen. Mabs used in the PS-RIP procedures: lane b, SP2/0 (negative control); lane c, 12D9/1; lane d, 6A2/1; lane e, 4C4/2; lane f, 5G8/2; lane g, 7B2; lane h, 6G12. A dark spot which barely migrated into the separating gel appeared on the autoradiograph in every immune precipitate in panel B. This may be DNA, RNA, or some other $^{32}P$-labeled cellular component that nonspecifically bound to the *Staphylococcus aureus* cells used as immunoadsorbent. LPS and molecular weight markers appear on the left.

system together with radioiodinated cells of group 1 Hib to determine whether other Hib LPS molecules exist which lack the epitopes recognized by the LPS-directed monoclonal antibodies and which also bind outer membrane proteins other than the 39K protein. The only radioiodinated Hib outer membrane protein immunoprecipitated by this poly-

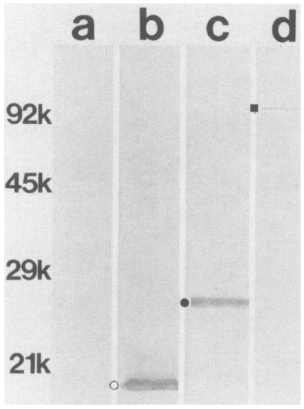


FIG. 6. Western blot analysis of the 27K protein-specific and 98K protein-specific mabs. LiCl-extracted outer membrane vesicles of group 3 Hib were resolved by SDS-PAGE in a 10% polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes, and probed with different mabs. Lanes: a, SP2/0 clone supernatant (negative control); b, 12D9/1 (anti-LPS); c, 7B2 (anti-27K protein); d, 6G12 (anti-98K protein). In this gel system, LPS migrated slightly behind the electrophoretic front. Symbols: ○, LPS; ●, 27K protein; ■, 98K protein. Molecular weight markers appear on the left.

clonal LPS-directed antiserum was the 39K protein (Fig. 7, lane d). This result further confirmed that the only Hib outer membrane protein which binds to Hib LPS in our RIP system is the 39K protein.

**LPS/39K protein interactions.** The immunoprecipitation experiments described above indicated that Hib LPS selectively formed detergent-stable complexes only with the 39K protein. In those experiments, the 39K protein and LPS components were present in cells of the same Hib strain. To determine whether LPS/39K protein complexes could form in detergent when the two components were derived from cells of different Hib strains, the following reconstitution

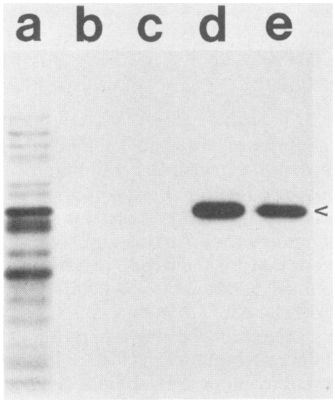


FIG. 7. RIP analysis of anti-LPS serum. Antiserum raised against group 1 Hib (DL26) LPS was used in the PS-RIP system together with radioiodinated, solubilized cells of group 1 Hib. Lane a, Solubilized preparation of radioiodinated group 1 Hib cells used as antigen; lane b, immune precipitate obtained with 100 μl of normal rat serum (negative control); lane c, immune precipitate obtained with 500 μl of SP2/0-Ag14 culture supernatant fluid (negative control); lane d, immune precipitate obtained with 100 μl of anti-LPS serum; lane e, immune precipitate obtained with 500 μl of 12D9/1 culture supernatant fluid. The arrow on the right indicates the position of the 39K protein.

experiments were performed. $^{125}$I-labeled cells of group 1 or group 2 Hib were mixed with unlabeled cells of either strain, solubilized in detergent, and used together with LPS-directed mabs in the PS-RIP system. The reconstitution of LPS/39K protein complexes was evidenced by the coprecipitation of $^{125}$I-labeled 39K protein by a mab directed against the LPS of the unlabeled Hib cells (Table 3).

