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Immunogenicity of Overlapping Synthetic Peptides Covering the Entire Sequence of *Haemophilus influenzae* Type b Outer Membrane Protein P2

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***Haemophilus influenzae* type b is a major cause of bacterial meningitis in young children. Antibodies against the outer membrane protein P2 are protective in the infant rat model of bacteremia. To identify conserved, surface-exposed, and protective epitopes of P2, 17 overlapping peptides covering the entire sequence of the protein were synthesized. Antisera from mice, guinea pigs, and rabbits raised against chromatographically purified P2 were tested for their reactivities to the peptides by enzyme-linked immunosorbent assays (ELISA). Three major linear immunodominant B-cell epitopes were mapped to residues 53 to 81, 241 to 265, and 314 to 341 of mature P2. Human convalescent-phase antisera also reacted strongly with these three epitopes. Rabbit antisera against all peptide-keyhole limpet hemocyanin conjugates except two peptides containing residues 8 to 19 and 302 to 319 recognized the corresponding peptides in ELISA and reacted with P2 on immunoblots. Immunization with all unconjugated peptides, except the 19 N-terminal residues, induced very strong peptide-specific antibody responses, and these antisera reacted with P2 on immunoblots. Rabbit antisera raised against peptides corresponding to residues 1 to 14, 125 to 150, 193 to 219, and 241 to 319 also recognized P2 purified from *H. influenzae* nontypeable isolates. Identification of these immunodominant B-cell epitopes and conserved regions is a first step toward the rational design of a universal *H. influenzae* vaccine.**

Infection by *Haemophilus influenzae* type b (Hib) is a major cause of bacterial meningitis in young children and can cause epiglottitis, cellulitis, and pneumonia (29, 31). Current Hib capsular polysaccharide (polyribosyl ribitol phosphate; PRP) conjugate vaccines selectively protect against meningitis caused by Hib (8, 11, 20, 35); they do not protect against other invasive typeable strains and nontypeable strains which are a common cause of otitis media.

Recent studies by Granoff and Munson (12) have shown that passive administration of antibodies directed against Hib outer membrane proteins (OMP) P1, P2, and P6 protects infant rats against live Hib challenge. Murphy and Bartos (26) also reported that a monoclonal antibody recognizing a surface-exposed epitope of a nontypeable *H. influenzae* P2 protein had bactericidal activity in vitro. In addition, several anti-P2 monoclonal antibodies were found to cross-react with typeable and nontypeable strains of *H. influenzae* (22, 33). Therefore, a promising strategy is to use a combination of OMP or their immunodominant epitopes as additional antigens and carriers for PRP to develop a new conjugate vaccine with enhanced protective ability and autologous T-cell priming. Such a vaccine may have advantages over the existing vaccines in which PRP is conjugated to either diphtheria toxoid, tetanus toxoid, or CRM197 or to OMP of *Neisseria meningitidis*. The use of OMP will reduce the vaccine formulation problems with diphtheria or tetanus toxoid in any future multivalent DTP-Hib combined vaccines and may be useful in protecting against otitis media, for which there is no vaccine.

OMP P2 (molecular weight, 38,000) is the most abundant protein in Hib outer membrane preparations (3, 21, 25). It has been shown to have porin activity when it is incorporated into liposomes (32). The gene encoding the P2 protein has been cloned from four different Hib subtypes. Although the nucleotide sequences of two subtypes (1H and 3L) were identical (14, 15, 24), some amino acid variabilities were found in the P2 sequences of two other Hib subtypes, 2L and 6U (23).

As a first step toward the design of an OMP-based *H. influenzae* vaccine candidate, 17 overlapping peptides covering the entire sequence of P2 were synthesized and used to map the conserved, surface-exposed, and protective epitopes of P2. Also, rabbits were immunized with either linear peptides or peptide-keyhole limpet hemocyanin (KLM) conjugates to find out whether synthetic peptides will induce functional antibody populations different from those obtained with the native protein.

(Portions of this work were presented at the 1990 Annual Meeting of the American Society for Microbiology in Anaheim, Calif. [6a].)

MATERIALS AND METHODS

P2 purification. The Hib strain used in this study was the Connaught production Eagan strain. Bacterial cells were precipitated from the culture broth by Cetavlon (0.1% [wt/vol]). P2 was purified from the cell paste as described previously (25). The purity of the chromatographically purified P2 was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and scanning densitometry. Protein concentration was determined by the Bio-Rad protein assay.

Selection of peptides and peptide synthesis. To map the functional epitopes of OMP P2, 17 peptides (12 to 30 residues long) were selected to cover the entire P2 sequence (Table

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TABLE 1. Hib OMP P2 overlapping peptides

Peptide	Residues	Sequence[a]
Porin-1	1–14	AVVYNNEGTNVELG(C)
HIBP2-26	8–19	GTNVELGGRLSI
HIBP2-25	17–32	LSIIAEQSNSTVDNQK
OMP2-1	28–55	VDNQKQQHGALRNQGSRFHIKATHNFGD(C)
		←——OMP2-2A——→
OMP2-2	53–81	FGDGFYAQGYLETRFVTKASENGSDNFGD(C)
OMP2-3	79–106	(C)FGDITSKYAYVTLGNKAFGEVKLGRAKT
OMP2-4	101–129	GRAKTIADGITSAEDKEYGVLNNSDYIP(C)
OMP2-5	125–150	SDYIPTSGNTVGYTFKGIDGLVLGAN(C)
OMP2-6	148–174	(C)GANYLLAQKREGAKGENKRPNDKAGEV
OMP2-7	171–196	AGEVRIGEINNGIQVGAKYDANDIVA(C)
OMP2-8	193–219	DIVAKIAYGRTNYKYNESDEHKQQLNG(C)
OMP2-9	219–244	(C)GVLATLGYRFSDLGLLVSLDSGYAKT
		←——OMP2-10A——→
OMP2-10	241–265	YAKTKNYKIKHEKRYFVSPGFQYEL(C)
OMP2-11	263–289	(C)YELMEDTNVYGNFKYERTSVDQGEKTR
OMP2-12	285–306	GEKTREQAVLFGVDHKLHKQLL(C)
OMP2-13	302–319	KQLLTYIEGAYARTRTT(C)
		←——CHIBP2A——→
CHIBP2	314–341	(C)ARTRTTETGKGVKTEKEKSVGVGLRVYF
OMP2-6U	148–174	(C)GANYLLAQKREGAKMANKLPNNKAGEV
		** * *
OMP2-6L	148–174	(C)GANYLLAQKREGAKGENKQPNDKAGEV
		*

[a] The asterisks indicate residues which are different from those found in the P2 protein of the *H. influenzae* strain 1H (23).

1). Peptides were synthesized with an Applied Biosystems model 430A automated peptide synthesizer and purified as described previously (22). Small portions of the peptide resin were removed at various stages of the synthesis to obtain truncated peptide analogs for fine epitope mapping.

