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Cloning and Expression in *Escherichia coli* of the Gene Encoding the Heat-Modifiable Major Outer Membrane Protein of *Haemophilus influenzae* Type b

FRANK R. GONZALES,[1] SANCY LEACHMAN,[1] MICHAEL V. NORGARD,[1] JUSTIN D. RADOLF,[2] GEORGE H. McCRACKEN, JR.,[3] CLAUDIA EVANS,[4] AND ERIC J. HANSEN[1]*

Department of Microbiology, Southwestern Graduate School of Biomedical Sciences,[1] Departments of Internal Medicine,[2] and Pediatrics,[3] Southwestern Medical School, University of Texas Health Science Center at Dallas, and the Pre-Clinical Science Unit, Veteran's Administration Medical Center,[4] Dallas, Texas 75235

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One major outer membrane protein (P1) of *Haemophilus influenzae* type b (Hib), with an apparent molecular weight of 34,000 (34K) as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), has been shown to be heat modifiable. After heating at 100°C for 5 min in 2% SDS, the P1 protein exhibits an apparent molecular weight of 49,000 (49K) in SDS-PAGE. Monoclonal antibodies (MAbs) reactive with P1 bound to the surface of Hib, and one of these MAbs had a protective effect against the development of Hib bacteremia in an animal model for invasive Hib disease. A 6-kilobase Hib DNA insert containing the gene encoding this P1 protein was cloned into *Escherichia coli* by using the λgt11 expression vector. Recombinant phage expressing P1 were identified by screening phage plaques with a MAb directed against the P1 protein. Expression of the P1 protein by an *E. coli* lysogen carrying the recombinant phage was independent of both vegetative phage growth and induction of *lacZ* gene-directed transcription of the Hib DNA insert. The Hib DNA insert encoding the P1 protein was subcloned into the plasmid vector pBR322, and a transformant containing the recombinant plasmid pFRG100 was identified with the P1 protein-directed MAb in a colony blot-radioimmunoassay. Western blot (immunoblot) analysis determined that the recombinant P1 protein possessed heat-modifiability characteristics identical to those of the native Hib protein. The P1 protein was expressed on the surface of both the *E. coli* lysogen containing the recombinant phage and the *E. coli* transformant containing pFRG100. Western blot analysis of acute- and convalescent-phase sera from infants with Hib meningitis showed that antibodies in the convalescent-phase sera recognized the P1 protein expressed by the *E. coli* transformant containing pFRG100. The availability of this cloned Hib DNA insert encoding the Hib P1 protein and the expression of this protein on the surface of recombinant *E. coli* should facilitate the investigation of P1 for both its vaccinogenic potential and its functional role in the outer membrane of Hib.

Haemophilus influenzae type b (Hib) remains the leading cause of bacterial meningitis in the United States (3). The polysaccharide capsule which surrounds Hib is both the primary virulence factor for this organism and a target for antibodies protective against systemic Hib disease (3, 8, 19, 24). Noncapsular surface antigens of this pathogen, however, also have been shown to bind protective antibodies. In particular, outer membrane proteins with epitopes exposed on the surface of Hib have been identified, and antibodies to some of these proteins protect against experimental Hib disease in active or passive immunization experiments (15, 21).

Recombinant DNA technology has the potential to facilitate investigation of Hib outer membrane proteins for both their vaccinogenic potential and their structure-function relationships. A gene encoding a 27,000-molecular-weight surface protein of Hib has been cloned and expressed in *Escherichia coli* (12), and the availability of monoclonal antibody (MAb) probes for this and other Hib outer membrane proteins (15, 23) should facilitate the use of molecular cloning techniques in the study of Hib surface proteins. Here we describe the use of the bacteriophage λgt11 cloning vector to prepare recombinant clones of *E. coli* that express the heat-modifiable major outer membrane protein of Hib. This protein (P1) has an apparent molecular weight (after heating at 100°C for 5 min in 2% sodium dodecyl sulfate [SDS]) of 49,000 (49K) in SDS-polyacrylamide gel electrophoresis (PAGE), whereas preparations of this protein incubated at 37°C for 20 min exhibit an apparent molecular weight of 34,000 (34K) (4, 29; Fig. 1). We also present data which indicate that a MAb to this Hib surface protein has protective efficacy against experimental Hib bacteremia.

MATERIALS AND METHODS

Bacterial strains and DNA cloning vectors. Two different Hib strains (DL41 and DL42) were used in most of the experiments in this study; both were isolated from the cerebrospinal fluid of infants with Hib meningitis and have been described (9). The source of Hib chromosomal DNA used in this study was Hib strain DL41. Hib strain DL42 was used for the production of P1-specific MAbs and in immunoprotection tests. Hib strain LA203, used in immunoprotection tests, is a cerebrospinal fluid isolate which belongs to the same lipooligosaccharide antigenic group (group 2) as Hib DL42 (9) but does not react with the P1-specific MAb 7C8 in the colony blot-radioimmunoassay (RIA). Media and growth conditions for Hib were identical to those used by Holmans et al. (12).

The bacteriophage expression vector λgt11 (*lac5 cI857 nin5 S100*) was used to construct Hib genomic DNA libraries (13). Plasmid subcloning was accomplished using pBR322 (18).

* Corresponding author.