As expected, when iodinated cells and unlabeled cells of the same strain (group 1 Hib) were mixed and solubilized, only the homologous LPS-directed mab (12D9/1) caused coprecipitation of the 39K protein. However, when mixtures of iodinated cells of one antigenic group (group 1 Hib) and unlabeled cells of the other antigenic group (group 2 Hib) were used as antigen, both the homologous (12D9/1) and heterologous (4C4/2) LPS-directed mabs caused coprecipitation of the 39K protein. This latter result indicated that the LPS of a group 1 Hib was able to form complexes in detergent with the 39K protein of a group 2 Hib. The converse experiment showed that LPS of a group 2 Hib could form a complex with the 39K protein of a group 1 Hib (Table 3).

**Interaction of purified 39K protein with purified LPS.** One limitation to the reconstitution experiments described above is that, because whole Hib cells were mixed and solubilized, it is possible that the resultant LPS/39K protein complexes possessed other cellular components which were not radioiodinated. To demonstrate definitively that LPS and the 39K protein form detergent-stable complexes, the reconstitution experiments were repeated with purified LPS and purified 39K protein preparations. Purified 39K proteins from group 1 and group 2 Hib were radioiodinated and mixed with purified LPS from either of the same two strains. The antigen mixtures were then probed with LPS-directed mabs in the PS-RIP system (Fig. 8).

In control experiments wherein $^{125}$I-labeled 39K protein alone was probed with the homologous LPS-directed mab, no 39K protein was immunoprecipitated (Fig. 8, lanes b). This result indicated that the purified 39K protein preparations did not possess sufficient amounts of LPS to permit coprecipitation to occur in the absence of exogenously supplied LPS. When pure 39K proteins were mixed with the homologous or heterologous LPS, coprecipitation of the 39K protein occurred only when the mab was directed against its homologous, purified LPS component. For example, when group 1 39K protein was mixed with group 1 LPS, only mab 12D9/1, which is directed against LPS of group 1 Hib, coprecipitated the 39K protein (Fig. 8A, lane d). However,

TABLE 3. Interaction of the 39K protein and LPS from different Hib strains as determined by coprecipitation of the 39K protein by LPS-specific mabs[a]

| Mab used for RIP | Antigenic group classification of $^{125}$I-labeled cells/unlabeled cells used as antigen[b] | | | |
|---|---|---|---|---|
| | 1/1 | 1/2 | 2/1 | 2/2 |
| 12D9/1 | + | + | + | – |
| 4C4/2 | – | + | + | + |
| None[c] | – | – | – | – |

[a] $^{125}$I-labeled Hib cells were mixed with unlabeled cells of the homologous or heterologous strain, solubilized, and used as antigen in the PS-RIP system. The reconstitution of LPS/39K protein complexes was evidenced by the coprecipitation of $^{125}$I-labeled 39K protein by a mab directed against the LPS of the unlabeled Hib cells.

[b] +, $^{125}$I-labeled 39K protein present in the immune precipitate; –, no $^{125}$I-labeled 39K protein present in the immune precipitate.

[c] SP2/0 culture supernatant fluid was used as a negative control.