Peptide-carrier conjugation. Individual peptides (except HIBP2-25 and HIBP2-26) were conjugated to KLH with sulfosuccinimyl(4-iodoacetyl)-aminobenzoate (Pierce) as cross-linker at a 10:1 molar ratio of peptide to KLH as described previously (6). Peptides HIBP2-25 and HIBP2-26 were conjugated to KLH with glutaraldehyde according to the method described by Askelof et al. (1).

Production of P2-specific antisera. P2-specific antisera were prepared as follows. Rabbits (Maple Farm, Clifford, Ontario, Canada), guinea pigs (Hazleton), and mice of six different strains (A/J, SJL/J, BALB/c, BL/6, C3H, and SWR/J) (Charles River, St. Constant, Quebec, Canada) were immunized intramuscularly or subcutaneously with 5 to 100 μg of purified P2 protein emulsified in complete Freund's adjuvant. Animals received two booster doses of the immunogen in incomplete Freund's adjuvant 2 and 4 weeks later. Blood samples were collected every 2 weeks after the second injection. Antisera were obtained from the clotted blood samples by centrifugation and were heat inactivated at 56°C for 30 min. Rabbit and guinea pig anti-P2 sera were further absorbed on a PRP affinity column and then on a Hib (Eagan) lipooligosaccharide affinity column. The specificities of anti-P2 antisera were assessed by P2-specific enzyme-linked immunosorbent assay (ELISA) and immunoblots with *H. influenzae* cell extract as antigen. Absorbed antisera were shown to be free of anti-lipooligosaccharide and anti-PRP antibodies. Three human convalescent-phase serum were generously provided by S. Halperin of Dalhousie University, Halifax, Nova Scotia, Canada.

Production of rabbit antipeptide antisera. Two rabbits were immunized intramuscularly with individual peptides (200 μg) or peptide-KLH conjugates containing 50 μg of peptide emulsified in complete Freund's adjuvant and then given two booster doses (half of the amount of the same immunogen in incomplete Freund's adjuvant) every 2 weeks. Antisera were collected and stored as described above.

P2- and peptide-specific ELISAs. Microtiter wells (Nunc-Immunoplate, Roskilde, Denmark) were coated with 200 ng of purified P2 or 500 ng of peptide in 50 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) for 16 h at room temperature. The plates were then blocked with 0.1% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature. Serially diluted antisera were added to the wells and incubated for 1 h at room temperature. After removal of the antisera, the plates were washed five times with PBS containing 0.1% (wt/vol) Tween 20 and 0.1% (wt/vol) BSA. Goat anti-rabbit, guinea pig, mouse, or human immunoglobulin G (IgG) $F(ab')_2$ fragments conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.) were diluted (1/8,000) with washing buffer and added to the microtiter plates. After 1 h of incubation at room temperature, the plates were washed five times with washing buffer. The plates were then developed with the substrate tetramethylbenzidine in H_2O_2 (ADI, Toronto, Ontario, Canada), and the optical density was measured at 450 nm with a Titretek Multiskan II (Flow Laboratories, McLean, Va.). Two pertussis toxin peptides, NAD-S1 (GALATYQSEYLAHRR IPP) and S3-P6 (FVRDGQSVIGACASPYEGRYRDMYDA LRRLLY) were included as negative controls in the peptide-specific ELISA. Assays were performed in triplicate, and the reactive titer of each antiserum was defined as the dilution which consistently showed a twofold increase in optical density over that obtained with the negative control.

Protection studies. The protective abilities of P2-specific rabbit or guinea pig antisera were assessed with the infant rat model of bacteremia using the Hib Eagan strain as the challenge strain (25).

Immunoblots. The specificity of each experimental antiserum was tested by the immunoblot technique described by Towbin et al. (30). Briefly, crude P2 extract was reduced and boiled in SDS-PAGE buffer containing 10 mM β-mercaptoethanol, electrophoresed in 12% polyacrylamide gels, and transferred onto an Immobilon membrane (Millipore Corp.). The membrane was blocked with 1% BSA for 1 h and then incubated with antisera diluted 500 times with washing buffer (0.1% Tween 20 in PBS) containing 0.1% BSA for 4 to 6 h at room temperature. The membrane was then washed four times with washing buffer and incubated with protein A conjugated to alkaline phosphatase (Bio-Rad) for 2 to 3 h at room temperature. The membrane was washed five times with washing buffer and then reacted with the 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt–*p*-nitroblue tetrazolium chloride color development kit (Bio-Rad) according to the manufacturer's instructions. The immunoblot strips were photographed for recording.

RESULTS

Selection of synthetic peptides. The surface-exposed regions of a protein often correlate with its antigenic determinants (18, 27). Thus, the peptides selected for this study correspond to the linear segments of the P2 protein sequence which contain hydrophilic β-turns predicted by conventional structure prediction algorithms (7, 18, 27) (Fig. 1). All peptides, except HIBP2-25 and HIBP2-26, were synthesized with an additional N-terminal or C-terminal cysteine residue

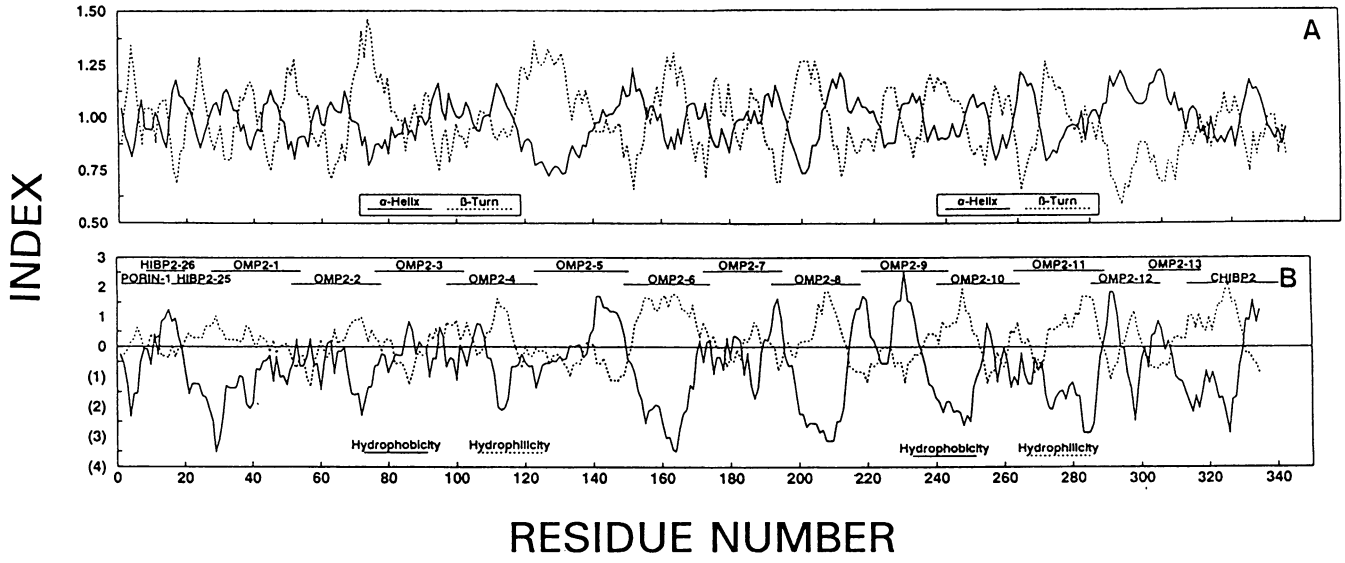


FIG. 1. Structure prediction analysis of Hib P2 protein with conventional algorithms. (A) Protein secondary-structure plots were obtained according to the method described by Chou and Fasman (7); (B) hydrophilicity and hydrophobicity plots were obtained according to the method described by Hopp (18). The values are derived from the averages of heptapeptide windows and are plotted at the midpoint of each fragment.

