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Identification of Surface-Exposed B-Cell Epitopes Recognized by *Haemophilus influenzae* Type b P1-Specific Monoclonal Antibodies

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A panel of P1 synthetic peptides was synthesized to map the surface-exposed epitopes of *Haemophilus influenzae* type b outer membrane protein P1 recognized by three murine monoclonal antibodies (MAbs 7C8, 3E12, and 6B1). By using peptide-specific enzyme-linked immunosorbent assays, MAbs 6B1, 7C8, and 3E12 were shown to recognize distinct epitopes localized within residues 60 to 88, 165 to 193, and 400 to 437 of mature P1, respectively. Since MAb 7C8 was shown previously to be protective against certain *H. influenzae* type b subtypes in the infant rat model of bacteremia, its cognate epitope was further characterized by using truncated peptide analogs. Fine mapping of the 7C8 epitope by competitive inhibition studies revealed that it was localized within residues 184 and 193.

Haemophilus influenzae type b (Hib) is a major cause of bacterial meningitis in children under 5 years of age (16, 17). Antibodies induced against the capsular polysaccharide, polyribosyl ribitol phosphate (PRP), of the organism are protective (19). Effective conjugate vaccines in which PRP is linked to different carrier proteins, such as diphtheria toxoid (PRP-D), tetanus toxoid (PRP-T), CRM 197, and the outer membrane protein (OMP) of *Neisseria meningitidis*, were subsequently developed (5, 9). However, these conjugate vaccines do not protect against other invasive typeable *H. influenzae* strains and, more importantly, against nonencapsulated nontypeable *H. influenzae* infection that is one of the common causes of otitis media, for which there is no vaccine. Therefore, the inclusion of selected nonencapsulated *H. influenzae* immunogens in current Hib vaccines may be advantageous (11).

Granoff and Munson (6) reported that antibodies directed against Hib OMPs P1, P2, and P6 were protective in the infant rat model of bacteremia. Recent studies also showed that a murine P1-specific monoclonal antibody (MAb 7C8) and rabbit antisera raised against purified P1 from either typeable or nontypeable *H. influenzae* strains were protective in animal models (7, 10, 13). Therefore, a promising strategy for designing a universal *H. influenzae* vaccine with enhanced protective ability would be to use either purified OMPs or their protective epitopes as additional immunogens and carriers for PRP.

The gene coding for P1 has been cloned from several different Hib subtypes (1H, 3L, and 6U) (4, 12). The comparative analysis of P1 protein sequences from these Hib isolates revealed the existence of three hypervariable regions. Indeed, the protective P1-specific MAb reported by Hansen's group recognizes only 50% of the Hib isolates tested (4, 7). The purpose of this study was to map P1 surface-exposed epitopes recognized by P1-specific MAbs as a first step towards the rational design of a synthetic peptide-based vaccine against both typeable and nontypeable *H. influenzae*.

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MATERIALS AND METHODS

Peptide synthesis. P1 peptides corresponding to the MinnA strain sequence (subtype 1H) (12) were chemically synthesized with an ABI 430A peptide synthesizer. A cysteine residue was added at either the C or N terminus of the peptide to facilitate the coupling to a carrier protein in one specific orientation. Small aliquots of peptide-resin were removed at various stages of synthesis to obtain truncated peptide analogs for fine epitope mapping. The peptides were cleaved from the resin by trifluoromethanesulfonic acid or hydrofluoric acid and purified by reversed-phase high-performance liquid chromatography (reversed-phase HPLC) using a semipreparative Vydac C_4 column and a 10 to 50% acetonitrile gradient in 0.1% trifluoroacetic acid developed over 40 min at a flow rate of 2 ml/min. The peptides were shown to be >95% pure by analytical HPLC. The amino acid compositions of the peptide hydrolysates were found to be in good agreement with the theoretical compositions.