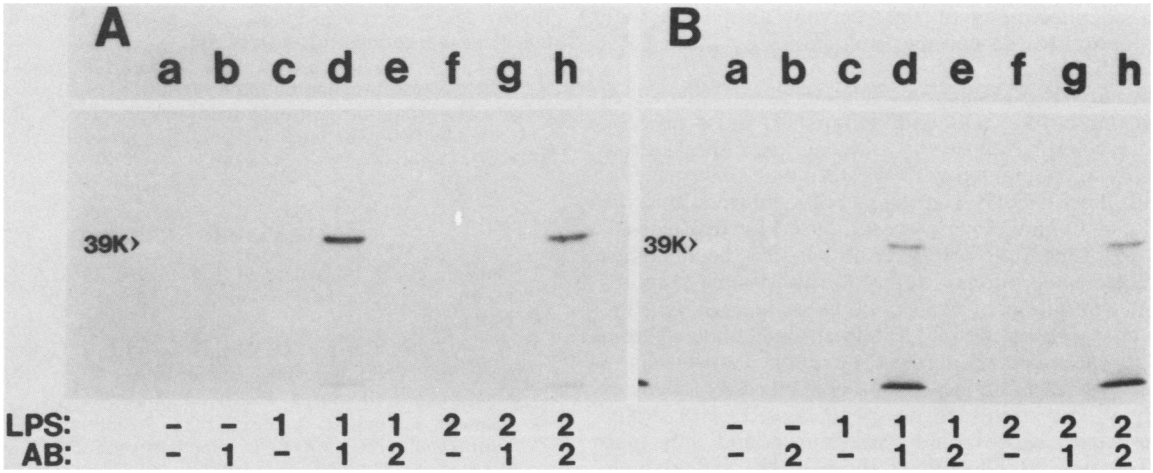


FIG. 8. RIP analysis of mixtures of $^{125}$I-labeled purified 39K protein and unlabeled purified LPS. $^{125}$I-labeled, purified 39K protein from either (A) group 1 Hib or (B) group 2 Hib was mixed with purified LPS of either strain and used as antigen in RIP analysis with various mabs. LPS, antigenic group classification of the Hib strain from which the LPS was obtained; AB, antigenic group classification of the Hib strain against which the LPS-specific mab was raised. The bands at the electrophoretic front in lanes d and h of each autoradiograph represent degradation products of the radiolabeled 39K protein.

when group 1 39K protein was mixed with LPS from group 2 Hib, only mab 4C4/2, which is directed against the LPS of group 2 Hib, coprecipitated the group 1 39K protein (Fig. 8A, lane h). Analogous results were obtained with mixtures of $^{125}$I-labeled 39K protein of group 2 Hib, purified LPS, and LPS-specific mabs (Fig. 8B). These results confirmed that the 39K protein and LPS form complexes in the detergent mixture used for RIP analysis, that the 39K protein and LPS are the only components necessary for complex formation, and that LPS from one antigenic group is able to form complexes with the 39K protein of a heterologous antigenic group. These experiments further established that the mabs used in our antigenic grouping system (4) are directed against LPS and not the 39K protein.

## DISCUSSION

The mabs which are directed against Hib LPS antigenic determinants and which immunoprecipitate the 39K protein in a complex with LPS were first identified as being 39K protein-specific by the use of RIP analysis (4, 20). Our recent use of the Western blot technique, in which the antigens are resolved by SDS-PAGE before analysis with antibody probes, permitted the elucidation of the true antigenic specificities of these mabs. That the observed interaction between Hib LPS and the 39K protein may be unique to this particular outer membrane protein was established by data from several experiments. The fact that the mabs which immunoprecipitated the 27K and 98K proteins in RIP analysis (Fig. 5) reacted with only their respective antigens in Western blot analysis (Fig. 6) confirmed the antigenic specificities of these two mabs. Neither of these two mabs immunoprecipitated $^{32}$P-labeled LPS in RIP analysis, indicating that the 27K and 98K proteins do not form complexes with LPS. More importantly, the fact that all of the LPS-specific mabs immunoprecipitated only the 39K protein in RIP analysis with radioiodinated Hib cells, which contain numerous other radioiodinated outer membrane proteins (7; Fig. 2), indicates that the LPS apparently interacted selectively with the 39K protein in this RIP system.

That certain outer membrane proteins associate with LPS is not a new finding. Studies of several outer membrane proteins of *Escherichia coli* demonstrated that association with LPS was necessary for these proteins to exhibit certain functional activities. For example, the OmpA protein needs to be associated with LPS to act as a bacteriophage receptor (22, 30). Many porin proteins similarly need to be associated with LPS to form functional pores and to act as bacteriophage receptors (21, 23). Studies on the immunogenicity of purified outer membrane proteins of *S. typhimurium* have shown that the major outer membrane protein needs to be mixed with LPS to induce the synthesis of protective antibodies (14). Finally, the 15K LPS-binding protein of *Salmonella minnesota* may provide a biochemical model for the LPS/39K protein interaction of Hib. The 15K LPS-binding protein forms insoluble complexes with LPS in double diffusion of the two components in agarose gels (2). This protein has a very basic pI (10.3) and is believed to bind LPS, which has a very acidic pI, through ionic interactions. The biochemical and biophysical characteristics of the Hib LPS/39K protein interaction have yet to be determined. Similarly, we do not have any information at this time as to whether the 39K protein and Hib LPS interact in the intact organism.