to facilitate one-orientational conjugation between the peptide and the carrier protein. Peptides containing more than 25 residues were synthesized to better mimic the conformation of native epitopes exposed on P2 as suggested by Van Regenmortel et al. (34).

Characterization of murine, guinea pig, and rabbit anti-P2 antisera. To identify the immunodominant B-cell epitopes of P2, rabbits, guinea pigs, and six strains of mice of different haplotypes (*H-2*^{a}, *H-2*^{b}, *H-2*^{d}, *H-2*^{k}, *H-2*^{q}, and *H-2*^{s}) were immunized with purified P2 in the presence of Freund's adjuvant. After both primary and secondary immunizations, all animals generated a strong and specific anti-P2 antibody response, as judged by P2-specific ELISA (Table 2) and immunoblots. As previously reported by Granoff and Munson (12), both rabbit and guinea pig anti-P2 antisera consistently protected infant rats against live Hib challenge. These results indicate that purified P2 is immunogenic and capable of inducing protective antibodies which will be useful for identification of protective epitopes.

Validation of P2 peptide-specific ELISA. Numerous studies have shown that peptide-specific ELISA can be performed with microtiter plates directly coated with synthetic peptides (6, 10, 28). However, negative results may be observed if peptides do not bind to polystyrene or if they bind through their antigenic determinants. To validate the ELISAs used in this study, polystyrene microtiter wells were coated with 500 ng of individual peptides and probed with peptide-specific rabbit antisera raised against peptide-KLH conjugates. All peptides, except HIBP2-26 (residues 8 to 19) and OMP2-13 (residues 302 to 319), were recognized by their respective antipeptide antisera (Table 3). No cross-reactivity was observed. The lack of reactivity between rabbit antisera and P2 peptides HIBP2-26 and OMP2-13 was due to the fact that both peptide-KLH conjugates failed to elicit an antipeptide antibody response. Further experiments showed that both plate-bound peptides (HIBP2-26 and OMP2-13) were recognized by rabbit anti-P2 antibodies (see below). These results establish that all P2 peptides were adsorbed onto the microtiter wells and that their antigenic determinants were accessible to peptide-specific antibodies. To eliminate the possibility of nonspecific interaction between synthetic peptides and serum immunoglobulins, synthetic peptides were screened with commercially available purified human, rab-

TABLE 2. Reactivities of antisera raised against native P2 as determined by P2-specific ELISA

Source	Immunogen	Titer[a]
Human		
1	Hib[b]	25,600
2	Hib	6,400
3	Hib	1,600
Guinea pig		
52	Native P2	409,000
RF3430	Native P2	1,638,400
RF3438	Native P2	6,553,600
Mouse[c]		
A/J[c]	Native P2	1,600
SJL/J	Native P2	25,600
BALB/c	Native P2	12,800
BL/6	Native P2	25,600
C3H	Native P2	12,800
SWR/J	Native P2	6,400
Rabbit		
RB-RF3428	Native P2	819,200
RB493	Native P2	6,533,600
RB-1	PRP-P2[d]	25,600
RB-2	PRP-P2	51,200
RB-3	PRP-P2	12,800
RB-4	PRP-P2	51,200
RB-5	PRP-P2	102,400

[a] Preimmunization titers were subtracted from postimmunization titers.
[b] Antisera were obtained from convalescent-phase patients.
[c] Two mice per group were used in immunogenicity studies.
[d] PRP-P2 was prepared by direct conjugation between cyanogen bromide-activated PRP and chromatographically purified P2. No linker was used, and the PRP/P2 (wt/wt) ratio was approximately 7.

TABLE 3. Immunological properties of rabbit antisera raised against P2 peptides and peptide-KLH conjugates

Immunogen	Reactive titer as determined by ELISAs[a]		Recognition of P2 in immunoblots	
	Native P2	Specific peptides	Hib	Nontypeable
Porin-1–KLH	3,200	104,800	Yes	Yes
Porin-1	<200	<200	No	No
HIBP2-25–KLH	<200	25,600	Yes	No
HIBP2-25	<200	102,400	Yes	No
HIBP2-26–KLH	<200	<200	No	No
HIBP2-26	<200	<200	No	No
OMP2-1–KLH	<200	6,400	Yes	No
OMP2-1	<200	3,200	Yes	No
OMP2-2–KLH	<200	409,600	Yes	No
OMP2-2	<200	204,800	Yes	No
OMP2-3–KLH	<200	3,200	Yes	No
OMP2-3	<200	102,400	Yes	No
OMP2-4–KLH	<200	6,400	Yes	No
OMP2-4	12,800	102,400	Yes	No
OMP2-5–KLH	25,600	204,800	Yes	Yes
OMP2-5	<200	102,400	Yes	Yes
OMP2-6–KLH	<200	6,400	Yes	No
OMP2-6	<200	204,800	Yes	No
OMP2-7–KLH	3,200	51,200	Yes	No
OMP2-7	<200	102,400	Yes	No
OMP2-8–KLH	6,400	51,200	Yes	Yes
OMP2-8	51,200	3276,800	Yes	Yes
OMP2-9–KLH	<200	6,400	Yes	No
OMP2-9	<200	409,600	Yes	No
OMP2-10–KLH	3,200	51,200	Yes	Yes
OMP2-10	12,800	409,600	Yes	Yes
OMP2-11–KLH	<200	800	Yes	Yes
OMP2-11	6,400	102,400	Yes	Yes
OMP2-12–KLH	51,200	3,276,800	Yes	Yes
OMP2-12	51,200	32,000	Yes	Yes
OMP2-13–KLH	<200	<200	No	No
OMP2-13	51,200	1,638,400	Yes	Yes
CHIBP2–KLH	12,800	204,800	Yes	No
CHIBP2	<200	1,600	Yes	No
OMP2-6U–KLH	204,800	3,276,800	Yes	Yes

[a] The data shown are those obtained with rabbit antisera having the highest antibody titers.

bit, guinea pig, and mouse IgGs. Only peptide OMP2-10 (residues 241 to 265) was found to react nonspecifically with IgGs at dilutions below 1/800. Therefore, the reactive titers for peptide OMP2-10 were corrected by subtracting the background titer obtained with normal IgGs. In addition, the interaction between each solid-phase peptide and its corresponding antiserum could be specifically blocked by preincubation of antiserum with free peptide (data not shown).

