INFECTION AND IMMUNITY, June 1990, p. 1909–1913
0019-9567/90/061909-05$02.00/0

Vol. 58, No. 6

Expression in *Escherichia coli* of a High-Molecular-Weight Protective Surface Antigen Found in Nontypeable and Type b *Haemophilus influenzae*†

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Received 13 July 1989/Accepted 8 January 1990

An *Escherichia coli* clone producing a high-molecular-weight surface antigen of *Haemophilus influenzae* type b (Hib) was isolated from a library of Hib DNA fragments cloned as lysogens in a lambda replacement vector. The antigen is found in sarcosyl-insoluble outer membrane protein preparations and was produced by all 36 *H. influenzae* isolates tested. Absorption studies indicated that the antigen is a surface determinant on all isolates tested. Antibodies to the antigen (D15) were found in eight of nine convalescent-phase sera from children with invasive Hib infection. Affinity-purified antibodies prepared against the cloned antigen gave protection against the development of bacteremia in a rat pup model.

There has been considerable interest in cloning and expressing genes coding for the outer membrane proteins (OMPs) of *Haemophilus influenzae* type b (Hib) (4–7, 12–14). These studies will help in structural and immunological investigations to subtype the organism (17) and perhaps to develop a vaccine. Not only would OMP vaccines be effective for young infants, but the cloning technology could permit the investigation of the development of live mucosal vaccines along the lines of the *Salmonella typhimurium* mutants that express cloned vaccine molecules (15). While type b infection is the major invasive bacterial disease for young children in industrial societies, infants in other regions have invasive disease from a broader range of *H. influenzae* isolates (10, 16). This has been well documented in the highlands of Papua New Guinea (16), where bacterial pneumonia accounts for about half of the pediatric hospital admissions and fatalities and half of these cases are due to infection with typeable and nontypeable isolates. It would, therefore, be advantageous to study immunity that is directed towards antigens shared by a wide range of *H. influenzae* isolates. In this study we report the expression of a high-molecular-weight surface antigen from Hib DNA cloned in *Escherichia coli* and present evidence that it is a target for protective immunity and is conserved in a range of typeable and nontypeable isolates.

MATERIALS AND METHODS

Bacterial strains and vectors. *E. coli* BTA282 used for lysogenic growth of recombinant bacteriophage lambda and the cloning vector lambda gt11 Ampl have been described (18). The *H. influenzae* isolates used in these studies were obtained from A. W. Cripps, Austpharm Institute for Mucosal Immunology, Newcastle, Australia; J. Montgomery, Institute of Medical Research, Goroka, Papua New Guinea; and J. O'Connor, Princess Margaret Hospital, Perth, Australia. They were grown on chocolate agar.

Isolation of *E. coli* expressing Hib antigen. The methods and library used in the study were described previously (18). Briefly, the replacement vector lambda gt11 Ampl was used to clone 6- to 9-kilobase fragments of Hib DNA (Ca isolate) lysogenized in *E. coli* BTA282. For immunoassays, colonies were replica plated on nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) and grown on L plates at 30°C, and, after a temperature switch to induce expression, colonies were lysed by placing the filters on 1% sodium dodecyl sulfate (SDS) in a chloroform atmosphere for 15 min. Colony radioimmunoassay was performed by using a 1/200 dilution of rabbit anti-Hib absorbed with *E. coli* lysate as previously reported (18). Skim milk was used for blocking, and radioiodinated staphylococcus protein A was used to develop the reaction for autoradiography. For some screening, the antiserum was first absorbed with induced lysates of *E. coli* clones, expressing Hib OMP, which had been previously isolated from the library. Successful absorption, monitored by dot blot, was usually accomplished by diluting the serum 1/50 in a lysate made after suspending the bacteria in 0.1 volume of an induced broth. Lysates were prepared by adding 100 ml of a 30°C overnight culture to 900 ml of L broth and incubating for 2 h with vigorous shaking. The cultures were then incubated at 42°C for 15 min and at 37°C for a further 2 h before being pelleted and suspended at the desired concentration. Lysates were made by freeze-thawing and sonication.

