Molecular Cloning of DNA Coding for Outer Membrane Proteins of
Haemophilus influenzae Type b

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DNA from Haemophilus influenzae type b was cloned in Escherichia coli with a vector lambda gt11 Amp1. Clones producing antigens reactive with hyperimmune rabbit antisera were identified by colony radioimmune assay. A second screening with hyperimmune serum adsorbed with intact H. influenzae type b bacteria was used to identify those clones producing surface-exposed outer membrane proteins. The proteins expressed in E. coli were coupled to Sepharose and used to affinity purify antibodies which were tested for reactivity with outer membrane vesicles. It was found by Western blotting that the clones were producing antigens corresponding to M, 49,000, 39,500, and 35,000 major outer membrane protein or antigens of H. influenzae type b. Additional clones could be detected by human serum, but their reactivity was not removed when serum was adsorbed with intact bacteria. One of these studied in more detail was found to produce an antigen present in H. influenzae type b lysate but not in outer membrane vesicle preparations.

Haemophilus influenzae type b (Hib) is the major cause of bacterial meningitis in industrialized countries (22). A vaccine to prevent systemic Hib infection has been produced from the capsular polysaccharide ribosylribotol phosphate (PRP), but it does not elicit immunity in the most susceptible age group, infants under 15 months old (19). There have therefore been attempts to improve the immunogenicity of PRP or develop alternate vaccines. Recent trials with multiple doses of a conjugate of PRP and diphtheria toxoid vaccine have shown improved antibody responses (7), although the anti-PRP titers achieved by half of the infants were still below the level used to predict long-term protection.

Outer membrane proteins may also be targets for protective immunity. At least some of these proteins are accessible to antibodies (8, 9), and monoclonal antibodies to two proteins have been shown to protect rat pups from systemic infection (10, 13). Given that infants convalescent from Hib meningitis produce antibodies to these proteins (8) and that surface-accessible outer membrane proteins from different isolates show extensive antigenic cross-reactivity (6), these proteins are potential vaccines. Because of the possibility of using recombinant DNA technology to produce large quantities of a vaccine molecule or to construct a live genetically engineered vaccine, we have begun to clone and express Hib antigens in Escherichia coli. In this paper we describe a strategy which has resulted in the cloning of DNA coding for and the expression of major surface-exposed Hib outer membrane proteins.

MATERIALS AND METHODS

Reagents. Restriction endonuclease EcoRI, T4 ligase, DNA polymerase I, DNase I, and pronase were from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Low-melting-point agar was from Seaplaque, FMC Corp., Rockland, Maine.

Bacteria. The E. coli strain used for lytic growth of bacteriophage lambda gt11 Amp1 (lambda Amp1) (see below) was RY1070 (22), and the hflA50 strain for lysogenic growth was BTA282 (4). These strains and lambda Amp1 were provided by D. Kemp, Walter and Eliza Hall Institute, Melbourne.

The Hib strain (Ca) was isolated from a meningitis patient in the Princess Margaret Hospital, Perth, and was provided by J. O'Conner. Hib was grown on chocolate agar.

Cloning of Hib DNA in E. coli. The lambda Amp1 vector used for cloning in E. coli was constructed by D. Kemp from lambda gt11 (23). This bacteriophage is the same as lambda gt11 Amp3 (12), except that the XbaI site at 24.8 kilobases of lambda gt11 Amp3 has been replaced by an EcoRI site. For cloning, the lambda Amp1 was digested with EcoRI, and the 5.2-kilobase fragment containing most of the lacZ gene was used as a stuffer. Religated arms grew to 20% the efficiency of arms religated with the stuffer. This vector does not produce a fused polypeptide like lambda gt11, but retains other features to increase production of foreign protein. Lambda Amp1 grows as a lysogen at 30°C in the hflA E. coli BTA282 and because of the cl857 mutation can be induced to expression at 42°C. BTA282 has a lon mutation to decrease proteolysis, and lambda Amp1 has an S100 mutation which prevents lysis of BTA282 bacteria so phage accumulates. Lysogenized E. coli BTA282 were selected by ampicillin resistance (10 μg/ml). To prepare vector, phage was produced by lytic growth at 37°C in E. coli RY1070 and purified by step and equilibrium cesium chloride gradients, and DNA was extracted (16). DNA was digested with EcoRI, and the arms were isolated after electrophoresis in 0.7% low-melting-point agar. DNA from Hib was isolated by a sodium dodecyl sulphate (SDS)-protease technique (3) and partially digested with EcoRI, and 6- to 9-kilobase fragments were isolated by electrophoresis in low-melting-point agar. The fragments were ligated with lambda Amp1 arms by incubation with T4 ligase at a 1:4 molar ratio. The ligation mix was packaged (1) and incubated with BTA282 at 20°C for 30 min. The infected bacteria were incubated with L broth for 15 min at 30°C to allow β-lactamase production and then grown on L plates containing 10 μg of ampicillin per ml at 30°C to allow colony growth.