TABLE 4. Reactivities of murine anti-P2 antisera with P2-overlapping peptides

Peptide	Mouse strain[a]					
	BL/6	BALB/c	SWR/J	SJL/J	A/J	C3H
OMP2-2	+	+	+	+	+	+
CHIBP2	+	+	+	+	+	+
OMP2-10	+	+	+	+	+	+
OMP2-3	+	+	+	−	+	−
OMP2-1	−	+	−	+	+	+
HIBP2-25	−	*	*	+	−	−
OMP2-7	+	−	*	−	−	*
OMP2-4	+	*	*	−	−	−
OMP2-11	+	*	−	−	−	−
OMP2-5	*	−	−	−	−	−
OMP2-9	−	*	−	−	−	−
OMP2-12	−	−	−	−	−	−
OMP2-6	−	−	−	−	−	−
OMP2-8	−	−	−	−	−	−
OMP2-13	−	−	−	−	−	−
HIBP2-26	−	−	−	−	−	−
Porin-1	−	−	−	−	−	−
S3-P6[b]	−	−	−	−	−	−
NAD-S1[b]	−	−	−	−	−	−

[a] Six strains of mice (two per group) were immunized with 75 μg of native P2. The antisera were analyzed by peptide-specific ELISA for antipeptide activity. +, antisera from two different mice reacted with the peptide; * and −, one of two and zero of two mouse antisera recognized the peptide, respectively.

[b] Peptides S3-P6 and NAD-S1 are irrelevant synthetic peptides from *Bordetella pertussis* used as negative controls.

Reactivities of anti-P2 antisera with P2 peptides. The 17 P2 peptides were reacted with mouse anti-P2 antisera in peptide-specific ELISAs. For comparison and ease of identification of the immunodominant B-cell determinants of P2, one dilution of antiserum (1/6,400) was chosen to be analyzed. The results are summarized in Table 4. Peptides OMP2-2 (residues 53 to 81), OMP2-10 (residues 241 to 265), and CHIBP2 (residues 314 to 341) were recognized by all mouse anti-P2 antisera. These peptides were also found to be more reactive than purified P2 as judged by ELISA titer. The reactive titers with most antisera were >102,400, whereas the highest anti-P2 titer was only 25,600. Synthetic peptides OMP2-1 and OMP2-3 were recognized by four of six strains of mice, but their reactive titers were much lower (12,800). At 1/6,400 dilution, antisera from both BL/6 and BALB/c mice reacted with 8 and 9 of 17 peptides, respectively. At this dilution (1/12,800) antisera from SWR/J, SJL/J, A/J, and C3H mice recognized only OMP2-2, OMP2-10, and CHIBP2.

The reactivities of P2 peptides with three different guinea pig anti-P2 antisera (1/6,400 dilution) were tested by a procedure similar to the one described above. As shown in Fig. 2, peptide CHIBP2 showed a relatively high reaction with all three guinea pig antisera.

P2 peptides were tested for their reactivities with two different rabbit anti-P2 antisera (1/6,400 dilution). Peptides OMP2-2 and CHIBP2 were well recognized by both antisera (Fig. 3A). In addition, peptides Porin-1, HIBP2-25, HIBP2-26, OMP2-5, OMP2-7, OMP2-9, OMP2-11, OMP2-12, and OMP2-13 reacted only with antibodies from rabbit no. 493.

Since one of our objectives is to use P2 as both a

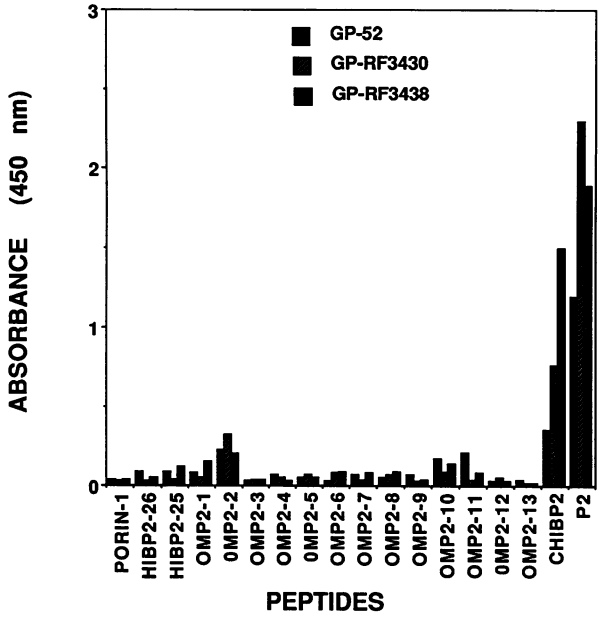


FIG. 2. ELISA reactivities of guinea pig (GP) anti-P2 antisera 52, RF3430, and RF3438 (1/6,400 dilution) with purified P2 and a panel of P2 synthetic peptides.

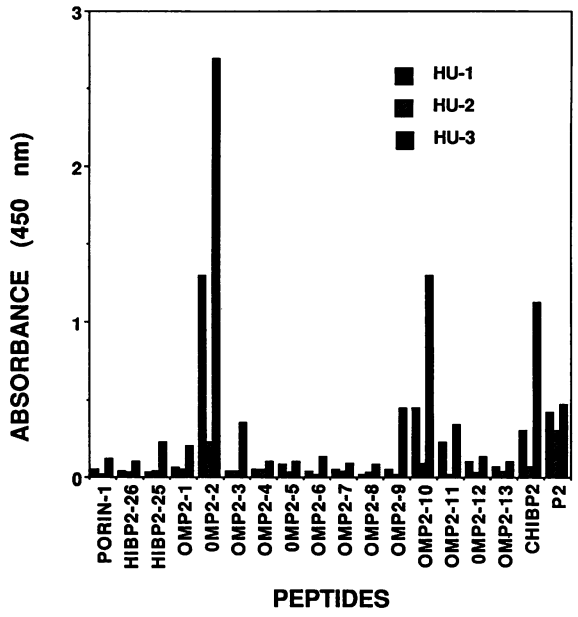
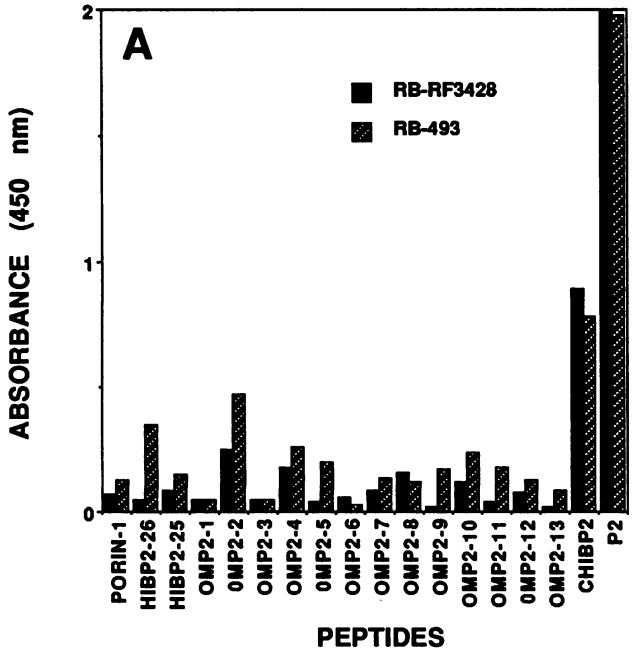