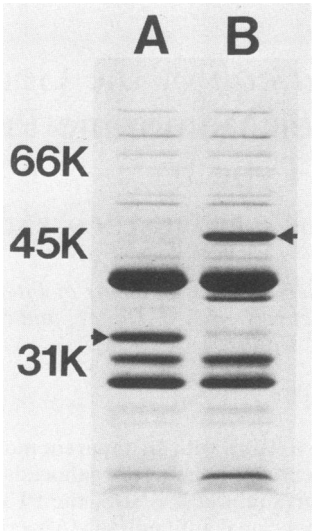

FIG. 1. Heat modifiability of the Hib P1 protein. Outer membrane vesicles (45 μg of protein) of Hib strain DL42 were suspended in digestion buffer (10) and incubated at 37°C for 20 min (lane A) or at 100°C for 5 min (lane B). These preparations were resolved by SDS-PAGE in a 10% (wt/vol) polyacrylamide gel, and the proteins were stained with Coomassie blue. Molecular weight reference markers are provided on the left side of this figure. Arrows: lane A, unmodified P1; lane B, heat-modified P1.

Recombinant λgt11 phage were plated on a derivative of *E. coli* Y1090 (Δ*lacU169 proA*$^+$ Δ*lon araD139 strA supF* [*trpC22*::Tn*10*]; pMC9) that had been rendered restriction negative and was obtained from Promega Biotech (Madison, Wis.) (13). Stable lysogens were established by using *E. coli* Y1089 (Δ*lacU169 proA*$^+$ Δ*lon araD139 strA hflA150* [*chr*::Tn*10*]; pMC9) (13). Transformation experiments involved the use of *E. coli* HB101 (F$^-$ *hsdS20* [r_B^- m_B^-] *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$) (18). All *E. coli* strains were grown in L broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) or on L agar plates.

Construction of genomic libraries. Chromosomal Hib DL41 DNA was purified from a 1-liter overnight culture as described by Bricker et al. (2). Partial *Eco*RI digestion of Hib strain DL41 chromosomal DNA was accomplished by incubating 126 μg of Hib DNA with 0.85 U of *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in a final volume of 224 μl under the conditions recommended by the manufacturer. The extent of digestion was evaluated by agarose gel electrophoresis (18). DNA fragments ranging in size from 2 to 7 kilobases (kb) were isolated by sucrose density gradient centrifugation (1) by using end-labeled λ-*Hin*dIII fragments as size markers in a separate gradient. Dephosphorylated, *Eco*RI-digested λgt11 arms (500 ng; Promega) were ligated to 20 ng of the size-fractionated Hib *Eco*RI fragments by using 1 U of T4 DNA ligase (Boehringer) in a final volume of 5 μl at 16°C for 2 h. This mixture was packaged into phage heads in vitro using 50 μl of packaging extract (Promega) for 2 h at room temperature (RT). The resulting phages were titered on *E. coli* Y1090 at 42°C; IPTG (isopropyl β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were added to determine the percentage of recombinants (13).

Production of P1 protein-directed MAbs. A BALB/c mouse was immunized with viable cells of Hib strain DL42, and the spleen from this mouse was used in the construction of plasma cell hybridomas as described by Robertson et al. (25). Hybridomas secreting MAbs reactive with Hib DL42 outer membrane antigens were identified in an enzyme-linked immunosorbent assay by using outer membrane vesicles of Hib strain DL42 as antigen (25). MAbs (6B1 and 7C8) specific for the P1 protein were identified in a Western blot (immunoblot) analysis performed as described by Kimura et al. (15), using Hib DL42 outer membrane vesicles solubilized at 100°C for 5 min in digestion buffer (10) as the source of P1 antigen. Isotype analysis of the P1-directed MAbs was performed by Ellen S. Vitetta of the Department of Microbiology at this institution, using affinity-purified polyclonal rabbit antisera specific for the heavy chains of the different murine immunoglobulins.

Immunoprotection tests. MAb 7C8 was affinity purified on protein A-Sepharose and used to passively immunize 7-day-old infant rats as described previously (15). MAb 6B5, an immunoglobulin G2a (IgG2a) MAb directed against an outer membrane protein of a nontypable *H. influenzae* strain, was purified on the same protein A-Sepharose column and was used as a negative control. Challenge of the animals with Hib and quantitation of bacteremia 24 h postchallenge was accomplished as described earlier (15). Results of the immunoprotection experiments were analyzed by using the Student's *t* test.

Immunoscreening of genomic libraries. Hib genomic libraries were plated on *E. coli* Y1090 at a density of approximately 800 PFU per plate (90-mm diameter) at 42°C for 4 h. Dry nitrocellulose filters (0.45 μm, pore size; Schleicher & Schuell, Inc., Keene, N.H.) previously impregnated with IPTG (13) were applied to the agar surface and incubated at 37°C overnight. The filters were removed and washed five times in Tris-buffered saline (TBS; 50 mM Tris hydrochloride, pH 7.5, containing 150 mM NaCl, 0.02% [wt/vol] NaN_3, and 0.008% [wt/vol] NaI) to remove debris. All washing and incubation steps throughout this procedure were performed at RT with gentle agitation for 10 min unless otherwise specified. Nonspecific protein-binding sites were blocked with TBS containing 1% (wt/vol) nonfat dry milk (TBS-milk) for 1 h at 4°C. Hybridoma culture supernatant (15 ml) containing the P1-specific MAb 6B1 was incubated with each filter for 4 h at 4°C. Unbound MAb was removed by washing once in TBS plus 0.1% (vol/vol) Nonidet P-40 followed by five TBS washes. Filter-bound MAb was detected with affinity-purified and radioiodinated goat anti-mouse immunoglobulin (9, 15) diluted in TBS-milk. Filters were washed five times in TBS, dried, and used for autoradiography (10). Plaques reactive with MAb 6B1 were picked, rescreened, and subjected to several cycles of single plaque purification until all plaques reacted with this MAb.