Colony radioimmune assay. Nitrocellulose filters (Schleicher & Schuell Co., Keene, N.H.) with replicated colonies of BTA282 lysogenized with recombinants produced from...
Lambda Amp1 were incubated on L plates (10 μg of ampicillin per ml) for 3 h at 30°C, induced at 42°C for 2 h, and assayed for antigen (12, 23). The induced colonies were lysed by placing the filters on MM paper saturated with 1% SDS in an atmosphere of chloroform for 15 min. The filter was rocked overnight in TNT buffer (10 mM Tris hydrochloride, 0.15 M NaCl, 0.05% Tween 20 [pH 8.0]) and then incubated with antiserum diluted in a phosphate-buffered saline (PBS) containing 5% skim milk powder and 0.02% sodium azide for 1 h at room temperature. Filters were then given three washes for 30 min with rocking in TNT and then incubated in TNT containing 106 cpm of 125I-staphylococcal protein A per ml (5 × 107 cpm/μg) for 1 h at room temperature, which was followed by three washes in TNT. The filters were dried and autoradiographed with intensifying screens at -70°C with Fugi RX film (Hanimex Pty Ltd., Brookvale, New South Wales). Radioiodination was performed by the chloramine T method (11) with [125]iodide (13 to 17 mCi/μg; Amersham Corp., Arlington Heights, Ill.).

Before use, antiserum was absorbed with a lystate of BTA282 infected with lambda Amp1. lysates were prepared by incubating 900 ml of L broth with 100 ml of an overnight culture of BTA282 lysogenized with lambda Amp1. After 2 h of incubation at 30°C with vigorous shaking, the culture was induced at 42°C for 15 min and incubated for a further 2 h at 37°C. Cells were sedimented (3,000 × g, 10 min) and suspended in 30 ml PBS, frozen, thawed, and then the solution was sonicated until its viscosity decreased. For absorption, serum was diluted 1/50 in the sonicate, incubated with rotation overnight at 4°C, and then clarified by centrifugation (3,000 × g, 15 min).

**Dot-blot assays.** Some immunoassays were performed with a dot-blot system. Lysates of *E. coli* BTA282 clones lysogenized with recombinant phage were induced and prepared with freeze-thawing and sonication as described above for lysates used to absorb antiserum. The bacteria were suspended in PBS to 0.1 the volume of the induced broths before sonication. An overnight culture of 2 ml of lystate was dotted onto nitrocellulose, air dried, and washed for 30 min in TNT before the addition of antiserum. Dot-blot assays were also performed with Hib isolates. Overnight cultures of Hib were grown on chocolate agar, suspended at 109 cells per ml in PBS, and then freeze-thawed and sonicated.

**Affinity purification of antibodies.** *E. coli* BTA282 cells with lysogenized recombinant phage were induced; lysates were prepared as described above, except bacteria from a 500-ml culture were suspended in 2 ml of a 0.1 M NaHCO₃-0.5 M NaCl (pH 9.5) solution before snap freezing. The sonicate was clarified by microfuging and passed through a 3-ml Sephadex G25 column and eluted with the NaHCO₃-NaCl buffer. The first 3 ml was collected and used for coupling to Sepharose 4B (Pharmacia Diagnostics, Piscataway, N.J.). The rapid cyanogen bromide activation method (17) was used to couple 50 mg of sonicate to 5 ml of Sepharose 4B.

Antibodies were affinity purified by first passing 20 ml of serum through a column coupled with a sonicate from bacteria infected with lambda Amp1 and then through a column coupled with a sonicate from bacteria with a recombinant phage. The column was washed with (i) 100 ml of PBS, (ii) 100 ml of 0.1 M borate-0.5 M NaCl-0.05% Tween 20 (pH 8.5), and (iii) 100 ml of PBS. Antibodies were eluted with 0.1 M glycine-0.15 M NaCl (pH 2.6).