Affinity purification of antibodies and Western blotting (immunoblotting). To help characterize Hib antigens expressed in *E. coli* clones, lysates were made from an induced broth culture (0.1 culture volume) (see above) and coupled to Sepharose 4B (Pharmacia Diagnostics, Piscataway, N.J. [Div. Pharmacia, Inc.]) by a cyanogen bromide method. This mixture was then used as an immunoabsorbent to prepare affinity-purified antibodies as described previously (18). The affinity-purified antibodies were tested for specificity by dot blot or by Western blotting against lysates from recombinant *E. coli* clones or *H. influenzae* OMPs. Lysates of *E. coli* were prepared after inducing broth cultures and suspending the pelleted bacteria in 0.1 culture volume (18). A 10-μl volume was loaded onto SDS-polyacrylamide gels. For *H. influenzae* OMP preparations, a rapid microprocedure was used to isolate sarcosyl (2%)-insoluble proteins (3). Detergent-insoluble pellets prepared from bacteria grown on

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† Publication no. 329 of the Clinical Immunology Unit, Princess Margaret Children's Medical Research Foundation.

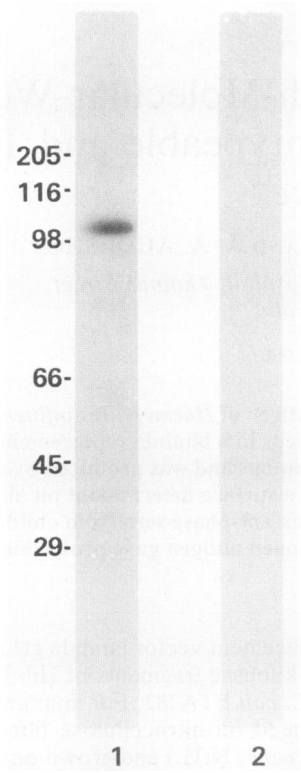

FIG. 1. Reactivity of affinity-purified antibody to clone D15 with Hib OMP. An OMP preparation (sarcosyl-insoluble fraction) was electrophoresed on an 8 to 18% SDS-polyacrylamide gel, and Western blotting was performed with rabbit antibodies that had been affinity purified from the D15 lysate coupled to Sepharose. Reactivity to a band of about 103 kDa was obtained (lane 1), and this was lost if the antibodies were absorbed with intact Hib before the immunoassay (lane 2).

confluent overnight chocolate plates were suspended in 100 μl of 10 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 25 μl was loaded onto a gel track. SDS-polyacrylamide gel electrophoresis was conducted by using standard procedures with 8 to 18% gradient gels and electroblotting onto nitrocellulose filters (2). Filters were assayed by colony radioimmunoassay and developed with radioiodinated protein A.

Absorption with intact Hib. Overnight cultures of Hib were harvested from chocolate agar and washed three times in phosphate-buffered saline. Five-milliliter volumes of a 1/100 dilution of rabbit antiserum were absorbed with 10^{10} CFU of Hib for 1 h at 4°C.

Rat protection assay. Eight-day-old Sprague-Dawley rats were injected intraperitoneally with doses of 10^3 to 10^5 Hib (Ca isolate, used for cloning), and bacteremia was measured after 24 h by culturing 20 μl of blood on chocolate agar. For protection assays, bacteria grown to mid-log phase were incubated in affinity-purified antibody against cloned OMP for 1 h at 4°C and injected into the rat pups. To test for nonspecific effects or contaminating antibody specificities, the affinity-purified antibody preparations (2 ml) were absorbed twice with 2.5 ml of Sepharose 4B coupled with the lysate of bacteria expressing the antigen being tested (D15) or an unrelated lysate (D12) to see if protective activity was removed. Later experiments were conducted to test for protective activity with other type b isolates and to test for a "serum shift" phenomenon like that described by Anderson et al. (1, 8). In these experiments, the isolates were incubated for 30 min at 37°C in rat pup serum at 10^7/ml before an equal volume of affinity-purified antibody was added. The concentration of the antibody solution used was approximately 20 μg/ml.