Lysogen formation. Stable lysogens of immunoreactive phage clones were established in *E. coli* Y1089 as described previously (13). Cells were infected with recombinant phage at a cell/phage ratio of 1:10 in L broth containing 10 mM $MgCl_2$ plus 0.20% (wt/vol) maltose at 32°C for 15 min and then plated overnight at 30°C. Random colonies were subsequently tested for temperature sensitivity at 37°C. Those strains which grew at 30°C and not at 37°C were assumed to be lysogens and were tested for antigen production by colony blot-RIA with MAb 6B1 as described earlier (15, 16).

Subcloning and restriction enzyme mapping of the Hib DNA insert. DNA prepared from recombinant phage by the plate lysate method (18) was digested to completion with *Eco*RI, and the fragments were separated by electrophoresis in 1% agarose (SeaKem GTG agarose; FMC Corp., Marine

Colloids Div., Rockland, Maine). The Hib DNA insert was extracted from the gel with 10 mM Tris hydrochloride (pH 8.0) containing 1 mM EDTA and was ligated to *Eco*RI-digested pBR322 (18). This ligation mixture was then used to transform *E. coli* HB101 made competent by the calcium chloride-rubidium chloride procedure (18). The resulting transformants were screened for antigen expression in the colony blot-RIA with MAb 6B1.

Plasmid DNA (pBR322 and pFRG100) was extracted and purified by cesium chloride density centrifugation as previously described (12, 18). The Hib DNA insert in pFRG100 was mapped by using various restriction endonucleases under the manufacturer's recommended conditions followed by electrophoresis in 1% (wt/vol) agarose gels (18).

Western blot procedures. Antigens in Hib and recombinant *E. coli* that were reactive with MAb 6B1 were characterized by the immunoblot method of Towbin et al. (27). Whole-cell lysates (5×10^8 CFU of Hib or *E. coli* solubilized in digestion buffer as described previously [15]) were subjected to SDS-PAGE in 10% (wt/vol) polyacrylamide slab gels (10, 11), and the proteins were electrophoretically transferred to nitrocellulose membranes. After nonspecific protein binding sites were saturated with TBS-milk, the membranes were reacted with MAb 6B1 or MAb 7C8 (15). The washing steps and the detection of filter-bound MAb with radiolabeled goat anti-mouse immunoglobulin were identical to the methods used to immunoscreen phage plaques (described above).

A modified Western blot procedure was used when human sera were used as the source of primary antibody. Acute- and convalescent-phase serum samples (150 μl) obtained from two infants with Hib meningitis were absorbed for 2 h at RT with 12 ml of an *E. coli* HB101(pBR322) suspension in pH 7.2 phosphate-buffered saline (PBS) plus 0.05% (vol/vol) Tween 20 (Tween). This *E. coli* suspension consisted of a 1:1 mixture of whole *E. coli* cells (0.5 g, wet weight) and disrupted (sonicated) *E. coli* cells (0.5 g, wet weight). Nitrocellulose membrane strips containing SDS-PAGE-resolved proteins were incubated in PBS-Tween for 1 h at RT to inhibit nonspecific protein binding. These membrane strips were then reacted with 4 ml of the *E. coli*-absorbed serum or with 4 ml of hybridoma culture supernatant containing MAb 6B1 for 6 h at RT. These strips were incubated next in PBS-Tween overnight at 4°C and then washed three times with PBS-Tween (20 min per wash). Human antibody bound to antigens on these strips was detected with a 1:3,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Cappel Laboratories, Cochranville, Pa.) in PBS-Tween containing 0.10% (wt/vol) bovine serum albumin. This secondary antibody probe was absorbed with *E. coli* HB101(pBR322) as described above prior to use in this system. MAb 6B1 bound to antigens on these strips was detected with HRP-conjugated goat anti-mouse IgG (Cappel) diluted 1:1,000 in PBS-Tween-bovine serum albumin. These secondary antibodies were applied for 4 h at RT, and unbound secondary antibody was removed by four 20-min PBS-Tween washes. A tertiary antibody probe was included to increase sensitivity. HRP-conjugated rabbit anti-goat IgG (Cappel) was diluted 1:3,000 in PBS-Tween-bovine serum albumin, absorbed with *E. coli* HB101(pBR322) as described above, and incubated with the nitrocellulose strips overnight at 4°C. After four 20-min washes with PBS-Tween, immunoreactive bands were detected by addition of the HRP substrate (4-chloro-1-naphthol) as described (15).