Growth of bacteria and preparation of OMP extracts. OMP extracts were obtained from both typeable (Eagan, MinnA, Durst, 1613, 8358, DL41, and DL42) and nontypeable (PAK12085, SB30, SB32, and SB33) *H. influenzae* strains. Bacteria were cultured in brain heart infusion broth supplemented with NAD (2 μg/ml; Difco) and hemin (2 μg/ml; Difco) at 37°C. Cell envelopes were obtained from ultrasonically disrupted cells by ultracentrifugation at 140,000 × *g* for 45 min. Pellets containing the OMP fractions were resuspended in 50 mM Tris-HCl (pH 7.5). Protein concentrations were determined by the BCA protein assay (Pierce), and the crude OMP extracts were stored at −20°C for immunoblot analysis and MAb reactivity studies.

Preparation of P1-specific MAbs. Three murine MAbs

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TABLE 1. Potential B-cell epitopes of Hib OMP P1

Peptide	Residues	Sequence[a]
HIBP1-2	60–88	GDVTSYAQIITNQIGMKAIKDGSASQRNV(C)
		<-----HIBP1-4A----->
HIBP1-4	165–193	YAKAQVERNAGLIADSVKDNQITSALSTQ(C)
		<----HIBP1-4B--->
HIBP1-4(L)	165–193	YAKAQVERNAGIIANSVNDTQVKTALSVL(C)
HIBP1-4(U)	165–193	YAKAQVERNAGIITESVKIAQNALKT(C)
HIBP1-5	189–218	ALSTQQEFRDLKKYLPSKDKSVVSLQDRA(C)
HIBP1-5E	184–218	NQITSALSTQQEFRDLKKYLPSKDKSVVSLQDRA(C)
HIBP1-13	400–437	(C)FKEVKTIGDKRTLTLNTTANYTSQAHANLYGLNLNYSF
HIBP1-14	400–437	(C)FKEAQQAAGGFITTTANYTSQAHANLYGLNLNYSF

[a] All peptide sequences except those of peptides HIBP1-4(L), -4(U), and -14 are based on the amino acid sequence of the mature P1 protein of the 1H isolate. Peptide sequences of HIBP1-4(L) and of HIBP1-4(U) and -14 are derived from the P1 sequences of the 3L and 6U Hib isolates, respectively (12). Hypervariable residues among P1 sequences are underlined.

(7C8, 6B1, and 3E12) were generated against the Hib strain DL42 as described previously (4, 7) and shown to be P1 specific by immunoblot analysis. These MAbs recognize three different epitopes on the P1 surface, and Gonzales et al. have reported that the passive administration of MAb 7C8 protected infant rats against challenge with certain live Hib bacteria (4). Hybridoma cell culture supernatants were used as the source of MAbs. MAb 7C8 was further purified by protein A-affinity chromatography and found to be of the immunoglobulin G2a (IgG2a) isotype.

Peptide-carrier conjugation. Individual peptides were conjugated to keyhole limpet hemocyanin with sulfosuccinimyl(4-iodoacetyl)-amino-benzoate (Pierce) at a 10:1 molar ratio of peptide to carrier protein by the method of Chong et al. (1).

Production of rabbit antipeptide antisera. Rabbits were immunized with either free peptides (50 μg) or peptide-keyhole limpet hemocyanin conjugates (200 μg) emulsified in complete Freund's adjuvant and then with two booster doses (half the amount of the same immunogen in incomplete Freund's adjuvant) at 2-week intervals. Antisera were collected, heat inactivated at 56°C for 30 min, and stored at −20°C.

Three human convalescent-phase serum samples were generously provided by S. Halperin of Dalhousie University, Halifax, Canada.

