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Detection of Antibody-Accessible Proteins on the Cell Surface of *Haemophilus influenzae* Type b

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A radioimmunoprecipitation method has been devised which permits the direct identification of those proteins which are both exposed on the cell surface of *Haemophilus influenzae* type b and accessible to antibody. Both extrinsically and intrinsically radiolabeled cells of this organism were employed as the sources of antigen in radioimmunoprecipitation experiments which involved incubation of intact bacterial cells with antisera. Several different proteins, ranging in apparent molecular weight from 33,000 to 160,000, were shown to be accessible to antibody on the cell surface of this pathogen.

Immunoprotection against systemic disease caused by *Haemophilus influenzae* type b (Hib) has not been achieved in those very young children at highest risk for *Haemophilus* disease (6). The failure of the existing Hib vaccine (purified capsular antigen) to induce the synthesis of protective antibodies in children less than 14 months of age has made necessary the evaluation of other Hib antigens for their possible efficacy as vaccinogens. That antibody directed against noncapsular Hib somatic antigens can protect against experimental Hib disease has been amply demonstrated with the infant rat model system (3, 11). The proteins present in the outer membrane of Hib represent one group of potential vaccinogen candidates, and previous studies from this laboratory have established that several proteins present in the outer membrane of Hib are immunogenic in very young mammals (4). However, for an outer membrane protein to be considered as a vaccinogen candidate, it must not only be immunogenic in very young mammals, but it must also be accessible to bactericidal or opsonizing antibodies or both. Therefore, we developed a radioimmunoprecipitation method which permits the direct identification of those Hib proteins which are accessible to antibody in their native state on the cell surface of this pathogen.

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Hib strain 26 cells grown in brain heart infusion broth (Difco Laboratories) supplemented with Levinthal base (1) were harvested by centrifugation at 4°C during the late logarithmic phase of growth. Extrinsic radiolabeling of Hib outer membrane proteins was accomplished by the use of a lactoperoxidase-catalyzed radioiodination procedure (4). Intrinsic radiolabeling of Hib proteins was accomplished by growing cells in the presence of 100 μCi of [^{35}S]methionine (specific activity, 900 Ci/mmol) per ml. All cells were washed three times with cold pH 7.2 phosphate-buffered saline subsequent to radiolabeling and were immediately employed in the radioimmunoprecipitation system. Three types of sera were employed as sources of antibody in the radioimmunoprecipitation system. Immune serum was pooled from adult Sprague-Dawley rats which had been injected intraperitoneally several times over a 3-month period with 10^6 colony-forming units of Hib strain 26. Preadsorbed serum was prepared by incubating immune serum with intact Hib cells four times sequentially at 4°C to remove antibodies directed against Hib cell surface antigens. Control serum was obtained from uninfected rats. Complement activity in all sera was inactivated by heating at 56°C for 30 min.

A portion of phosphate-buffered saline-suspended Hib cells containing 1×10^7 cpm of [^{125}I]iodine (specific activity, 0.01 cpm per colony-forming unit) or 5×10^7 cpm of [^{35}S]methionine (specific activity, 0.006 cpm per colony-forming unit) was incubated with 100 μl of serum at 4°C for 90 min with gentle agitation to allow antibodies specific for Hib cell surface components to attach to their respective antigens. No significant cell lysis occurred during this procedure, as evidenced by the fact that less than 0.2% of the radioactivity associated with intrinsically radiolabeled Hib cells was released into the suspension buffer. The cells were then col-