Serum samples. The sera used were convalescent-phase sera taken from children 3 weeks after meningitis or epiglottitis infection with type b isolate. The sera were from children aged 1.5 to 3 years and were supplied by D. M. Roberton, Royal Children's Hospital, Parkville, Victoria, Australia.

RESULTS

Cloning of high-molecular-weight antigens. A library of Hib DNA fragments in lambda gt11 Ampl lysogenized in *E. coli* (18) was grown on replica-plated nitrocellulose filters. To detect colonies producing antigens, the colonies were grown on the filters and, after temperature induction, were screened by colony radioimmunoassay with a 1/200 dilution of rabbit anti-Hib. This serum, however, had been absorbed with lysates of clones previously isolated (18) and shown to express Hib antigens with molecular masses corresponding to those of the 49- and 39-kilodalton (kDa) major OMPs and a 35-kDa OMP. Several clones with positive reactivity to the absorbed antiserum were isolated, and lysates were examined by using Western blotting for rabbit anti-Hib. It was found that the positive colonies included two clones that expressed high-molecular-weight antigens. The lysate from one clone (D12) had bands with M_rs of 93,500 and 99,000 that were reactive with rabbit anti-Hib. The lysate of the other clone (D15), to be examined in more detail, had a single band with an M_r of about 103,000. To examine the expression of the D15 antigen in Hib, the lysate of *E. coli* clone D15 was coupled to Sepharose 4B and used as an immunoabsorbent to prepare affinity-purified rabbit anti-D15. When used in Western blotting with Hib OMP preparations, this affinity-purified antibody reacted with a single band with an M_r of about 103,000 (Fig. 1), the same as the band found in *E. coli* clone D15. Affinity-purified antiserum absorbed with intact

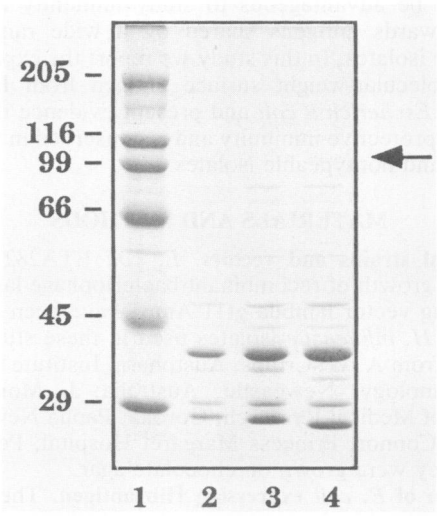

FIG. 2. Coomassie blue-stained polyacrylamide gel. OMP preparations from three different isolates stained with high-molecular-weight markers are shown. The arrow indicates the position at which clone D15 stains in Western blotting and which is occupied by two close bands.

TABLE 1. Effect of anti-D15 antibody on bacteremia in rat pups injected with Hib

Expt[a]	Treatment of Hib	Amt of Hib injected	No. of pups with bacteremia/ total	Mean bacterial count (range)
1	PBS	10^3	5/8	3,960 (450–11,000)
		10^2	6/8	7,400 (450–11,400)
		10	3/10	1,250 (150–3,150)
	Anti-D15 absorbed with D15	10^3	7/10	2,225 (50–5,750)
		10^2	7/9	3,865 (300–12,500)
	Anti-D15 absorbed with D12	10^3	0/10	0[b]
		10^2	0/9	0[b]
2[c]	NRS	10^3	9/9	10,744 (2,000–25,000)
	NRS	10	4/9	5,910 (1,950–9,450)
	NRS + anti-D15	10^3	0/10	0[d]
	NRS + anti-D15	10	0/10	0[d]
3	NRS	10^3	8/10	5,300 (1,900–12,000)
	NRS	10	9/10	1,861 (700–5,200)
	NRS + anti-D15	10^3	0/10	0[d]
		10	0/10	0[d]

[a] Hib isolates used: experiment 1, Ca; experiment 2, A1; experiment 3, 84:134.

[b] For experiment 1, groups injected with Hib that was treated with anti-D15 absorbed with D12 had less bacteremia than groups injected with Hib treated with phosphate-buffered saline or anti-D15 absorbed with D15 ($P < 0.001$; Mann-Whitney analysis).