Immunoassays. The reactivities of MAbs to membrane-bound P1 and P1 peptides were tested by enzyme-linked immunosorbent assays (ELISAs). Briefly, microtiter wells (Immulon; Dynatech Industries, Inc., McLean, Va.) were coated with either 5 μg of crude OMP extract per well or 1 μg of individual peptide in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) per well for 16 h at 4°C. Unreacted sites were blocked by incubation with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 60 min at 21°C. One hundred microliters of serially diluted P1-specific MAb (either culture supernatant or purified) was added to the wells, and the plates were incubated for 2 to 3 h at 21°C. Antibodies were removed, and the plates were washed four times with PBS containing 0.1% Tween 20 (washing buffer). Horseradish peroxidase-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG antibodies (Jackson Immuno-Research Labs, Inc.) were diluted (1/10,000) with washing buffer and added to the microtiter plates. After 1 to 2 h of incubation at 21°C, the plates were washed four times and then developed with tetramethylbenzidine and H_2O_2 (ADI), and the optical density was measured at 450 nm in a Titertek Multiskan II. Since peptide HIBP1-4(L) reacted nonspecifically with the goat $F(ab')_2$-peroxidase conjugate, a protein A-peroxidase conjugate was used instead in the ELISA. Irrelevant peptides derived from pertussis toxin and IgG antibodies purified from normal rabbit, guinea pig, and mouse sera purchased from BioCan (Toronto, Canada) were used as negative controls.

Immunoblot analyses were performed as described previously (1). Briefly, 30 to 50 μg of crude OMP extracts was electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, electroblotted, and reacted with purified MAb 7C8. An alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) antibody (Bio-Rad) was used as the second antibody.

Peptide-specific competitive binding inhibition assay. To evaluate the ability of a peptide to inhibit the binding of the MAbs to their cognate P1 epitopes, MAb 7C8 was adjusted to concentrations corresponding to its 50% maximum binding to P1 or HIBP1-5E and preincubated overnight at 4°C with increasing amounts of either OMP extract, target peptides, or a negative control peptide. Microtiter wells were coated with either 400 ng of HIBP1-5E per well or 5 μg of crude OMP extract in coating buffer per well for 16 h at 4°C. Uncoated sites were blocked with 3% BSA in PBS. The peptide-MAb mixtures were added to the wells, and the plates were incubated for 15 min at 21°C. Unbound MAb was removed, and the wells were washed four times with washing buffer. Horseradish peroxidase-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG antibodies were added to the microtiter plates. After 1 to 2 h of incubation at 21°C, the plates were washed four times and then developed with tetramethylbenzidine in H_2O_2. The optical density was measured at 450 nm in a Titertek Multiskan II.

RESULTS AND DISCUSSION

Selection of synthetic peptides. The objective of this study was to identify the surface-exposed epitopes of Hib P1 protein by using three P1-specific murine MAbs and to fine map the antigenic determinant recognized by the protective MAb 7C8 as a first step towards the design of a new generation of synthetic peptide-based cross-protective *H. influenzae* vaccine. A panel of peptides (Table 1) covering 90% of the P1 sequence was synthesized to assess the reactivity of the peptides with three P1-specific murine