**Preparation of outer membrane vesicles.** Outer membrane vesicles were prepared by the lithium chloride-lithium acetate method (8). The protein in the vesicles were analyzed by SDS-polyacrylamide gel electrophoresis in 10% gels (14); the major proteins were limited and in the molecular weight range described previously (2, 15). Vesicles from 0.5 ml of packed Hib were prepared and suspended in 1 ml of water.

**Electrophoretic transfer and immunoblotting.** Outer membrane vesicles were electrophoresed in 10% SDS-polyacrylamide gels, transferred electrophoretically to nitrocellulose (5), and reacted with anti-Hib and 125I-labeled protein A by the procedures used for the colony radioimmune assay.

**Colony hybridization and Southern blotting.** Southern hybridization and colony hybridization were performed as described by Maniatis et al. (16) with deoxycytidine 5'-[α-32P]triphosphate triethylammonium salt (approximately 3,000 Ci/mmol; Amersham) for nick translation. For colony hybridization the NaOH method of in situ lysis was used.

**Antiserum.** Hib cells were cultured overnight on chocolate agar, washed three times in PBS, suspended at 1010 organisms per ml in PBS containing 0.5% formaldehyde, and then incubated for 1 h at room temperature. The formalinized bacteria were washed three times in PBS and stored at 4°C. Rabbits were given fortnightly injections of 1010 Hib intravenously, and serum was taken after six injections.

**Adsorption of antiserum with intact Hib.** Overnight cultures of Hib were washed three times in PBS, and 5 ml of a 1/100 dilution of serum was given three successive absorptions with 1010 Hib organisms for 1 h at 4°C with rotation. Bacteria used for absorption were grown directly from stocks made as soon as possible after clinical isolation.

**RESULTS**

**Construction of clones reactive with rabbit anti-Hib and screening for surface antigens.** Hib DNA (Ca isolate) was partially digested with EcoRI, and 6- to 9-kilobase fragments were cloned into *E. coli* BTA282 by using the vector lambda Amp1. About 106 colonies lysogenized with recombinant lambda Amp1 were grown and replicated at high density on nitrocellulose filters. The lysogens were induced by a temperature switch to phage expression and lysed in situ on the filters and assayed for reactivity to a 1/200 dilution of hyperimmune rabbit anti-Hib by colony radioimmune assay. Colonies in areas showing positive reactions were plated in triplicate on nitrocellulose and reassayed. The triplicate samples were also tested with the rabbit anti-Hib after they had been adsorbed with intact Hib. The same Hib isolate (Ca) was used for immunizing, cloning, and adsorption. About 30 clones reacting with anti-Hib were detected, and reactivity to most of them was abolished by absorption with intact Hib, indicating that they may be surface antigens (see Fig. 2). Serum taken from the rabbit before immunization did not react with clones (data not shown).

**Screening with human serum.** The high-density library was also screened with a pool of serum from five laboratory personnel. The screen identified four more positive clones that were not reactive with rabbit anti-Hib. These were designated H4, H5, H6, and H7.

**Colony hybridization.** Colonies positive in the immunoassay were colony purified and examined for relatedness by DNA colony hybridization. The inserts from clones 2, 10, 33, and H5 were isolated, nick translated, and used as probes on an array of clones which included those detected by either the rabbit or human serum. The DNA from colony 2 showed identical reactivity to probe 10 and hybridized with most of the clones (Fig. 1). Probe 33 reacted with a colonies numbered 13, 33, 34, and Q4, whereas the H5 probe only
hybridized with H5. By assuming that cross-hybridizing colonies contain DNA coding for the same antigens, the number of potentially different clones was reduced to those designated 2, 8, 12, 25, 27, 28, 33, Q7, Q9, H4, H5, H6, and H7.

Southern analysis was also conducted to check that Hib sequences had been cloned. DNA from two Hib isolates, Ca and Png, and from E. coli was digested with EcoRI, and Southern blotting was performed with the 2, 10, 33, and H5 probes. No insert reacted with E. coli DNA, and all reacted with the Hib DNA.