[c] In experiment 2, Hib was incubated in normal rat pup serum (NRS) for 30 min at 10^7 CFU/ml before being diluted with an equal volume of anti-D15 or phosphate-buffered saline.

[d] For experiments 2 and 3, groups injected with anti-D15-treated Hib had significantly less bacteremia ($P < 0.001$; Mann-Whitney analysis).

Hib lost almost all reactivity to this band (Fig. 1). Coomassie blue-stained SDS-polyacrylamide gels of Hib OMP preparations showed two close bands in the expected area (Fig. 2). The phage DNA isolated from clone D15 was shown to release a 5.7-kilobase fragment from the *Eco*RI cloning site.

Protective activity of anti-D15 antibody. To test for the possibility that antibody to clone D15 could have a protective function, affinity-purified rabbit anti-D15 prepared as described above was incubated at 4°C for 1 h with a Hib suspension (10^6 CFU/ml), which was then divided into portions, diluted, and injected intraperitoneally into 8-day-old rat pups. As a control, the affinity-purified antibody was absorbed with Sepharose coupled to *E. coli* clone D15 lysate or with Sepharose coupled to the unrelated clone D12 lysate. Bacteremia, measured after 24 h, was not detected if the inoculum had been incubated with anti-D15 absorbed with D12 lysate, but normal bacteremia was found if the antibodies were absorbed with the homologous D15 lysate. To test for activity against other bacteria, two further Hib isolates (84:134 and A1) were used. Protection was achieved for both isolates (Table 1). The first of these isolates was obtained from Papua New Guinea, in 1984, and the second was obtained from Princess Margaret Hospital, Western Australia, in 1989. In addition, the bacteria in these experiments were preincubated in rat pup serum for 30 min before antibody was added. This was to test the possibility that the serum shift phenomenon described by Anderson et al. (1, 8) affected protection mediated through the D15 antigen.

Presence of D15 antigen on *Haemophilus* isolates and anti-D15 antibody in convalescent-phase serum. The production of

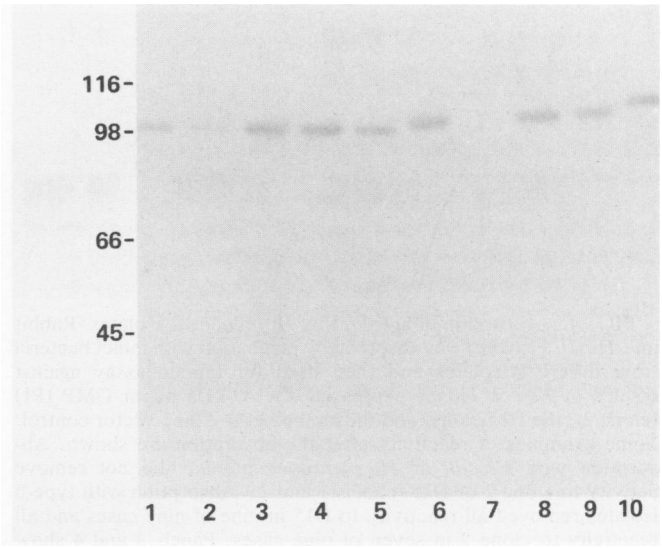

FIG. 3. Reactivity of affinity-purified anti-D15 with OMPs of different *H. influenzae* isolates. Sarcosyl-insoluble OMP preparations of different isolates were electrophoresed on an 8 to 18% SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-D15 antibodies that had been affinity purified from clone D15 lysate coupled to Sepharose. The isolates are tybe b (lanes 1 through 5) and nontypeable (lanes 6 through 10). The low reactivity of lane 7 in this gel resulted from the inadvertent loading of a low concentration of membrane proteins as demonstrated by a comparison with a concurrent Coomassie blue-stained gel.

D15 antigen by 16 Hib and 16 nontypeable isolates was examined by Western blotting of SDS-polyacrylamide gel OMP preparations. The antigen was found in all Hib isolates, with reactivity at an M_r of about 103,000 (Fig. 3). Nontypeable isolates also reacted strongly with a band of similar M_r (for examples, see Fig. 3), although reactivity for 2 of 16 isolates was low.