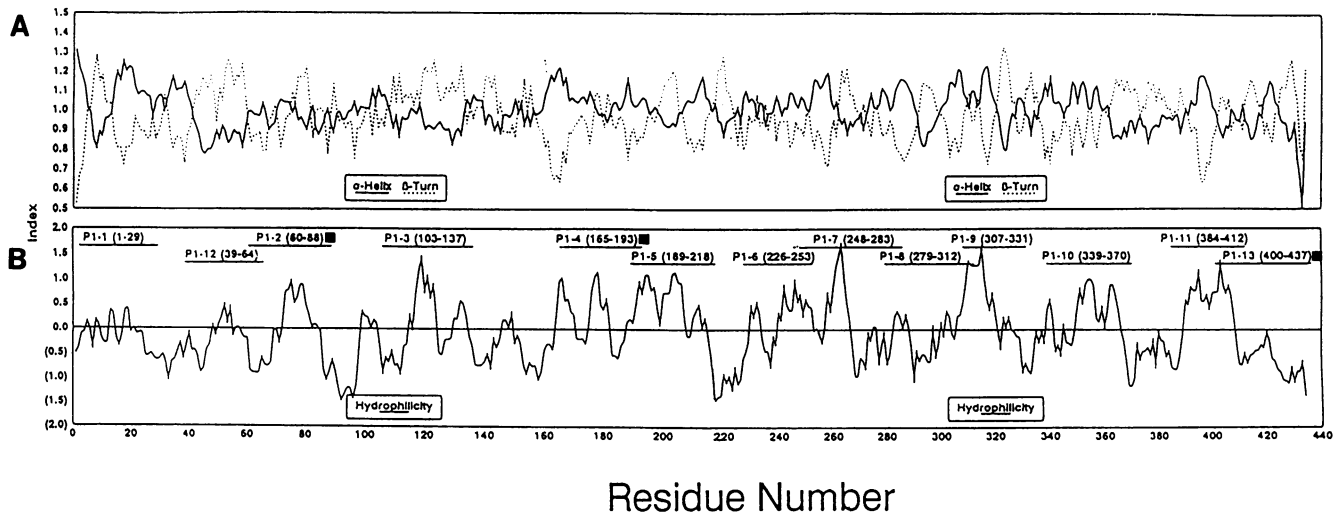

FIG. 1. Structure prediction analysis of Hib P1 protein by using conventional algorithms. (A) Secondary structure plots obtained by the method of Chou and Fasman (2); (B) hydrophilicity plot obtained by the method of Hopp (8). The values are derived from the average of heptapeptide windows and are plotted at the midpoint of each fragment. Symbol: ■, P1 peptides found to have strain-specific sequences.

MAbs, 7C8, 6B1, and 3E12. Since surface-exposed regions of a protein usually correlate with its antigenic determinants (8), peptides selected for this study correspond to linear segments of the mature P1 sequence containing hydrophilic β-turns identified by conventional structure prediction algorithms (2, 8) (Fig. 1). Peptides more than 20 residues in length were synthesized since it had been suggested that native linear epitopes were better mimicked by long peptides than by short peptides (18). Indeed, the advantage of using long peptides was well demonstrated when the antigenicity of HIBP1-4 was compared with that of its truncated analog HIBP1-4A in the peptide-specific ELISAs (described below).

Validation of P1 peptide-specific ELISAs. Numerous studies have demonstrated that reliable ELISA results can be obtained by coating synthetic peptides directly onto microtiter wells (1, 3, 15). However, negative results may be obtained if peptides are not absorbed or if they bind through their antigenic determinants. To validate the assays, individual peptides were coated onto microtiter wells and probed with peptide-specific rabbit antisera raised against corresponding peptide-keyhole limpet hemocyanin conjugates. All peptides except HIBP1-8 (residues 279 to 312) were recognized by their respective antipeptide antisera with reactive titers in the range of 10,000. The lack of reactivity of peptide HIBP1-8 with its rabbit antiserum was not due to its inability to bind to polystyrene microtiter wells since it was recognized by human convalescent-phase sera (data not shown). It is most likely that the HIBP1-8–keyhole limpet hemocyanin conjugate used for immunization failed to elicit an antibody response. Nevertheless, it was established that all P1 peptides were adsorbed onto microtiter plates and that their antigenic determinants were accessible to peptide-specific antibodies. The synthetic peptides were also screened with control IgGs isolated from normal rabbits and mice. Nonspecific binding was not observed.

Reactivity of anti-P1 MAbs with a panel of P1 synthetic peptides. MAbs 6B1 and 7C8 were previously shown to recognize two distinct surface-exposed B-cell epitopes of P1 and reacted with 50% of Hib subtypes as judged by immunoblot analysis (7). Only MAb 7C8 was found to be protective in the infant rat model of bacteremia (4, 7). MAb 3E12 had not been fully characterized. Therefore, P1 peptides were screened with the three murine MAbs in peptide-specific ELISAs. Each MAb recognized a distinct linear P1 epitope (Fig. 2). MAb 3E12 recognized peptide HIBP1-13 (residues 400 to 437), whereas MAb 6B1 reacted strongly with peptide HIBP1-2 (residues 60 to 88) and MAb 7C8 reacted specifically with peptide HIBP1-4 (residues 165 to 193). Interestingly, these three epitopes correspond to regions of P1 which have been found to be highly variable among Hib isolates (12). These results are consistent with