FIG. 4. ELISA reactivities of human convalescent-phase sera HU-1, -2, and -3 (1/1,000 dilution) with purified P2 and its synthetic peptides.

protective antigen and a carrier, it was of interest to identify the epitopes recognized by antisera raised against a PRP-P2 conjugate. Peptides OMP2-2 and CHIBP2 were recognized by all five antisera in the peptide-specific ELISA. These results are very similar to those obtained with antisera raised against P2, suggesting that the coupling of PRP to P2 did not destroy the immunodominant epitopes of P2.

Two of three human convalescent-phase serum samples (1/1,000 dilution) reacted strongly with peptides OMP2-2, OMP2-10, and CHIBP2 (Fig. 4). Thus, these results suggest that there are three immunodominant linear human B-cell epitopes located within peptides OMP2-2, OMP2-10, and CHIBP2.

It is surprising that both rabbit and guinea pig anti-P2 antisera showed antipeptide-reactive titers in the peptide-specific ELISAs against peptide-KLH conjugates that were



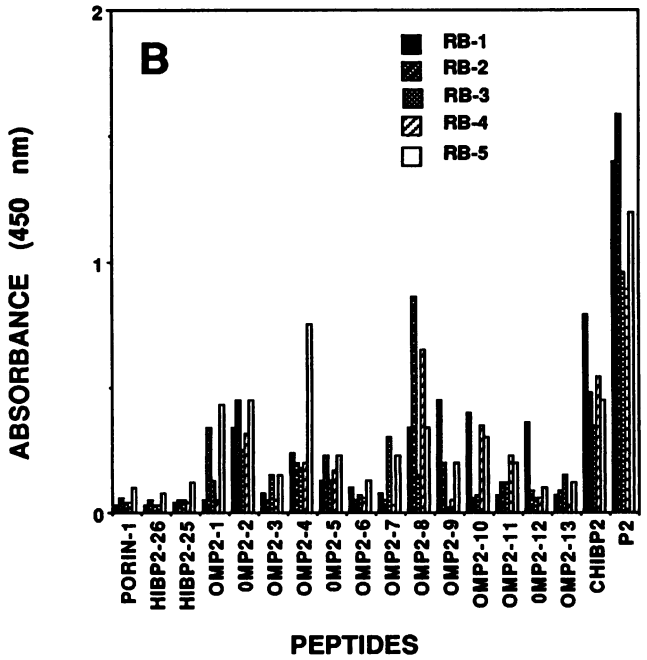


FIG. 3. ELISA reactivities of rabbit anti-P2 antisera RB-RF3428 and RB493 (A) and rabbit anti-PRP-P2 conjugate antisera RB-1, -2, -3, -4, and -5 (B) with purified P2 and its synthetic peptides. One dilution (1/6,400) was used in all assays.

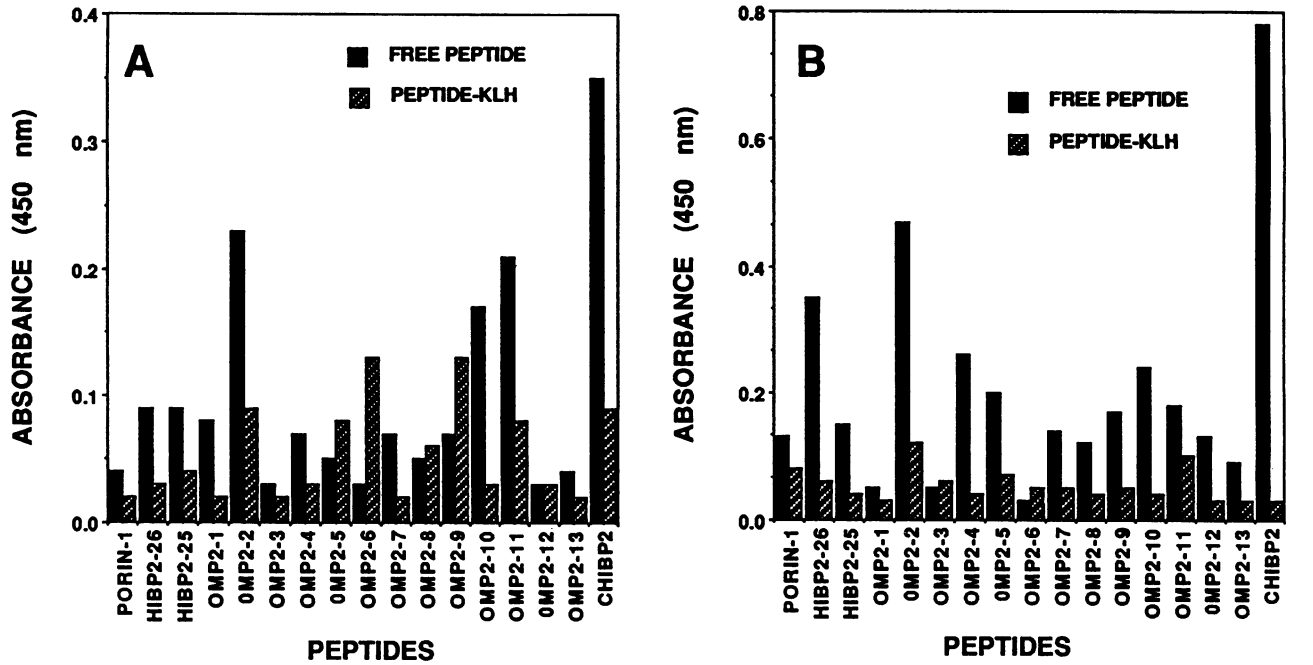


FIG. 5. ELISA reactivities of the panels of P2 synthetic peptides and peptide-KLH conjugates with guinea pig antiserum GP-52 (A) and rabbit antiserum RB-493 (B).

lower than those against free peptides (Fig. 5). Such an observation may be explained by previous reports in which the orientation of the peptide and the conjugation procedures would influence the binding pattern of antipeptide antibodies (5, 9, 10). Therefore, the current results demonstrate that coating microtiter wells with unconjugated peptide has an advantage over using a peptide carrier, which is time-consuming and not very cost-effective.