To test for the presence of D15 antigen on the surface of different isolates, rabbit anti-Hib serum was absorbed with 10^{10} intact bacteria from various isolates and then tested for reactivity with *E. coli* clone D15 lysate by radioimmune dot blot. The absorption procedure, with nine type b and eight nontypeable isolates, showed that the reactivity was removed totally for type b and also for nontypeable isolates, except for the Dover isolate (Fig. 4), for which some reactivity remained. Absorption with a *Haemophilus pleuropneumoniae* isolate or *E. coli* did not remove reactivity. As another control, reactivity to clone 2, which expressed the 49-kDa major OMP, was included, and reactivity to this protein was completely removed by most type b isolates and was not affected by nontypeable isolates. Figure 4 shows some examples of the absorption procedure. As another method of observing the distribution of D15 antigen among isolates, antibody reactivity in sera of nine convalescent-phase children was examined by a dot blot radioimmunoassay developed with radioiodinated protein A. Sera taken from children 3 weeks after recovery from meningitis or epiglottitis were tested for reactivity against lysates of clone D15 (Fig. 5). Also included in this assay were clone 10, which expresses the 49-kDa major OMP, and two less-well-characterized clones, namely, D12 and D13, which are known to express Hib antigens. The clone 10 lysate reacted with nine of nine sera, D15 reacted with eight of nine sera, D12 reacted with one of nine sera, and D13 reacted with zero of nine sera.

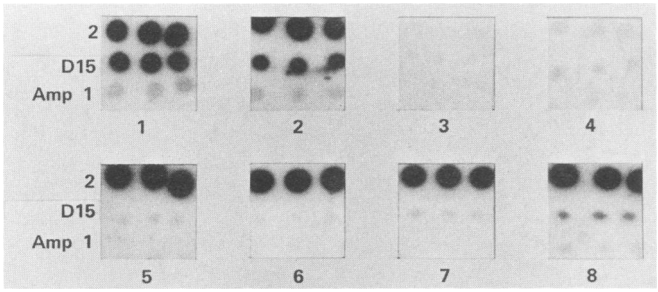

FIG. 4. Absorption of anti-D15 by *H. influenzae* isolates. Rabbit anti-Hib (Ca isolate) was absorbed by incubation with intact bacteria from different isolates and then used for immunoassay against lysates of *E. coli* clones expressing the 49-kDa major OMP (P1) (clone 2), the D15 clone, and the lambda gt11 Amp1 vector control. Some examples of reactivity after the absorption are shown. Absorption with *E. coli* or *H. pleuropneumoniae* did not remove activity to clone 2 or D15 (panels 1 and 2). Absorption with type b isolates removed all reactivity to D15 in nine of nine cases and all reactivity to clone 2 in seven of nine cases. Panels 3 and 4 show examples of removal of reactivity, and panel 5 shows the retention of activity. Panels 6 through 8 show reactivity after absorption with nontypeable isolates. Nontypeable isolates did not absorb out reactivity to clone 2 but removed reactivity to D15 in eight of eight cases. Panel 8 shows the 1 isolate of the 17 tested in which absorption of anti-D15 was not complete.

DISCUSSION

In this study we have presented evidence that *H. influenzae* produces a high-molecular-weight surface antigen with an M_r of about 103,000 in SDS-polyacrylamide gels which is conserved among type b and nontypeable isolates and is a target for protective antibody. The antigen is almost certainly a protein because the 5.7-kilobase Hib DNA fragment in lambda gt11 Amp1 clone D15 expresses this antigen with an M_r identical to that found in the Hib used for cloning, and it is unlikely that the two organisms could produce other types of surface antigen with the same fidelity. The antigen is also probably an OMP because it is found in the sarcosyl-insoluble fraction typical of these proteins, and intact Hib can absorb antibody to the antigen. We have previously investigated this absorption process by examining its effects on reactivity of antiserum to cloned antigens not found in outer membrane vesicles. Under the conditions used, reactivity to OMP antigens was abolished, while reactivity to antigens not found in the outer membrane was unaffected (18). The finding that the D15 antigen can function as a determinant for protective antibody is also consistent with it being a surface moiety. Coomassie blue staining of SDS-polyacrylamide gels shows two close bands in the area of the M_r, but further resolution could not be achieved with current techniques.