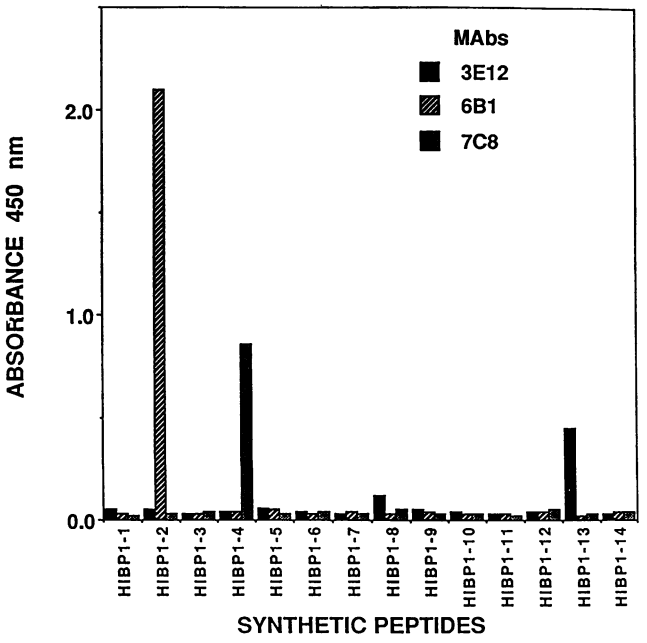

FIG. 2. Histogram showing ELISA reactivity of Hib P1 peptides with P1-specific MAbs 7C8, 6B1, and 3E12.

the observation that MAbs 6B1 and 7C8 recognize OMP P1 from only 50% of the Hib isolates previously tested (7).

MAb 3E12 recognizes a subtype-specific epitope. To determine whether MAb 3E12 recognized a subtype-specific epitope, peptide HIBP1-14 (Table 1) corresponding to the C-terminal end of the sequence of Hib subtype 6U (12) was synthesized and tested for its reactivity with MAb 3E12 in a peptide-specific ELISA. The results shown in Fig. 2 indicate that MAb 3E12 reacted strongly with peptide HIBP1-13 (subtype 1H) but failed to recognize HIBP1-14 (subtype 6U). This binding suggests that the highly hydrophilic sequence **VKTIGDKRTLTLN** (Table 1) of the variable segment of the C-terminal end of P1 is critical for MAb 3E12 recognition. Indeed, a synthetic peptide corresponding to the N-terminal half of HIBP1-13, **VKTIGDKRTLTLN**TTANYT, could specifically inhibit the binding of MAb 3E12 to solid-phase-bound HIBP1-13, whereas no inhibition was observed with its C-terminal half, TTANYTSQAHANLYGLNLNYSF (data not shown). These results are in good agreement with the finding of Proulx et al. (14), who, by deletion mapping experiments, identified an immunodominant and surface-exposed epitope located within the 35 carboxy-terminal residues (residues 402 to 437) of P1 from Hib 1H isolates which are responsible for the majority of invasive diseases in North America. Their MAbs and MAb 3E12 may recognize the same epitope since these MAbs reacted only with the P1 C-terminal sequence of the 1H subtype and not with the corresponding peptide derived from the 6U subtype sequence.