AA-RIA. The indirect antibody-accessibility RIA (AA-RIA) (16) was used to evaluate the surface exposure of the epitopes recognized by P1-directed MAbs on both Hib and recombinant *E. coli* cells. The indirect AA-RIA was performed with Hib strains DL41 and DL42 and MAbs 7C8 and 6B1 as described by Kimura et al. (16). The *E. coli* Y1089 lysogens tested in the AA-RIA were grown overnight at 30°C on L-agar plates. Similarly, *E. coli* transformants harboring plasmids were grown on L-agar plates containing 100 μg ampicillin per ml at 37°C overnight. Cell suspensions were made in cold (4°C) PBS containing 10% (vol/vol) heat-inactivated fetal calf serum (PBS-FCS) and were diluted to 10^8 CFU/ml.

Triplicate determinations were made with each strain tested. A 0.20-ml portion of the cell suspension was incubated with 1 ml of hybridoma culture supernatant at 4°C for 1 h with gentle agitation. Cells were collected by centrifugation (12,000 × *g*, 5 min), suspended in PBS-FCS, and centrifuged again to remove unattached MAb. MAb bound to the surface of the bacterial cells was detected by incubating the washed cells with radioiodinated and affinity-purified goat anti-mouse immunoglobulin (10^6 cpm per sample) for 90 min at 4°C. The bacteria were then washed twice with 1 ml of PBS-FCS and finally suspended in 0.50 ml of solubilization buffer (16). Radioactivity associated with these cells was measured with a Searle gamma counter (Searle Analytic Inc., Chicago, Ill.).

Electron microscopy. Visualization of immune complexes on bacterial cell surfaces was performed as described previously by Holmans et al. (12) with the following modification. After unattached MAbs were removed by washing in PBS-FCS, the cells were suspended in 1.0 ml of PBS-FCS containing 30 μl of a complex of colloidal gold and goat anti-mouse immunoglobulin (E.Y. Laboratories, San Mateo, Calif.). The cells were incubated in this mixture for 1 h at 23°C and then washed twice with PBS-FCS, twice with PBS, resuspended in 0.5 ml PBS, and applied to carbon-coated grids, followed by negative staining and electron microscopy (12). Cells were photographed at a magnification of ×20,000 to ×32,000.

RESULTS

Characterization of P1 protein-directed MAbs. Hib strain DL42 was used to immunize mice for the production of plasma cell hybridomas secreting MAbs directed against Hib outer membrane antigens. MAbs reactive with both the unheated and heat-modified forms of the P1 protein were identified in Western blot analysis using outer membrane vesicles of Hib strain DL42 as the source of this antigen. MAbs 7C8 and 6B1 reacted with both the heat-modified and the unheated forms of this major outer membrane protein of Hib DL42 (Fig. 2). Colony blot-RIA analysis of the strain specificity of these two MAbs revealed that MAbs 7C8 and 6B1 each reacted with 63 (52%) of 120 Hib strains (data not shown). The use of the indirect AA-RIA to detect antigenic determinants on the surface of Hib revealed that both of these MAbs bound to the surface of the homologous immunizing strain of Hib (DL42) and to the surface of Hib strain DL41 (Table 1).

Protective ability of a P1 protein-directed MAb. Isotype analysis determined that MAb 6B1 was a murine IgG1 antibody, whereas MAb 7C8 was shown to be a murine IgG2a antibody. MAb 7C8 was purified and used to passively immunize infant rats in an immunoprotection test (15). Although MAb 7C8 was unable to prevent the development of bacteremia in most animals immunized with this MAb, infant rats passively immunized with purified MAb 7C8

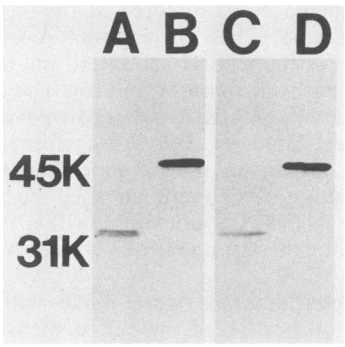

FIG. 2. Western blot analysis of the reactivity of MAbs 6B1 and 7C8 with the unheated and heat-modified forms of P1. Outer membrane vesicles (10 μg of protein) of Hib strain DL42 suspended in digestion buffer (10) were incubated at 37°C for 20 min (lanes A and C) or at 100°C for 5 min (lanes B and D). The proteins in these preparations were resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with MAb 6B1 (lanes A and B) or MAb 7C8 (lanes C and D).

developed significantly lower levels of bacteremia after challenge with Hib strain DL42 than did animals immunized with only PBS or with an irrelevant IgG2a MAb (Table 2). In contrast, no protective effect was observed when infant rats passively immunized with MAb 7C8 were challenged with Hib strain LA203 (Table 2), which was shown by colony blot-RIA to lack the antigenic determinant recognized by MAb 7C8. This last result confirms the antigenic specificity of the protective effect of MAb 7C8 against Hib strain DL42.

Molecular cloning of the gene encoding the P1 protein. The fact that our MAb to the P1 protein had protective activity against experimental Hib disease, together with similar data concerning the protective activity of P1-directed polyclonal antiserum (8), prompted us to clone the Hib gene encoding the P1 protein into *E. coli* for further analysis. The genomic library consisted of 10^5 phage, and the efficiency of insertion of Hib DNA into λgt11 was 80%. MAb 6B1 was used to screen 3×10^4 phage plaques for expression of the P1 protein. Seventeen different antibody-reactive plaques were identified, and the phage from one of these was purified by repeated single-plaque isolation. The DNA from this recombinant phage expressing an antigen reactive with MAb 6B1 was digested to completion with *Eco*RI and shown to contain a single 6-kb insert of Hib DNA (data not shown).