Two pertussis toxin peptides, NAD-S1 and S3-P6, were included as negative controls in the ELISAs. Neither peptide showed any cross-reactivity with anti-P2 antibodies.

Fine mapping of immunodominant B-cell epitopes. To further identify the residues in the immunodominant epitopes (OMP2-2, OMP2-10, and CHIBP2) critical for antibody recognition, N-terminal truncated peptides were prepared (Table 1) and tested in the peptide-specific ELISA (Table 5). The N-terminal residues (53 to 64) of OMP2-2 appear to be critical for human antibody binding, since all human antisera exhibited a fourfold reduced reactivity with the N-terminal truncated peptide OMP2-2A. Only guinea pig RF3438 anti-P2 antiserum showed less reactivity with synthetic peptide OMP2-10A, an N-terminal truncated peptide analog of OMP2-10. Therefore, the N terminus (residues 241 to 251) of OMP2-10 may not constitute the antibody-binding site which may be located within residues 252 to 265. The low reactivities between CHIBP2A (residues 322 to 341) and anti-P2 antisera from both rabbits and guinea pigs indicate that the N-terminal sequence of CHIBP2 (residues 314 to 341) is important for antibody binding (Table 5). In fact, residues 320 to 322 may be the most critical amino acids, since peptide OMP2-13, which contains the first six residues of CHIBP2, reacted poorly with all anti-P2 antisera. CHIBP2A also reacted less well with human convalescent-phase sera.

Immunogenicity of P2 peptides. Rabbit antisera raised

TABLE 5. Reactivities of human convalescent-phase sera and guinea pig and rabbit anti-P2 antisera with P2 immunodominant linear B-cell epitopes as determined by ELISA

Antiserum	Reactive titer against the following synthetic peptides:					
	OMP2-2	OMP2-2A	OMP2-10	OMP2-10A	CHIBP2	CHIBP2A
Guinea pig						
RF3430	800	<200	<200	<200	51,200	<200
RF3438	3,200	1,600	25,600	800	204,800	<200
52	12,800	1,600	800	1,600	25,600	<200
Rabbit						
RB493	25,600	6,400	25,600	6,400	102,400	12,800
RB-RF3428	12,800	6,400	12,800	6,400	102,400	<200
Human convalescent phase						
1	12,800	3,200	3,200	3,200	6,400	3,200
2	12,800	3,200	3,200	6,400	6,400	3,200
3	12,800	1,600	12,800	3,200	6,400	1,600

against P2 peptide-KLH conjugates were further assayed for reactivity against P2 by P2-specific ELISAs and by immunoblot analysis. Although all peptide-specific antisera, except antisera raised against HIBP2-26–KLH and OMP2-13–KLH conjugates, recognized P2 in the immunoblots, only Porin-1, OMP2-5, -7, -8, -10, and -12, and CHIBP2 peptide–KLH conjugates were found to elicit antibodies that cross-reacted with native P2 in the P2-specific ELISA (Table 3). All unconjugated peptides, except Porin-1 and HIBP2-26, emulsified in complete Freund's adjuvant induced very strong peptide-specific antibody responses against P2 in the immunoblot (Table 3). In addition, antisera raised against unconjugated peptides OMP2-4, -8, -10, -11, -12, and -13 reacted strongly with purified P2 in the P2-specific ELISA. These data indicate that these peptides contain potent functional T-helper-cell epitopes and that the T-cell epitopes are distributed throughout the P2 protein, with the exception of the first 19 N-terminal residues.

One objective of this study is to identify conserved protective epitope(s). Thus, it is of interest to know whether these peptide-specific antisera would cross-react with P2 from nontypeable *H. influenzae* isolates. P2 was purified from three different nontypeable isolates, SB30, SB32, and SB33 (kindly provided by S. Barenkamp of Washington University), and used as target antigen in immunoblots. Rabbit anti-Porin-1 and anti-OMP2-5, -8, -10, -11, -12, and -13 antisera reacted strongly with P2 from all three nontypeable isolates. These results suggest that the peptides corresponding to residues 1 to 19, 125 to 150, 183 to 219, 241 to 265, 263 to 289, 285 to 306, and 302 to 319 contain epitopes conserved between typeable and nontypeable strains of *H. influenzae*.

DISCUSSION

Synthetic peptides are convenient tools to map epitopes within linear segments of immunogens (1, 6, 10, 18, 28). Synthetic peptides corresponding to fragments of bacterial toxins have been shown to elicit high titers of specific antibodies which react with the native toxin and sometimes neutralize its biological activities (2, 6, 16, 19). Thus, synthetic peptides conceivably can be used as synthetic vaccines against diseases. As a first step toward the design of an OMP-based Hib vaccine, the antigenic determinants of P2 were mapped with overlapping synthetic peptides and the immunogenicities of the peptides were assessed for possible inclusion in a new generation of cross-protective *H. influenzae* vaccine against typeable and nontypeable strains. Berzosky (4) has pointed out that both the genetic background and the environmental histories of the host dictate the specificity in the uptake, processing, and presentation of an antigen for any specific antibody response. It was, therefore, of interest to examine the antibody profiles against P2 generated in various mouse strains and in rabbits, guinea pigs, and humans. In the present study, anti-P2 antisera and a set of 17 overlapping synthetic peptides were used to identify the immunodominant B-cell epitopes of P2. Our observations show that P2 peptides fall into one of the four following categories according to their reactivities with anti-P2 antisera: (i) high titer-high frequency of recognition; (ii) low titer-high frequency of recognition; (iii) low titer-low frequency of recognition; and (iv) nonreactive. Peptides with a high frequency of recognition and high titer of reactivity with the antisera represent the immunodominant linear B-cell epitopes of the antigen. The low titer-low frequency reactivity pattern may represent immunorecessive regions of the antigen. It has become widely accepted that there exists in many complex antigens a proportion of antigenic determinants which are dominant targets for the immune response. P2 appears to be no exception to this concept. Antisera from all species reacted strongly and with high frequencies to synthetic peptides OMP2-2 and OMP2-10 and the C-terminal peptide CHIBP2. These peptides thus represent immunodominant B-cell epitopes of *H. influenzae* P2 protein. These results are consistent with the hydrophilicity plots generated by computer analysis of the P2 protein sequence (Fig. 1). Regions encompassing residues 53 to 81 (OMP2-2), 241 to 265 (OMP2-10), and 314 to 341 (CHIBP2) exhibit features of hydrophilic β-turns which are considered to be antigenic properties. In fact, the C-terminal end of P2 (residues 314 to 341) has previously been identified as a surface-exposed epitope by using a P2-specific monoclonal antibody (22). Fine epitope mapping with truncated peptides revealed that residues 53 to 64, 252 to 265, and 314 to 322 of P2 are critical for generating anti-P2 antibodies that bind to peptides OMP2-2, OMP2-10, and CHIBP2, respectively.