Subtype specificity and fine mapping of the MAb 7C8 cognate epitope. As previously mentioned, MAb 7C8 recognizes peptide HIBP1-4 and protects infant rats against in vivo challenge with certain live Hib subtypes (4, 7). Although the sequence of this peptide varies among Hib subtypes, it was of interest to determine whether MAb 7C8 would cross-react with P1 from nontypeable isolates. Crude OMP extracts from different typeable (Eagan, MinnA, Durst, 1613, 8358, DL41, and DL42) and nontypeable (PAK12085, SB30, SB32 and SB33) *H. influenzae* strains were prepared and used as target antigens in an immunoblot analysis and an ELISA. Immunoblot analysis revealed that MAb 7C8 specifically recognized the P1 protein from Hib strains DL41, DL42, and Minn A and from the nontypeable strain SB32. To eliminate the possibility that the denatured P1 was not recognized in the immunoblot, a P1-specific ELISA was performed. MAb 7C8 failed to recognize P1 from strain Eagan in the ELISA (Fig. 3), but it cross-reacted with P1 in the OMP extract isolated from the nontypeable strain SB32. It has been reported that antibodies raised against membrane-bound P1 from a nontypeable *H. influenzae* strain have in vitro bactericidal properties (13). This result suggests that a cocktail of selected P1 epitopes could potentially serve as both cross-protective antigens and carriers for PRP in a new generation of synthetic peptide-based conjugate *H. influenzae* vaccine.

To fine map the epitope recognized by the protective MAb 7C8, two HIBP1-4 peptide analogs, HIBP1-4(U) and -4(L) (Table 1), corresponding to the P1 sequences of Hib subtypes 6U and 3L, respectively, were synthesized and purified for immunochemical analysis. These peptides differ from HIBP1-4 by several amino acid substitutions localized within residues 175 to 193. MAb 7C8 reacted poorly with both HIBP1-4(U) and -4(L) (Fig. 3). This lack of significant reactivity was not due to the inability of the analogs to bind to polystyrene, since subsequent experiments revealed that they were recognized by their respective monospecific rabbit antisera (data not shown). The lack of antibody reactivity was also not due to the masking of HIBP1-4(L) and -4(U) antigenic determinants as a result of physical adsorption to microtiter wells, since further studies (described below) showed that both peptides could not inhibit the binding of MAb 7C8 to peptide HIBP1-4. Therefore, MAb 7C8 most likely recognizes a nonconserved region.

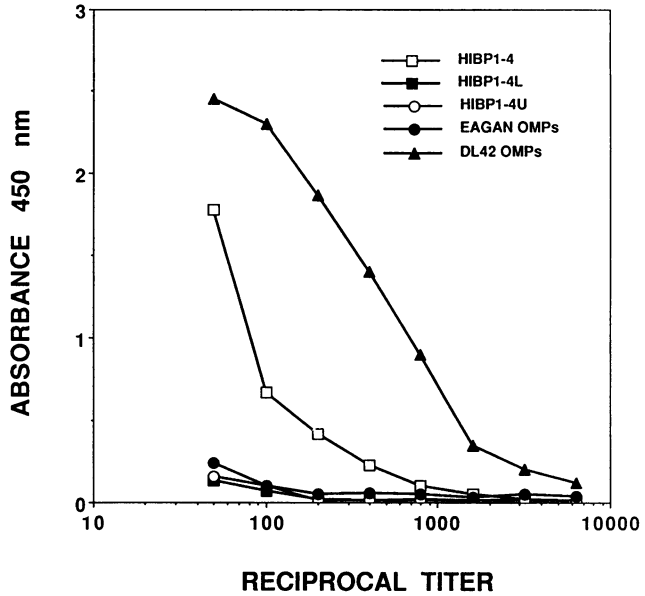

FIG. 3. ELISA reactivity of MAb 7C8 with Hib OMP extracts and synthetic peptides HIBP1-4, HIBP1-4(L), and HIBP1-4(U).

To further identify the critical residues of HIBP1-4 (residues 165 to 193) involved in antibody-peptide interaction, three peptides, HIBP1-4A (a C-terminal analog of HIBP1-4; residues 175 to 193), HIBP1-4B (an N-terminal analog of HIBP1-4; residues 165 to 181), and HIBP1-5E (an N-terminal extension analog of HIBP1-5; residues 184 to 218) (Table 1),