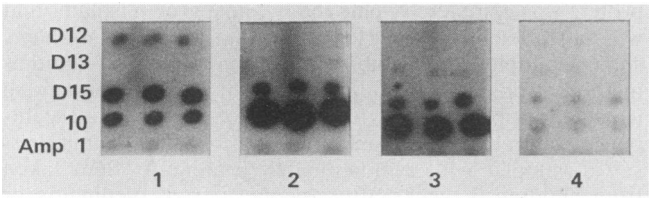

FIG. 5. Reactivity of convalescent-phase serum from children with invasive Hib disease. Panels 1 through 3, Examples of reactivity of convalescent-phase serum with Hib antigen expressed in *E. coli*; panel 4, control serum. Reactivity to dot-blotted lysates of clones was developed by ^{125}I-protein A. Of the sera tested, nine of nine and eight of nine showed reactivity to clones 10 and D15, respectively, as shown in the examples here, while one of nine and zero of nine reacted with clones D12 and D13, respectively. Panel 1 shows the only reaction found to D12.

The concept that antibodies to OMPs can provide protection from infection with type b organisms is well established. Of particular note is the report of protection mediated via the 49-kDa major OMP (P1 or a) (11) and protection mediated via a 98-kDa minor OMP (9). Because of the similar M_rs reported for the latter protein and the D15 antigen, it is possible that they are the same entity. The argument against this is that 30% of Hib lacked the 98-kDa OMP, as judged by monoclonal antibody, and negative isolates lacked a 98-kDa protein. All of the type b isolates we examined by Western blotting had a reactive protein at our estimate of about 103 kDa. These isolates were from diverse sources, namely, Papua New Guinea and Perth, Western Australia, and were taken over a number of years. The question of whether the protein and the D15 antigen are the same entity should, however, be resolved.

There have now been several reports of Hib antigens expressed in *E. coli*, including the major OMPs P1 (a) (5, 13), P2 (b/c) (6), and P6 (g) (4, 14). P1 and P6 have been sequenced. Some less-well-defined OMPs have also been cloned (7, 18), although clones 2 and 10 reported by our laboratories (18) express P1 and clone 33 expresses P2 as judged by an association of variations in migration of the antigens with corresponding protein bands in SDS-polyacrylamide gels (unpublished data). Evidence has been presented that most of these proteins are expressed in *E. coli* independent of the vector promoters. In this context it should be noted that LacZ genes are removed from the lambda gt11 Amp1 vector used for cloning.

The P6 OMP is also of some interest as discussed previously (14) because it has an epitope shared by all *H. influenzae* isolates and is a target for bacteriocidal antibody to nontypeable isolates. The D15 antigen is also widely distributed, being in all 16 Hib and the 16 nontypeable isolates tested. In most cases most of the antigenic determinants were conserved because absorption with all type b isolates removed all reactivity to the D15 antigen. Absorption with nontypeable isolates removed all reactivity in seven of eight cases and most of the reactivity in the remaining absorption. The presence of antibody to this antigen in eight of nine convalescent-phase sera confirms its conservation among the type b isolates as well as its immunogenicity in young children. The experiments showing that affinity-purified antibodies to this antigen could markedly protect rat pups from bacteremia demonstrate that this antigen can, at least under some circumstances, act as a target for protective immunity. The protection was mediated by anti-D15 antibodies and not contaminating specificities because it could be absorbed out with clone D15 antigen but not by *E. coli* expressing another Hib protein. Because of limitations in the amount of material available, protection assays were carried out by incubating the bacteria with affinity-purified antibody in vitro before injection. Since incubating *H. influenzae* in serum can render in-vitro-grown bacteria resistant to some bacteriocidal antibodies, experiments were included in which the bacteria were incubated for 30 min in rat serum by the method of Anderson et al. (1, 8). No serum-induced resistance was observed. Experiments to determine the ability of D15 to immunize against infection with other Hib and nontypeable isolates will be pursued as well as further studies of its expression and structure in different isolates. It is encouraging at this stage, however, to

note that protection has been obtained with isolates made 5 years apart and in both Australia and Papua New Guinea.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia, the TVW Telethon Foundation, and the Princess Margaret Children's Medical Research Foundation.