lected by centrifugation at 13,000 × g for 2 min, washed once with phosphate-buffered saline to remove unadsorbed antibodies, and suspended in solubilization buffer consisting of 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 1% (vol/vol) Triton X-100, 0.2% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate. The final concentration of cells in solubilization buffer was adjusted to 10^9 cells per ml, and the cell suspension was incubated at 37°C for 60 min. Control experiments determined that this protocol resulted in the solubilization of 95% of the radioactivity associated with either extrinsically or intrinsically radiolabeled Hib cells (E. J. Hansen and C. F. Frisch, unpublished data). Insoluble material was removed from the solubilized cell preparation by centrifugation at 45,000 × g which was performed for 1 h at 20°C. A representative repertoire of the solubilized cell surface antigen-antibody complexes involving immunoglobulin G antibodies was precipitated from the resultant supernatant by the addition of 1 ml of a 10% (wt/vol) Formalin-fixed suspension of *Staphylococcus aureus* (8) bearing protein A (Staph A) on its surface (10). Staph A was washed twice with solubilization buffer immediately before its addition to the supernatant. After incubation at 4°C for 60 min, the Staph A-antibody-antigen complexes were washed five times at room temperature by repeated centrifugation and resuspension in 1-ml volumes of solubilization buffer. The cell surface antigen-antibody complexes were released from Staph A, dissociated, reduced with 2-mercaptoethanol, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). Radiolabeled protein antigens present in the gels were identified by autoradiography (5) or fluorography (2).

Immune serum raised against intact Hib cells contained considerable antibody activity directed against specific cell surface proteins of this organism, as evidenced by the fact that this serum immunoprecipitated at least seven different radioiodinated Hib cell surface proteins (Fig. 1, lane b). One of these proteins, with an apparent molecular weight of approximately 39,000, represents a major Hib outer membrane protein (4). In contrast to immune serum, control serum from uninfected rats did not immunoprecipitate any Hib cell surface proteins (Fig. 1, lane d). That the seven radioiodinated proteins detected with immune serum are exposed on the cell surface of Hib was confirmed by the fact that preadsorption of immune serum with intact Hib cells removed all antibody activity directed against these proteins (Fig. 1, lane c). An additional control experiment, in which radioiodinated Hib cells were solubilized before the addition of immune serum, showed that this immune serum contained antibody activity directed against essentially all of the radioiodinated proteins present in the outer membrane of the Hib cells which were employed as a source of antigen in this experiment (Fig. 1, lane e). This latter control experiment also demon-

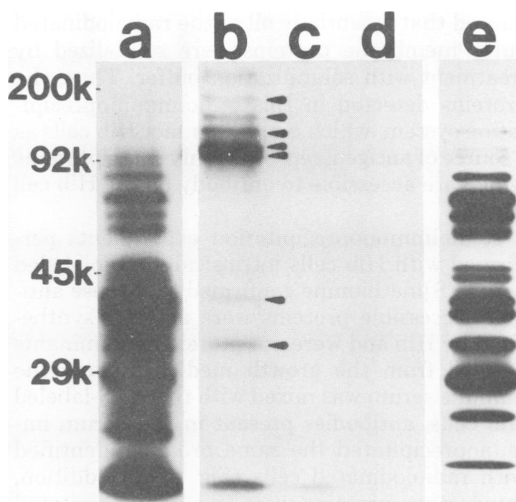

FIG. 1. *Radioimmunoprecipitation of ^{125}I-labeled Hib cell surface proteins with immune and control rat sera. Sera were mixed with intact ^{125}I-labeled Hib cells. The cell proteins and any adherent antibodies were then extracted from the cells by treatment with the detergent-ethylenediaminetetraacetic acid mixture. The control experiment in which radioiodinated Hib cells were solubilized before the addition of antibody is depicted in lane e. Antigen-antibody complexes were isolated by adsorption to staph A. The total immune precipitate from each reaction mixture was then processed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography to detect radioiodinated protein antigens as described in the text. (a) ^{125}I-labeled proteins present on the Hib cells employed as a source of antigen in this experiment; (b) ^{125}I-labeled proteins precipitated by 100 µl of pooled immune rat serum; (c) ^{125}I-labeled proteins precipitated by 100 µl of pooled immune rat serum preadsorbed with intact Hib cells; (d) ^{125}I-labeled proteins precipitated by 100 µl of pooled serum from uninfected control rats; (e) ^{125}I-labeled proteins precipitated when 10 µl of pooled immune rat serum was incubated with solubilized radioiodinated Hib cells. The arrows in lane b indicate those radioiodinated Hib cell surface proteins which were accessible to antibody and precipitated by the immune rat serum. Molecular weight reference markers were provided by the co-electrophoresis of purified myosin (200,000), phosphorylase b (92,000), ovalbumin (45,000), and carbonic anhydrase (29,000).*

strated that essentially all of the radioiodinated outer membrane proteins were solubilized by treatment with solubilization buffer. Thus, the proteins detected in this radioimmunoprecipitation system, which employs intact Hib cells as a source of antigen, represent only those proteins which are accessible to antibody on the Hib cell surface.