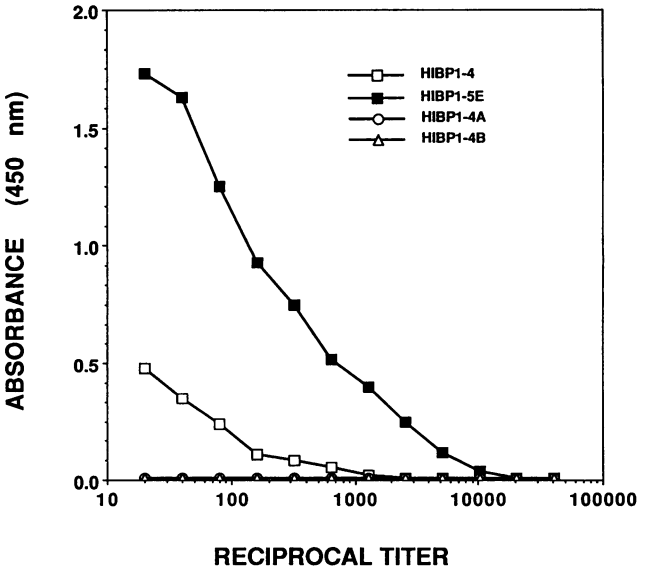

FIG. 4. ELISA reactivity of MAb 7C8 with synthetic peptides HIBP1-4, HIBP1-4A, HIBP1-4B, and HIBP1-5E.

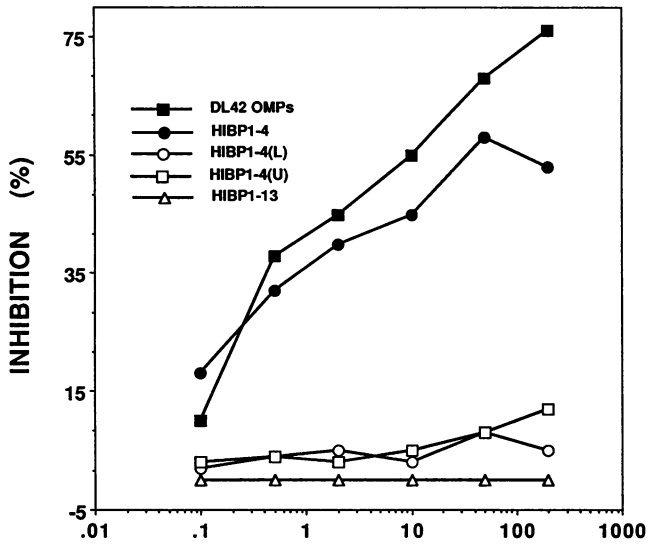

FIG. 5. Competitive inhibition of MAb 7C8 binding to DL42 OMP-coated plate by DL42 OMP extract, target peptides HIBP1-4, -4(L), and -4(U), and the control peptide HIBP1-13. The concentration of MAb used in the experiment was 40 ng/ml, and the amount of inhibitors used ranged from 0.01 to 200 μg. The percent inhibition was calculated from the absorbance (A) by using the following formula: percent inhibition = $[A(\text{HIBP1-13}) - A(x)]/A(\text{HIBP1-13})$, where x is either a peptide or the OMP extract. Each point represents the mean of duplicate determinations. The experiment was repeated twice.