Preliminary experiments have been performed to determine whether P2 peptides block the protective ability of rabbit anti-P2 antibodies in the infant rat model of bacteremia. These studies revealed that the protective ability of rabbit anti-P2 antisera could not be inhibited by adsorption with the immunodominant P2 peptides alone or in mixture (all 17 peptides) at concentrations of up to 200 μg of peptide per ml of antiserum. Rabbit antisera raised against native P2 protein contain antibodies to conformational and linear epitopes. In fact, the preliminary results described above suggest that the protective epitope(s) of P2 may be conformational.

Regions around peptides OMP2-4 (101 to 129), OMP2-6 (148 to 174), OMP2-8 (193 to 219), OMP2-11 (263 to 289), and OMP2-13 (302 to 319) are also predicted to be potential B-cell epitopes (Fig. 1). Also, Martin et al. (22) have previously reported that the region around OMP2-6 (residues 158 to 174) is a surface-exposed immunodominant B-cell epitope, as identified by several P2-specific monoclonal antibodies. In the present study, peptides OMP2-4 and -8 reacted strongly with antisera from rabbits immunized with either P2 or PRP-P2 conjugate, but the other potential B-cell epitopes were found to be immunorecessive when antisera from other animal species were used. On the basis of the diversity of the protein sequences between *H. influenzae* subtypes and the observation that polyclonal antisera generated against P2 of an OMP subtype 1H strain did not protect against challenge with an OMP subtype 2L in the infant rat model of bacteremia, Munson et al. (23) suggested that a subtype-specific protective epitope on P2 could be located in residues 160 to 170. The nonreactivity of OMP2-6 could be due to the fact that the polyclonal antibodies generated against the Eagan strain are strain specific. Thus, two peptides, OMP2-6L and OMP2-6U, were synthesized according to the protein sequences of the 2L and 6U subtypes (Table 1), respectively. No significant binding between these two peptides and anti-P2 antisera from rabbits, guinea pigs, and human convalescent-phase sera were observed (data not shown). It should be cautioned that a lack of binding cannot be taken as proof of absence of antibody to these sequences. However, the possibility that the peptides were adsorbed to the microtiter plate through their antigenic determinants is eliminated by two observations. Firstly, peptide OMP2-6 reacted strongly with several mouse monclonal antibodies in the peptide-specific ELISA (22). Secondly, when peptide-KLH conjugates were used as antigens in the ELISA, no

binding was observed between the OMP2-6–KLH conjugate and all antisera tested. Musser et al. (26a) have reported that the Eagan strain is a highly atypical strain among Hib. Thus, it is possible that the P2 protein sequence of the Eagan strain at this region differs from the other three known P2 sequences. Without the aid of three-dimensional structural data, it is not possible to predict the presence of assembled epitopes in this region of P2.

Our present work and those of others (12, 26) have demonstrated that antibodies directed against P2 are protective against strain-specific live *H. influenzae* challenge. A recent clinical trial showed that PRP conjugated to OMP of *N. meningitidis* appeared to be very immunogenic in 2- to 4-month-old infants (13, 17, 36). Also, the PRP-P2 conjugates used in this study elicited strong anti-PRP IgG responses, which were four- to fivefold higher than that obtained with the PRP-diphtheria toxoid vaccine (5a). Thus, PRP conjugated to the conserved surface-exposed and protective P2 epitope(s) would be very useful as a universal vaccine protecting against typeable and nontypeable *H. influenzae* infections. It is of interest to identify such conserved surface-exposed epitopes. To this end, rabbit P2 peptide-specific antisera were tested against typeable and nontypeable *H. influenzae* isolates. All peptide-specific antisera recognized Hib P2 on immunoblot analysis regardless of whether they had been raised against conjugated or unconjugated peptides. However, their abilities to react with nontypeable P2 were significantly different. Only antisera raised against peptides corresponding to residues 1 to 19, 125 to 150, 241 to 265, 263 to 289, 285 to 306, and 302 to 319 recognized nontypeable P2 in the immunoblot analyses. These results strongly suggest that these peptides contain conserved epitopes. Therefore, these peptides alone or in combination will be useful in the preparation of prototype cross-reactive conjugate vaccines against *H. influenzae*.

All peptides, with the exception of the first two N-terminal P2 peptides (residues 1 to 14 and 8 to 19), induced strong anti-peptide IgG antibody responses, indicating that potent functional T-helper-cell epitopes are located throughout the P2 protein, with the exclusion of the first N-terminal 19 residues. In preliminary experiments on T-cell epitope mapping, OMP2-5 (residues 125 to 150), OMP2-8 (residues 193 to 219), OMP2-9 (residues 219 to 244), and OMP2-10 (residues 241 to 265) were found to be capable of stimulating the proliferation of P2-specific T-cell lines generated from several strains of mice (5a). These results further support the idea that P2 or its peptide fragments can serve as both protective antigens and carriers for PRP.

In conclusion, we have identified three linear immunodominant B-cell epitopes (resides 53 to 81, 241 to 265, and 314 to 341) on P2. Also, on the basis of the information obtained from the cross-reactivities of the peptide-specific antibodies with P2 isolated from either typeable or nontypeable strains, the N-terminal first 14 residues and regions around residues 125 to 150 and 241 to 319 of P2 contain conserved epitopes among *H. influenzae* isolates. Identification of these immunodominant B-cell epitopes and conserved regions represents a first step toward the rational design of a cross-protective *H. influenzae* vaccine.

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REFERENCES

1. **Askelof, P., K. Rodmalm, J. Abens, A. Unden, and T. Bartiai.** 1988. Use of synthetic peptides to map antigenic sites of *Bordetella pertussis* toxin subunit S1. J. Infect. Dis. **157:**738–742.
2. **Audibert, F., M. Jolivet, L. Chedid, J. E. Alouf, P. Boquet, P. Rivaille, and O. Siffert.** 1981. Active anti-toxic immunization by a diphtheria toxin synthetic oligopeptide. Nature (London) **289:**593–594.
3. **Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J. Infect. Dis. **143:**668–676.
4. **Berzosky, J. A.** 1985. Intrinsic and extrinsic factors in protein antigenic structure. Science **229:**932–940.
5. **Briand, J. P., S. Muller, and M. H. V. Van Regenmortel.** 1985. Synthetic peptides as antigens: pitfalls of conjugation methods. J. Immunol. Methods **78:**59–69.

5a. **Chong, P., et al.** Unpublished data.

6. **Chong, P., M. Sydor, G. Zobrist, H. Boux, and M. Klein.** 1991. Structural and functional analysis of the S1 subunit of pertussis toxin using synthetic peptides. Mol. Immunol. **28:**239–245.