TABLE 1. Reactivity of MAbs 6B1 and 7C8 with whole cells of Hib strains DL41 and DL42[a]

Bacterial strain and MAb	Amt (cpm) of probe bound[b]
DL41	
6B1	9,880
7C8	8,415
5D6[c]	210
Sp2/0[d]	103
DL42	
6B1	13,960
7C8	14,022
5D6[c]	339
Sp2/0[d]	214

[a] Binding of MAbs to whole cells of these Hib strains was measured in the AA-RIA as described in Materials and Methods.
[b] Counts per minute of radioiodinated goat anti-mouse immunoglobulin bound to Hib cells incubated with the designated MAb. Data represent the average of triplicate samples.
[c] MAb 5D6 is an IgG MAb which recognizes *T. pallidum*; it is included here as a negative control for nonspecific binding of an IgG MAb to Hib.
[d] Negative control involving supernatant from Sp2/0-Ag14 cells (25) to check for nonspecific binding of the radioiodinated probe to Hib cells.

TABLE 2. Effect of passive immunization with MAb 7C8 on development of Hib bacteremia in an animal mode

Immunization agent[a]	Challenge strain[b]	Animals (no. bacteremic/no. challenged)	Level of bacteremia (CFU/10 μl of blood [mean ± SEM])[c]
PBS	DL42	9/9	221 ± 46
MAb 6B5[d]	DL42	10/10	299 ± 27
MAb 7C8	DL42	7/8	6 ± 2[f]
PBS	LA203[e]	10/10	163 ± 17
MAb 6B5	LA203	9/9	146 ± 24
MAb 7C8	LA203	8/8	179 ± 31[g]

[a] Infant rats were passively immunized with 0.1 ml of PBS or with 100 μg of MAb 6B5 or MAb 7C8 in 0.1 ml of PBS.
[b] Infant rats were challenged intraperitoneally with 100 to 150 CFU of Hib.
[c] Bacteremia was quantitated 24 h after challenge as described previously (15).
[d] Negative control. MAb 6B5 is an IgG2a MAb directed against a surface epitope of the major outer membrane protein of a nontypable *H. influenzae* strain and does not bind to Hib.
[e] Hib strain LA203 does not react with MAb 7C8 in the colony blot-RIA.
[f] Significantly different from results obtained with MAb 6B5 and Hib strain DL42 ($P < 0.01$).
[g] Not significantly different from results obtained with MAb 6B5 and Hib strain LA203.

Characterization of the Hib gene product synthesized in *E. coli*. *E. coli* Y1089 was lysogenized with this recombinant phage by standard methods (13). We next determined whether this Hib gene product synthesized in *E. coli* exhibited the heat-modifiability characteristic of the native Hib P1 protein. The lysogen carrying the recombinant phage was heat shocked, and samples of these cells obtained at the start of the experiment and 1 h later were solubilized in SDS and heated at 37°C for 20 min or at 100°C for 5 min. The Hib gene product synthesized in *E. coli* (Fig. 3, lanes C to F) was

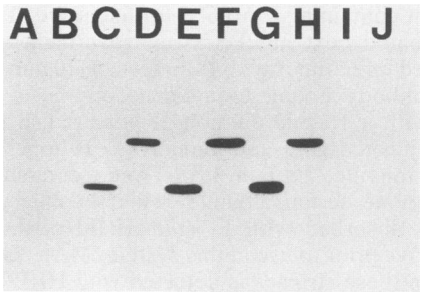

FIG. 3. Autoradiograph of Western blot analysis of the reactivity of MAb 6B1 with the unmodified and heat-modified forms of the recombinant P1 protein. A lysogen of λgt11 (negative control) and a lysogen of the recombinant phage were heat shocked at 42°C and then incubated at 37°C, and whole-cell lysates were prepared from these cells at 0 and 60 min. These lysates and a whole-cell lysate of Hib strain DL41 were solubilized in digestion buffer (10) and heated at 37°C for 20 min or at 100°C for 5 min before being subjected to SDS-PAGE and Western blot analysis as described in the legend to Fig. 5. Lanes: A, λgt11 lysogen (t = 0 [min], 37°C); B, λgt11 lysogen (t = 0, 100°C); C, recombinant lysogen (t = 0, 37°C); D, recombinant lysogen (t = 0, 100°C); E, recombinant lysogen (t = 60, 37°C); F, recombinant lysogen (t = 60, 100°C); G, Hib strain DL41 (positive control, 37°C); H, Hib strain DL41 (100°C); I, λgt11 lysogen (t = 60, 37°C); J, λgt11 lysogen (t = 60, 100°C).

indistinguishable from the native Hib protein (Fig. 3, lanes G and H) with regard to its heat modifiability. These data also indicate that the MAb 6B1-reactive gene product synthesized by this recombinant lysogen was not part of a fusion protein with β-galactosidase. Additional control experiments determined that the addition of IPTG after thermal inactivation of the temperature-sensitive λ repressor did not increase the level of expression of the Hib P1 protein by the lysogen (data not shown). Also, the Hib P1 protein synthesized by the recombinant lysogen growing at 30°C (to prevent prophage induction) was expressed on the *E. coli* cell surface (data not shown).