LITERATURE CITED

1. **Anderson, P., A. Flesher, S. Shaw, A. L. Harding, and D. H. Smith.** 1980. Phenotypic and genetic variation in the susceptibility of *Haemophilus influenzae* type b to antibodies to somatic antigens. J. Clin. Invest. **65**:885–891.
2. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecylsulfate gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
3. **Carlone, G. M., M. L. Thomas, H. S. Rumschlag, and F. O. Sottnek.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. J. Clin. Microbiol. **24**:330–331.
4. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170**:489–498.
5. **Gonzales, F. R., S. Leachman, M. V. Norgard, J. D. Radolf, G. M. McCracken, Jr., C. Evans, and E. J. Hansen.** 1987. Cloning and expression in *Escherichia coli* of the gene encoding the heat-modifiable major outer membrane protein of *Haemophilus influenzae* type b. Infect. Immun. **55**:2993–3000.
6. **Hansen, E. J., F. R. Gonzales, N. R. Chamberlain, M. V. Norgard, E. E. Miller, L. D. Cope, S. E. Pelzel, B. Gaddy, and A. Clausell.** 1988. Cloning of the gene encoding the major outer membrane protein of *Haemophilus influenzae* type b. Infect. Immun. **56**:2709–2716.
7. **Holmans, P. L., T. A. Loftus, and E. J. Hansen.** 1985. Cloning and surface expression in *Escherichia coli* of a structural gene encoding a surface protein of *Haemophilus influenzae* type b. Infect. Immun. **50**:236–242.
8. **Inzana, T. J., and P. Anderson.** 1985. Serum factor-dependent resistance of *Haemophilus influenzae* type b to antibody to lipopolysaccharide. J. Infect. Dis. **151**:869–877.
9. **Kimura, A., P. A. Gulig, G. H. McCracken, T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47**:253-259.
10. **Lancet.** 1985. Acute respiratory infections in under-fives: 15 million deaths a year. Lancet **ii**:699–701.
11. **Loeb, M. R.** 1987. Protection of infant rats from *Haemophilus influenzae* type b infection by antiserum to purified outer membrane protein. Infect. Immun. **55**:2612–2618.
12. **Loeb, M. R., and K. A. Woodin.** 1987. Cross-reactivity of surface-exposed epitopes of outer membrane antigens of *Haemophilus influenzae* type b. Infect. Immun. **55**:2977–2983.
13. **Munson, R., and S. Grass.** 1988. Purification, cloning and sequence of outer membrane protein P1 of *Haemophilus influenzae* type b. Infect. Immun. **56**:2235–2242.
14. **Nelson, N. B., M. A. Apicella, T. F. Murphy, H. Vankeulen, L. D. Spotila, and D. Rekosh.** 1988. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect. Immun. **56**:128–134.
15. **Poirier, T. P., M. A. Kehoe, and E. H. Beachy.** 1988. Protective immunity evoked by oral administration of attenuated aroA *Salmonella typhimurium* expressing cloned streptococcal M protein. J. Exp. Med. **168**:25–32.
16. **Shann, F., S. Germer, D. Hazlett, M. Gratten, V. Linnemann, and R. Payne.** 1984. Aetiology of pneumonia in children in Goroka Hospital, Papua New Guinea. Lancet **ii**:537–541.
17. **Takala, A. K., J. Escola, P. Bol, L. van Alphen, J. Palmgren, and P. H. Makela.** 1987. *Haemophilus influenzae* type b strains of outer membrane subtypes 1 and 1c cause different types of invasive disease. Lancet **ii**:647–650.
18. **Thomas, W. R., and A. A. Rossi.** 1986. Molecular cloning of DNA coding for outer membrane proteins of *Haemophilus influenzae* type b. Infect. Immun. **52**:812–817.