Adsorption studies. Some clones designated above were examined for reactivity by dot-blot assay to rabbit anti-Hib (Fig. 2). This assay was essentially the same as the colony immunoassay, except lysates from colonies were dotted onto the nitrocellulose rather than lysing colonies in situ. The colonies 2, 8, 10, 12, 25, 27, 33, and Q7 gave the expected positive reactions to rabbit anti-Hib. Adsorption of the anti-Hib (Ca) with the homologous intact Hib (Ca) abrogated all reactivity, as found in the colony immunoassay. The clones H4, H6, and H7 (Fig. 2) characteristically did not react with hyperimmune rabbit anti-Hib. These clones reacted with antibodies found in some normal human serum. Figure 3 shows the reaction of clones H5 and H7 with serum AR, which was from a laboratory employee. Attempts to diminish this reactivity with three adsorptions of intact Hib (Ca) failed (Fig. 3). It therefore appears that the antigens H5 and H7 are internal.

Characterization of cloned antigens. Lysates were prepared from clones 2, 8, 25, and H5 and attached to Sepharose 4B (Pharmacia) to affinity purify antibody from rabbit anti-Hib antiserum (clones 2, 8, and 25) or human serum (clone H5). The affinity-purified antibody was tested by dot-blot assay for reactivity to the clones (Fig. 4). Affinity-purified anti-H5 only reacted with H5. The affinity-purified anti-2 reacted with 2 and 10, which are related by colony hybridization, but not other clones. Affinity-purified anti-8 and anti-25, however, both reacted with 8, 12, and 25. These clones therefore probably express the same antigen. This is also consistent with the finding that when an individual human serum sample reacts with one of these clones it also reacts with the others.

The affinity-purified antibodies were then examined for reactivity with outer membrane vesicles from Hib (Ca) by dot-blot immunoassay (Fig. 5). The outer membrane vesicles were prepared with the chaotropic lithium chloride-lithium acetate buffer. Anti-2, anti-25, and anti-H5 were reacted with lysates of Hib and membrane vesicle preparations. The anti-2 and anti-25 reacted with both the lysate and vesicle preparations. The anti-H5 reacted strongly with the lysate, but did not react to the vesicle preparation.
The reactivity of the proteins of the outer membrane vesicle preparation with affinity-purified antibodies was then examined by Western blotting. The vesicle preparations were electrophoresed in 10% SDS-polyacrylamide gels. Coomassie-stained gels are shown in Fig. 6. Major bands were found at \( M_r 49,000, 39,500, \) and 33,000. The band at \( M_r 35,000 \) (first above the 33,000) is of interest because it stains strongly by Western blotting. For Western blotting the antigens were transferred electrophoretically to nitrocellulose and then blotted with affinity-purified antiserum and developed with \(^{125}\)I-labeled protein A by using the procedure from the colony immunoassay (Fig. 7). Anti-2 reacted with an \( M_r 49,000 \) band corresponding to a major band by Coomassie staining, and the anti-25 reacted with a major band of \( M_r 35,000 \) which showed strong reactions with rabbit and Hib antiserum (Fig. 7). Affinity-purified rabbit anti-25 was also prepared and tested by Western blotting (Fig. 8). These antibodies reacted with a band of \( M_r 39,500 \). Figure 8 shows Western blotting of outer membrane vesicle proteins exposed to anti-33 (track a), to anti-25 (track b), and to both anti-25 and anti-33 (track c). These antibodies recognize different outer membrane proteins.

To test the size of the Hib antigen produced by \( E. coli \) clones, lysates were similarly electrophoresed and examined by Western blotting with rabbit anti-Hib antiserum. The clones 2, 25, and 33 produced Hib antigens which migrated with an \( M_r \) almost identical to that found in Hib membrane vesicles (Fig. 9).

**DISCUSSION**

B cells can be divided in those responsive to type 1 thymus-independent antigens and those responsive to type 2 thymus-independent antigens. In the mouse these populations have been defined by the CBA/N Xid mutation and the Lyb 5 surface antigen (19). Responsiveness to the Hib capsular PRP antigen is almost entirely mediated by the populations responsive to type 2 antigen, a population of cells which does not mature until late in ontogeny. Furthermore, although soluble T-cell factors can augment their responses, the type 2-responsive B cells do not participate in classical major histocompatibility complex-restricted T-B cell collaboration, even when the antigen is coupled to a protein (21). Thus, although progress has been made in attempts to increase the immunogenicity of PRP-based Hib vaccines for infants (7), the exploration of protein vaccines is warranted.

There have been considerable advances in the characterization and classification of outer membrane proteins of Hib (2, 15), and it has been demonstrated that purified outer membrane proteins can induce protective immunity (18). The experiments of Hansen and collaborators showing that monoclonal antibodies to outer membrane proteins can protect rat pups from systemic disease have also been particularly encouraging (10, 13). These investigations have identified a 98-kilodalton minor outer membrane protein which is very immunogenic in infants and is a suitable target for protective antibodies (13), although the distribution of this protein among different isolates needs further study.