The fact that these LPS-directed mabs were originally identified as being directed against the 39K protein necessitates reinterpretation of published data involving them (4, 8, 20). The ability of mab 6A2 to both protect against and eradicate systemic Hib disease in the infant rat model system (8) must now be interpreted as providing evidence that mab directed against Hib LPS can have a protective effect. This development is of interest because a previous study from another laboratory indicated that Hib LPS-directed polyclonal (serum) antibody is not protective against experimental Hib disease (24). These divergent results may be due to differences in effector functions between the polyclonal rabbit LPS-directed antibodies obtained in the cited study (24) and our immunoglobulin G3 mab. Alternatively, the B cells of rabbits and mice may respond to different epitopes on Hib LPS molecules, and both the immunogens and the methods of immunization used for production of these two sets of antibodies were quite different (20, 24). Resolution of these disparate findings will require careful definition of the

isotypes and subclasses of the relevant antibodies with special attention to the epitopes recognized by these LPS-directed antibodies.

In addition, our classification scheme which divided all Hib strains into four antigenic groups (4) must now be regarded as being based on the antigenic characteristics of Hib LPS. Results obtained with Western blot analyses (Fig. 4) and with the ELISA system (Table 2) confirmed that the colony blot-radioimmunoassay results used for this classification system reflect the reactivity of the LPS-specific mabs with LPS molecules in the different Hib strains. Accordingly, reinterpretation of the previous study involving the antigenic characterization of 126 Hib strains collected from pediatric research centers across the country showed that a set of just two LPS-directed mabs collectively recognizes essentially all Hib strains. More specifically, 17 Hib strains bound only mab 12D9/1, and 78 strains bound only mab 4C4/2; 30 strains bound both of these mabs, and a single strain failed to bind either mab. These results indicate that certain Hib LPS antigenic determinants are highly conserved among strains of this pathogen.

Similarly, selected aspects of data obtained in other studies which used RIP procedures to analyze the antibody content of human (5) and animal (6, 7) sera must be reevaluated in light of the demonstrated coprecipitation of Hib LPS and 39K protein in these RIP systems. Because this coprecipitation phenomenon does not apparently involve any other Hib outer membrane proteins, the principal point to be considered is whether the immunoprecipitation of the 39K protein by these various sera was mediated by 39K protein-specific antibodies, LPS-specific antibodies, or both. This question is currently under investigation in our laboratory.

It must be emphasized that we have recently shown that a mab directed against the 98K outer membrane protein of Hib can protect against experimental Hib disease (13). This finding confirms the protective ability of antibody directed against Hib cell surface-exposed proteins (18). The data contained in the present report extend our knowledge about the interaction of Hib somatic noncapsular antigens with the host immune system and prove that a mab against LPS, the other principal antigenic constituent of the Hib outer membrane, can exert a protective effect against experimental Hib disease. This protective ability of an LPS-directed mab (8) and the highly conserved nature of the antigenic determinants of Hib LPS (4) further reinforce the necessity for thorough experimental investigation of the vaccinogenic potential of Hib LPS. The failure of an LPS-directed polyclonal antiserum to protect against experimental Hib disease suggests that Hib LPS in its native state in whole bacteria either may not be capable of inducing the synthesis of protective antibodies or may not induce a sufficient level of antibody to provide protection (24), while the inherent toxicity of the native LPS moieties of most gram-negative bacteria precludes the utilization of an intact LPS molecule as a vaccine in infants. However, recent progress in producing immunogens by coupling nontoxic, LPS-derived oligosaccharide or polysaccharide molecules from *Neisseria meningitidis* (10), *Neisseria gonorrhoeae* (15), *S. typhimurium* (12, 25, 26), and *Pseudomonas aeruginosa* (29) to protein carrier molecules warrants further evaluation of Hib LPS as a possible source of vaccine components.

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