Subcloning of the Hib DNA insert. The 6-kb Hib DNA insert encoding the P1 protein was excised from the recombinant phage and subcloned into the unique *Eco*RI restriction site in the plasmid vector pBR322. After ligation, the reaction mixture was used to transform *E. coli* HB101 and transformants expressing the P1 protein were identified by screening with MAb 6B1 in the colony blot-RIA. A transformant reactive with this MAb was shown to contain a plasmid containing the 6-kb insert of Hib DNA (Fig. 4). This plasmid was designated pFRG100. The P1 protein expressed by the transformant containing pFRG100 was heat modifiable, just like the native Hib protein (data not shown). The use of the indirect AA-RIA indicated that the P1 protein synthesized by the transformant containing pFRG100 was expressed on the surface of this recombinant *E. coli* strain (Table 3). This finding was confirmed by the use of immunoelectron microscopy in which a colloidal gold-labeled goat anti-mouse immunoglobulin probe was used to detect the P1-specific MAb 7C8 bound to the surface of the recombinant *E. coli* strain containing pFRG100 (Fig. 5).

Restriction enzyme mapping of the Hib DNA insert. Restriction enzyme analysis of pFRG100 determined that several restriction enzymes had unique sites within the Hib DNA inserts. *Ava*I, *Bam*HI, *Hin*dIII, *Pvu*I, and *Xba*I each had single recognition sites in the Hib DNA insert (Fig. 6). Two restriction enzymes (*Sal*I and *Cla*I) did not cleave the Hib DNA insert.

Reactivity of the recombinant P1 gene product with polyclonal human sera. The experiments described above suggested that the Hib P1 protein synthesized in *E. coli* was similar, if not identical, to the native Hib protein. All of these data were obtained, however, by using MAbs which have a high degree of specificity and which each recognize only a single epitope on this relatively large polypeptide. To deter-

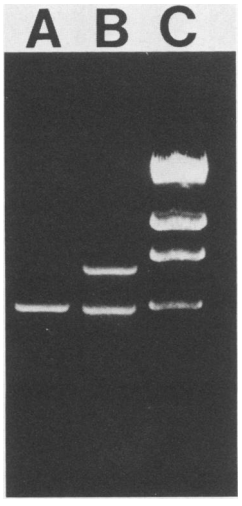

FIG. 4. Characterization of the Hib DNA insert in pFRG100. DNA samples were subjected to electrophoresis in 1% (wt/vol) agarose. Lanes: A, pBR322 digested with *Eco*RI; B, pFRG100 digested with *Eco*RI; C, bacteriophage λ-*Hin*dIII molecular weight markers (23.3 [top], 9.5, 6.4, 4.2, 2.2, and 2.0 kb [bottom]).

TABLE 3. Binding of MAb 7C8 to the surface of Hib and *E. coli* transformants carrying pBR322 or pFRG100[a]

Bacterial strain and antibody	Amt (cpm) of probe bound[b]
Hib DL41	
Sp2/0[c]	1,653
5D6[d]	2,058
7C8	56,219
E. coli HB101(pBR322)	
Sp2/0	4,682
5D6	5,981
7C8	4,058
E. coli HB101(pFRG100)	
Sp2/0	4,203
5D6	4,882
7C8	76,647

[a] Binding of MAbs to these whole bacterial cells was measured in the indirect AA-RIA.

[b] Counts per minute of radioiodinated goat anti-mouse immunoglobulin bound to Hib cells incubated with the designated MAb. Data represent the average of triplicate samples.

[c] Negative control involving supernatant from Sp2/0-Ag14 cells (25) to check for nonspecific binding of the radioiodinated probe to Hib cells.

[d] MAb 5D6 is an IgG MAb which recognizes *T. pallidum*; it is included here as a negative control for nonspecific sticking of an IgG MAb to the *E. coli* cells.