Given that the production of a vaccine is likely to involve recombinant DNA technology or a genetically engineered live vaccine, we have begun to study the expression of Hib outer membrane proteins in \( E. coli \). We report here a strategy which has led to the cloning of DNA coding for three surface-exposed outer membrane proteins and possible other surface antigens. The strategy was to (i) clone Hib DNA in \( E. coli \) by using an expression vector, lambda gt11 Amp1, a derivative of lambda gt11 (23), (ii) screen for antigen expression by colony radioimmunoassay with rabbit serum.
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Thomas and Rossi
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Antibody reactivity shown electrophoresed on anti-2 and purified Outer rabbit anti-Hib. reacts munoblotted with Coomassie blue. The right hand track has molecular mass standards of 66, 45, 36, 29, 24, and 20 kilodaltons. All other tracks are vesicles at different concentrations.

The clones 2, 33, and 25 produced antigens which were identified as proteins from outer membrane vesicles. They corresponded to the major antigen bands of 49,000, 39,500, and 35,000 as determined by Western blotting with antibody affinity purified from the cloned antigens. These were in positions of major outer membrane proteins as shown by Coomassie staining (clones 2, and 33) or by Western blotting (clone 25) with polyclonal rabbit anti-Hib (Fig. 9). The finding that intact bacteria completely absorbed out antibody reactivity to these proteins in immunoassays indicates that most of the antibody in the hyperimmune serum was produced to the surface-exposed determinants. The size of these proteins also corresponds well to that of the Hib major outer membrane proteins a, b/c, and d (2, 15). Antibody reactivity to the antigen of clones 28 and Q7 was also adsorbed by intact bacteria, indicating that they may also produce outer membrane proteins or other surface antigens. The finding that the intact Hib completely absorbed out the reactivity of the rabbit anti-Hib to the clones suggests that the rabbits only responded to surface antigens or that only surface determinants were expressed by the clones. However, the production of antibody only to surface determinants is consistent with the report of Gulig et al. (8), showing that after systemic Hib infection children develop antibodies only to surface determinants.

The lambda gt11 Amp1 is a replacement vector which lysogenizes hRA50 E. coli BTA282 at 30°C to grow as a single copy. Because of the temperature-sensitive cl857 mutation and the S100 lysis mutation it can be induced to accumulate phage products and antigen at 42°C. Once colonies producing antigen were isolated, colony DNA hybridization testing was performed to find other antigen-producing clones containing the same or related Hib sequences. When such relationships where determined, these colonies were assumed to produce the same protein. The correctness of this assumption could have been tested by Southern blotting. However, as it turned out most of the
colonies formed a single group (antigen 2) and anti-2 affinity-purified antibody reacted with all clones related by DNA hybridization and only reacted with a single outer membrane band on Western blotting. The conclusion is that the Hib (Ca) isolates used in this study produce only one protein of this antigenic type. However, the production of antigenically related and cross-hybridizing membrane proteins could be envisaged, although anti-25 and anti-33 similarly only reacted with one band on Western blotting.

The clones H4, H5, H6, and H7 were detected by reaction with some human sera. Unlike the clones recognized by hyperimmune rabbit anti-Hib, the reactivity of serum to these clones could not be absorbed out by intact Hib. Correspondingly, affinity-purified antibody to clone H5 did not react with outer membrane vesicles in dot-blot assays and so is presumably not an outer membrane protein. That H5 does in fact produce an Hib antigen was determined by showing that H5 DNA hybridized with Hib DNA in Southern analysis and affinity-purified anti-H5 reacted with Hib lysates in dot-blot assays. The antigens produced by clones H4, H6, and H7 (it is not known whether H4, H6, and H7 are related) are also apparently internal proteins of Hib and appear to be unusual in that several rabbits, rats, and mice hyperimmunized with live or formalinized Hib cells have not produced detectable antibodies to them (data not shown).

They are however detected by antibodies in the sera of about 30% of laboratory personnel. The antigens originally detected by rabbit anti-Hib, including the surface proteins in clone 2, 33, and 25, are also detected by antibodies in normal adult human sera and sera from children convalescent from meningitis (manuscript in preparation). Studies to determine isolate distribution and the suitability of the cloned outer membrane surface protein for protective immunization are in progress.

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LITERATURE CITED