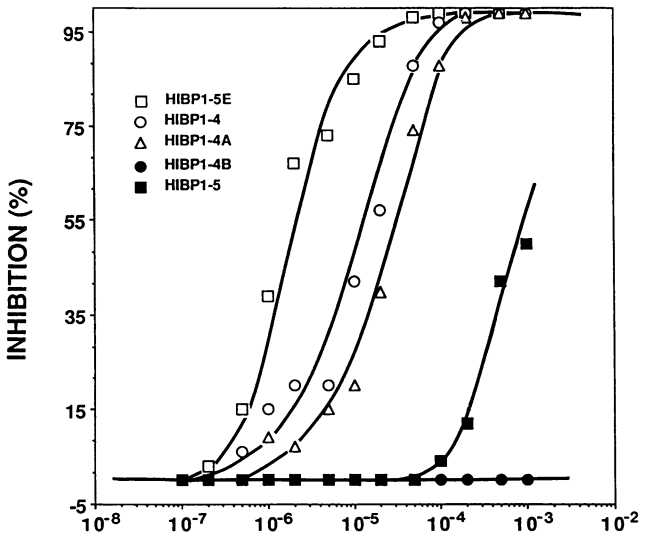

FIG. 6. Competitive inhibition of MAb 7C8 binding to HIBP1-5E-coated plate by target peptides HIBP1-4, -4A, -4B, -5, and -5E and the control peptide HIBP1-13. The concentration of MAb used in the experiment was 40 ng/ml, and the amount of inhibitors used varied from 0.1 to 2,000 μg. The percent inhibition was calculated from the absorbance (A) by using the following formula: percent inhibition = $[A(\text{HIBP1-13}) - A(x)]/A(\text{HIBP1-13})$, where x is the inhibitor. Each point represents the mean of two determinations. The experiment was repeated twice. The affinities (K_a) of MAb 7C8 for HIBP1-5E and -4 were calculated from the concentrations of inhibitors corresponding to 50% inhibition and found to be 10^6 M^{-1} and 10^5 M^{-1}, respectively.

were synthesized, purified, and used as solid-phase antigens in peptide-specific ELISAs. Only HIBP1-4 and -5E were recognized by MAb 7C8 (Fig. 4). On the basis of the peptide-specific ELISA reactivity, it appears that MAb 7C8 binds to HIBP1-5E with a higher affinity than it does to HIBP1-4 (Fig. 4). That both HIBP1-4 and -5E react with MAb 7C8 and overlap by 10 amino acids (**NQITSALSTQ**) suggests that the protective epitope may be located within these 10 residues. The surprising lack of reactivity with peptide HIBP1-4A, which contains these 10 amino acids, may be explained by conformational restriction in epitope accessibility due to physical adsorption of peptides onto the polystyrene plate (see below).

Competitive binding inhibition assays. The ability of a peptide to inhibit the binding of a MAb to either solid-phase-bound P1 or its cognate epitope was evaluated in a competitive binding inhibition assay. This inhibition assay gives valuable information about the relationship between the conformation adopted by the soluble peptide and its antigenicity. OMP extracts from Hib strain DL42 and peptide HIBP1-4 were both capable of blocking the reaction of MAb 7C8 with membrane-bound P1 (Fig. 5). In contrast, the OMP extract from strain Eagan and peptides HIBP1-13, -4(L), and -4(U) were not inhibitory. These results confirmed the previous ELISA data (Fig. 3).

A similar assay was also performed to determine whether the lack of binding of MAb 7C8 to the peptides HIBP1-4A, -4B, and -5 was due to a masking of their antigenic determinants as a result of coating. Peptides HIBP1-5E, -4, and -4A specifically inhibited MAb 7C8 binding to solid-phase HIBP1-5E at micromolar concentrations, and HIBP1-5 could block the MAb 7C8-HIBP1-5E binding at a millimolar concentration, whereas HIBP1-4B was completely inactive (Fig. 6). Therefore, the 7C8 epitope can be localized between residues 184 and 193, which is the most variable region of P1 among Hib subtypes (12). This observation also suggests that the adsorption of peptide HIBP1-4A onto polystyrene either masks its antigenic determinant or alters its conformation. In addition, these results confirm that MAb 7C8 has very low reactivity with both HIBP1-4(U) and -4(L), whose peptide sequences are very similar within residues 165 to 181 but diverge at residues 175 to 193. Epitope fine mapping on HIBP1-4A and alanine substitution studies for the precise identification of antigenic residue are in progress. To determine whether the surface-exposed epitopes (residues 60 to 88, 165 to 193, and 400 to 437) identified in this study will be suitable for inclusion in a synthetic peptide-based conjugate vaccine against nontypeable *H. influenzae* disease, further studies are required. Studies are in progress to determine whether these epitopes can elicit protective antibodies and function as carriers for PRP.

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