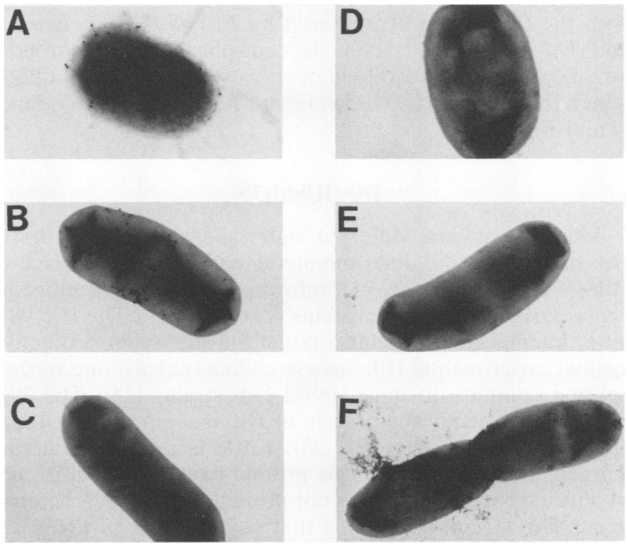

FIG. 5. Electron microscopic visualization of immune complexes on the bacterial cell surface. Bacterial cells (Hib or *E. coli*) were incubated with MAbs and then probed with colloidal gold-labeled goat anti-mouse immunoglobulin to detect MAbs bound to the cell surface, as described in Materials and Methods. MAb 7C8 is directed against the Hib P1 protein; Mab 5D6 is an irrelevant control MAb directed against *Treponema pallidum*. Panels: A, Hib DL42 with MAb 7C8; B, *E. coli*(pFRG100) with MAb 7C8; C, *E. coli*(pBR322) with MAb 7C8; D, Hib DL42 with MAb 5D6; E, *E. coli*(pFRG100) with MAb 5D6; F, *E. coli*(pBR322) with MAb 5D6.

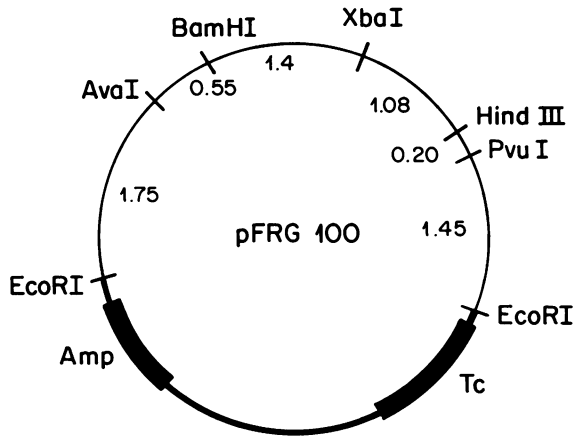

FIG. 6. Partial restriction enzyme map of the 6-kb insert in pFRG100 that encodes the Hib P1 protein. Distances between restriction sites are given in kilobases. Amp and Tc refer to the ampicillin and tetracycline resistance genes from pBR322.

mine whether this recombinant gene product would react with polyclonal antibodies originally raised against the native Hib P1 protein, we proceeded to use the *E. coli* transformant containing the recombinant plasmid pFRG100 to study the immune response of infants convalescing from Hib meningitis. It has been previously shown that infants convalescing from Hib meningitis produce an antibody response to Hib outer membrane proteins and that most of these antibodies are directed against surface epitopes of these proteins (10). Acute- and convalescent-phase sera from two infants with Hib meningitis were used in Western blot analysis together with the *E. coli* transformant containing pFRG100 as the source of recombinant P1 protein. Acute-phase sera from both infants lacked antibody reactive with the P1 protein synthesized by *E. coli* (Fig. 7, lanes C and E). In contrast, convalescent-phase sera from both infants contained readily detectable amounts of antibody which reacted with the recombinant P1 protein (Fig. 7, lanes D and F).

DISCUSSION

Although several different outer membrane proteins of Hib have antigenic determinants exposed on the surface of Hib (10, 12, 15, 20, 21, 23), information about the antigenic characteristics of these proteins is still limited. The Hib 98K outer membrane protein is a target for antibodies protective against experimental Hib disease and has at least one surface epitope common to most (69%) Hib strains (15). The 39K major outer membrane protein of Hib is a porin as well as a protective immunogen (21, 28). Little is known about the antigenic composition of this protein except that antiserum to purified 39K protein was not protective against a heterologous Hib strain, suggesting that some degree of heterogeneity exists among Hib strains with regard to surface epitopes of this protein (21). The first Hib outer membrane protein shown to possess a surface epitope common to all Hib strains, as well as nontypable *H. influenzae*, is the P6 or 14K to 16K protein described by Murphy et al. (23). A MAb to this protein blocked serum bactericidal activity in normal human serum directed against nontypable *H. influenzae* (22), and Munson et al. (20) have shown that immunization with a P6-cell wall complex induced the synthesis of antibodies protective against experimental Hib disease.

The results of the present study indicate that a MAb to the heat-modifiable major outer membrane protein (P1) of Hib was effective in significantly reducing the level of bacteremia which developed in infant rats after challenge with Hib (Table 2). This same P1 protein was recently purified by Granoff and Munson (8), who showed it could induce a low level of immunity to experimental Hib disease. The inability of these polyclonal antibodies and our MAb to protect completely against experimental Hib disease may be explained in several ways. The particular purification protocol employed by Granoff and Munson (8) may have destroyed immunogenic surface epitopes of the P1 protein. Similarly, the use in passive immunization tests of a MAb which recognizes only a single epitope in this large polypeptide limits the number of opsonizing or bactericidal antibodies which can attach to the Hib cell. In addition, this particular MAb may simply be relatively ineffective in opsonizing or lysing Hib. A precedent for this situation has been reported with *Neisseria gonorrhoeae* in which two MAbs of the same subclass which bound to surface epitopes on the same antigen differed markedly in their bactericidal activities (14).