Radioimmunoprecipitation experiments performed with Hib cells intrinsically radiolabeled with [^{35}S]methionine confirmed that these antibody-accessible proteins were actually synthesized by Hib and were not protein contaminants derived from the growth medium. When the immune serum was mixed with these ^{35}S-labeled Hib cells, antibodies present in this serum immunoprecipitated the same proteins identified with radioiodinated cells (Fig. 2). In addition, three other proteins were immunoprecipitated by this serum. These latter three proteins, which were not immunoprecipitated with ^{125}I-labeled cells, may be deficient in exposed tyrosine residues which are necessary for protein iodination by the lactoperoxidase-catalyzed method (12). Taken together, these data indicate that this particular strain of Hib possesses at least 10 proteins which are both exposed on the Hib cell surface and accessible to immunoglobulin G antibody.

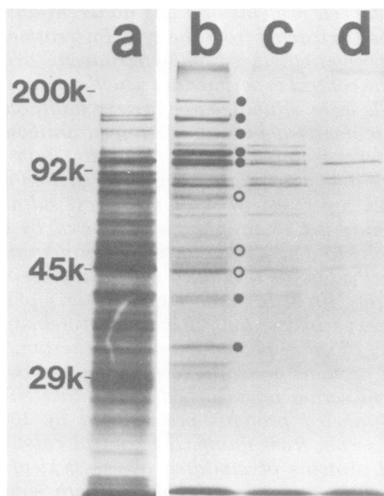

FIG. 2. *Radioimmmunoprecipitation of ^{35}S-labeled Hib cell surface proteins with immune and control rat sera. The experimental procedure employed here was identical to that described in the legend to Fig. 1. (a) ^{35}S-labeled proteins present in the Hib cells employed as a source of antigen in this experiment; (b) ^{35}S-labeled proteins precipitated by 100 µl of pooled immune rat serum; (c) ^{35}S-labeled proteins precipitated by 100 µl of pooled immune rat serum preadsorbed with intact Hib cells; (d) ^{35}S-labeled proteins precipitated by 100 µl of pooled serum from uninfected control rats. The closed circles in lane b indicate those antibody-accessible Hib cell surface proteins which were also detected when radioiodinated Hib cells were employed as the source of antigen (Fig. 1). The open circles in lane b indicate those antibody-accessible Hib cell surface proteins which were not evident when radioiodinated Hib cells were used as the source of antigen.*

It is important to note that a few of these antibody-accessible proteins with apparent molecular weights above 100,000 are only faintly visible or not visible at all in the fluorogram of ^{35}S-labeled Hib cells (Fig. 2, lane a). Long-term exposure of this fluorogram did result in the appearance of bands corresponding to all of the high-molecular-weight proteins recovered in the immunoprecipitates (Hansen and Frisch, unpublished data). This finding suggests that some of these cell surface proteins are present in only a relatively few copies per cell. Detection of these proteins in the radioimmunoprecipitation system was possible because this method selects out and enriches for only the antibody-accessible proteins present on the Hib cell surface.

The ability to identify antibody-accessible proteins on the cell surface of Hib is critically important in the selection of potential vaccinogen candidates from the numerous proteins present in the outer membrane of Hib. Similarly, the radioimmunoprecipitation technique described in this paper may be amenable to studies on the cell surface location or relative exposure of outer membrane proteins of other gram-negative bacteria, such as proteins involved in iron transport (9) or as bacteriophage receptors (7). We are currently employing this technique together with the infant rat model system (13) to study the ontogeny of the immune response against these cell surface-exposed, antibody-accessible Hib proteins. These studies, coupled with the use of monoclonal antibodies directed against Hib outer membrane protein antigens (S. M. Robertson, C. F. Frisch, J. R. Kettman, and E. J. Hansen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B167, p. 42), will permit the rapid and accurate identification of Hib outer membrane proteins which are both immunogenic in very young mammals and accessible to antibody on the Hib cell surface.

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