6a. **Chong, P., G. Zobrist, B. Tripet, Y. Choi, M. Sydor, C. Sia, R. Fahim, and M. Klein.** 1990. Mapping of the B-cell epitopes of the outer membrane protein P2 of *H. influenzae* type b using overlapping synthetic peptides, abstr. E-77. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990. American Society for Microbiology, Washington, D.C.

7. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predication of protein conformation. Annu. Rev. Biochem. **47:**251–276.
8. **Cochi, S. L., and C. V. Broome.** 1986. Vaccine prevention of Haemophilus influenzae type b disease: past, present, and future. Pediatr. Infect. Dis. J. **5:**12–19.
9. **Dyrberg, T., and M. B. Oldstone.** 1986. Peptides as antigens: importance of orientation. J. Exp. Med. **164:**1344–1349.
10. **Geerligs, H. J., W. J. Weijer, W. Bloemhoff, G. W. Welling, and S. Welling-Wester.** 1988. The influence of pH and ionic strength on the coating of peptides of herpes simplex virus type 1 in an enzyme-linked immunoabsorbent assay. J. Immunol. Methods **106:**239–244.
11. **Gordon, L. K.** 1984. Characterization of a hapten-carrier conjugate vaccine, p. 393–396. *In* R. M. Chanock and R. A. Lerner (ed.), Modern approaches to vaccines. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
12. **Granoff, D. M., and R. S. Munson, Jr.** 1986. Prospects for prevention of *Haemophilus influenzae* type b disease by immunization. J. Infect. Dis. **153:**448–461.
13. **Granoff, D. M., G. A. Weinberg, and P. G. Shackelford.** 1989. IgG subclass response to immunization with H. influenzae type b polysaccharide-outer membrane protein conjugate vaccine. Pediatr. Res. **24:**180–185.
14. **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.
15. **Hansen, E. J., C. Hasemann, A. Clausell, J. D. Capra, K. Orth, C. R. Moomaw, C. A. Slaughter, J. L. Latimer, and E. E. Miller.** 1989. Primary structure of the porin protein of *Haemophilus influenzae* type b as determined by nucleotide sequence analysis. Infect. Immun. **57:**1100–1107.
16. **Harari, I., A. Donohue-Rolfe, G. Keusch, and R. Arnon.** 1988. Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity. Infect. Immun. **56:**1618–1624.
17. **Hetherington, S. V., and A. F. Rutkowski.** 1990. Antibody affinity in infants after immunization with conjugated capsular polysaccharide from *H. influenzae* type b. J. Infect. Dis. **162:**1185–1188.
18. **Hopp, T. P.** 1986. Protein surface analysis. Methods for identifying antigenic determinants and other interaction sites. J. Immunol. Methods. **88:**1–18.
19. **Jacob, C. O., M. Sela, and R. Arnon.** 1983. Antibodies against synthetic peptides of the B subunit of cholera toxin: cross-

reaction and neutralization of the toxin. Proc. Natl. Acad. Sci. USA **80:**7611–7615.

20. **Kayhty, H., H. Peltola, J. Eskola, P. R. Ronnberg, E. Kela, V. Varanko, and P. H. Makela.** 1989. Immunogenicity of *Haemophilus influenzae* oligosaccharide-protein and polysaccharide-protein conjugate vaccination of children at 4, 6, and 14 months of age. Pediatrics **84:**995–999.
21. **Loeb, M. R., and D. H. Smith.** 1980. Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. Infect. Immun. **30:**709–717.
22. **Martin, D., R. Munson, Jr., S. Grass, P. Chong, J. Hamel, G. Zobrist, M. Klein, and B. R. Brodeur.** 1991. Mapping of B-cell epitopes on the outer membrane P2 porin protein of *Haemophilus influenzae* using recombinant proteins and synthetic peptides. Infect. Immun. **59:**1457–1464.
23. **Munson, R., Jr., C. Bailey, and S. Grass.** 1989. Diversity of the outer membrane protein P2 gene from major clones of *Haemophilus influenzae* type b. Mol. Microbiol. **3:**1797–1803.
24. **Munson, R., Jr., and R. W. Tolan, Jr.** 1989. Molecular cloning, expression, and primary sequence of outer membrane protein P2 of *Haemophilus influenzae* type b. Infect. Immun. **57:**88–94.
25. **Munson, R. S., Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff.** 1983. Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. J. Clin. Invest. **72:**677–684.
26. **Murphy, T. F., and L. C. Bartos.** 1988. Purification and analysis with monoclonal antibodies of P2, the outer membrane protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **56:** 1084–1089.

26a. **Musser, J. M., J. S. Simon, E. R. Moxon, and R. K. Selander.** 1988. Evolutionary genetics of the encapsulated strains of *Haemophilus influenzae*. Proc. Natl. Acad. Sci. USA **85:**7758–7762.

27. **Parker, J. M. R., D. Guo, and R. S. Hodges.** 1986. New hydrophilicity scale derived from high performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. Biochemistry **25:**5425–5432.
28. **Rhodes, H., R. Houghten, J. P. Taulane, D. Carson, and J. Vaughan.** 1985. The immune response to Epstein-Barr nuclear antigen: conformational and structural features of antibody binding to synthetic peptides. Mol. Immunol. **21:**1047–1054.
29. **Terr, P. I., and G. Peter.** 1978. Demographic factors in the epidemiology of Haemophilus influenzae meningitis in young children. J. Pediatr. **92:**884–888.
30. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
31. **Turk, D. C.** 1984. The pathogenicity of *Haemophilus influenzae*. J. Med. Microbiol. **18:**1–16.
32. **Vachon, V., R. Laprade, and J. W. Coulton.** 1986. Properties of the porin of *Haemophilus influenzae* type b in planar lipid bilayer membranes. Biochim. Biophys. Acta **861:**74–82.
33. **Vanalphen, L., P. Eijk, L. Geehen-van den Broek, and J. Dankert.** 1991. Immunochemical characterization of variable epitopes of outer membrane protein P2 of nontypeable *Haemophilus influenzae*. Infect. Immun. **59:**247–252.
34. **Van Regenmortel, M. H. V., S. Muller, V. F. Quesniaux, D. Altchuh, and J. P. Briand.** 1988. Operational aspects of epitope identification: structural features of proteins recognized by antibodies, p. 113–122. *In* H. Kohler and P. T. LaVerde (ed.), Vaccines: new concepts and developments. Longman, London.
35. **Weinberg, G. A., and D. M. Granoff.** 1988. Polysaccharide-protein conjugate vaccines for the prevention of *Haemophilus influenzae* type b disease. J. Pediatr. **113:**621–631.
36. **Weinberg, G. A., S. Menachem, S. Einhorn, A. A. Lenoir, P. D. Granoff, and D. M. Granoff.** 1987. Immunological priming to capsular polysaccharide in infants immunized with *H. influenzae* type b polysaccharide-*Neisseria meningitidis* outer membrane protein conjugate vaccine. J. Pediatr. **111:**22–27.