Van Alphen et al. (30) showed that this P1 protein was consistently immunogenic in infants convalescing from systemic Hib disease. In contrast, Loeb and Smith (17) reported

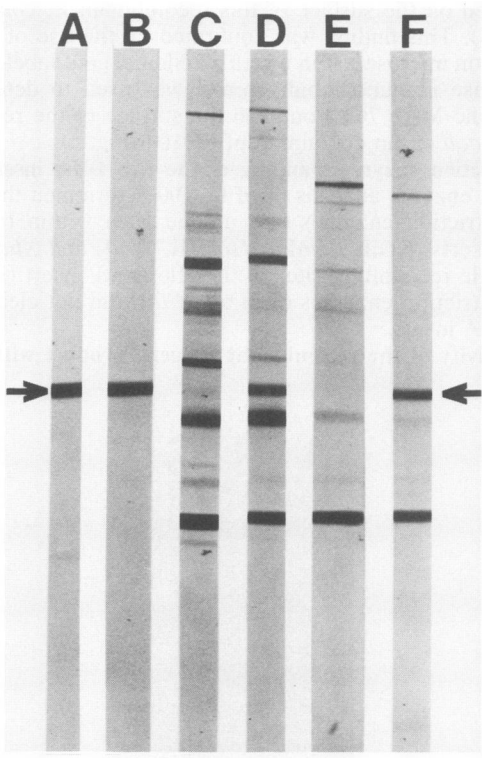

FIG. 7. Western blot analysis of the reactivity of human polyclonal antibodies with the recombinant form of P1. Whole-cell lysates of the *E. coli* transformant containing pFRG100 were used as antigen in lanes B to F, and a whole-cell lysate of Hib strain DL41 was used as antigen in lane A. Western blot analysis with MAb 6B1 and with acute- and convalescent-phase serum from infants with Hib meningitis was performed as described in Materials and Methods. The primary antibodies were as follows (lanes): A and B, MAb 6B1; C, acute-phase serum from infant 1; D, convalescent-phase serum from infant 1; E, acute-phase serum from infant 2; F, convalescent-phase serum from infant 2. The arrows indicate the position of the P1 protein.

that infants with Hib meningitis did not consistently produce a convalescent-phase antibody response to the P1 protein, while Erwin and Kenny (6) have reported that this protein exhibits some degree of antigenic heterogeneity among Hib strains. We chose to employ recombinant DNA methods to clone the Hib gene encoding this protein in order to facilitate further analysis of the immune response to this major surface antigen of Hib. In addition, the availability of the cloned structural gene for the P1 protein would permit eventual genetic and structure-function analyses of this protein and its role in the outer membrane of Hib.

The λgt11 expression vector used in our experiments previously has been successfully used by Gotschlich et al. (7) to clone the genes encoding the H8 antigens and protein III of *N. gonorrhoeae*. This same vector was also used recently to clone three Hib genes which encode surface antigens of this pathogen (26). In the latter report, one of these Hib gene products corresponded in size to the P1 protein, but the absence of additional data in this previous report concerning the heat-modifiability and antigenic characteristics of this protein preclude accurate identification of this antigen (26).

The expression of the P1 protein by the recombinant phage containing a 6-kb Hib DNA insert did not require either vegetative growth of this phage or induction of *lacZ*-directed transcription of the Hib DNA insert. In fact, thermal induction of the prophage and the presence of IPTG apparently did not significantly increase the amount of P1 protein expressed by the *E. coli* lysogen carrying the recombinant phage. This finding implied that the native Hib promoter was being used for transcription in *E. coli*. Although the orientation of the Hib DNA insert in either the recombinant phage or plasmid pFRG100 was not determined in the present study, previous work from our laboratory showed that a 27,000-molecular-weight surface protein of Hib is expressed equally well from a Hib DNA insert regardless of its orientation in a recombinant plasmid (12). Whether the expression in *E. coli* of gonococcal protein III (7) or the Hib gene products described by Rossi and Thomas (26) are dependent on transcription of the *lacZ* gene region of the λgt11 vector has not been reported, although some gonococcal promoters are known to be functional in *E. coli* (Janne G. Cannon, personal communication).

In addition to being expressed constitutively by both our recombinant lysogen and the transformant containing pFRG100, the recombinant P1 protein was localized on the surface of both of these *E. coli* strains. Surface expression of foreign protein molecules by recombinant *E. coli* has been reported to occur with certain *Legionella pneumophila* antigens (5) and with another Hib surface protein (12). The availability of *E. coli* cells expressing the Hib P1 protein on their surfaces will make it possible to study the immunogenicity of this protein in a different antigenic background, as well as the interaction of this antigen with P1 protein-directed antibodies. Surface expression of this P1 protein in *E. coli* will also permit the use of these recombinant cells in the affinity purification of P1 protein-directed antibodies from polyclonal sera (5).

It is interesting to note that the heat-modifiability characteristics of the recombinant P1 protein and those of the native Hib protein were apparently identical. Gotschlich et al. (7) have similarly reported that gonococcal protein III synthesized in *E. coli* is reduction modifiable just like the native gonococcal protein. These data reinforce the fact that the Hib gene encoding the P1 protein is transcribed and translated with a high degree of accuracy in *E. coli*.

A recombinant *E. coli* strain expressing the Hib P1 protein also proved useful in the characterization of the immune response of infants to systemic Hib disease. Two infants convalescing from Hib meningitis lacked detectable acute-phase serum antibody to the recombinant P1 protein, but these same infants were shown to produce readily detectable antibody to this recombinant protein during convalescence. These data suggest that these particular recombinant *E. coli* may be useful in the study of immune response to the P1 protein.

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