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Immunogenicity of Synthetic Peptides of *Haemophilus influenzae* Type b Outer Membrane Protein P1

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To identify the B- and T-cell epitopes of P1 of *Haemophilus influenzae* type b, 13 peptides covering 90% of the protein were chemically synthesized. Mouse, guinea pig, and rabbit antisera raised against purified native P1 were tested for their reactivities against the peptides in peptide-specific enzyme-linked immunosorbent assays (ELISAs). Six immunodominant linear B-cell epitopes were mapped to residues 103 to 137, 189 to 218, 248 to 283, 307 to 331, 384 to 412, and 400 to 437 of the mature P1 protein. When P1 peptides were screened for their reactivities with three human convalescent-phase serum specimens, peptides corresponding to residues 39 to 64, 226 to 253, and 400 to 437 reacted strongly with the antisera. Four regions (residues 39 to 64, 226 to 253, 339 to 370, and 400 to 437) contained murine T-cell epitopes. Rabbit antipeptide antisera were tested for their reactivities with the immunizing peptides and P1 protein by ELISA and immunoblots. All anti-P1 peptide antisera except those raised against peptide HIBP1-8 (residues 279 to 312) or HIBP1-8–keyhole limpet hemocyanin conjugate were shown to be specific for their respective immunizing peptides by ELISA. In addition, rabbit antisera raised against the synthetic peptides corresponding to residues 1 to 29, 39 to 64, 103 to 137, 189 to 218, 226 to 253, 248 to 283, 307 to 331, and 400 to 437 of the mature P1 protein recognized the P1 protein from both typeable and nontypeable isolates. These results suggest that these peptides contain epitopes highly conserved among typeable and nontypeable strains of *H. influenzae*. However, none of the antipeptide antisera have bactericidal activity, nor were they protective against *H. influenzae* type b in the infant rat model of bacteremia.

Current capsular polysaccharide (polyribosyl ribitol phosphate [PRP]) conjugate vaccines are protective against meningitis caused by *Haemophilus influenzae* type b (Hib). However, they are ineffective against other invasive typeable and nontypeable strains of *H. influenzae* (NTHi) which are a common cause of otitis media in children (14, 30, 32). Recent studies have indicated that antibodies raised against the outer membrane protein (OMP) P1 of Hib are protective in the infant rat model of bacteremia (9, 11, 16). Furthermore, monoclonal antibodies raised against P1 of Hib strain DL42 were found to cross-react with P1 from other typeable and NTHi strains (21, 24). A recent study (23) reported that P1 purified from NTHi elicited a protective immune responses in the chinchilla model of experimental otitis media. Therefore, the use of either P1 or its immunodominant epitopes as both additional immunogens and carriers for PRP could represent a strategy to develop a new generation of Hib conjugate vaccines with enhanced protective ability and cellular T-cell-priming capability.

The gene encoding P1 has been cloned from several different Hib subtypes (8, 19). The comparative analysis of the gene-derived P1 protein sequences from Hib subtypes 1H, 2L, 3L, and 6U revealed the existence of three hypervariable regions comprising 15% of the coding region (19). A protective monoclonal antibody, 7C8, reported by Hansen's group was found to recognize 50% of the Hib isolates tested, and its epitope was mapped to residues 165 to 193 of P1 (10, 21). To characterize further the biochemical and immunological properties of P1, 13 peptides covering 90% of the P1 sequence of the 1H subtype were synthesized and used to map its immunodominant T- and B-cell epitopes. Rabbits were immunized with either linear peptides or peptide-keyhole limpet hemocyanin (KLH) conjugates to investigate whether synthetic P1 epitopes could induce protective antibody responses.

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MATERIALS AND METHODS

Bacteria. The Hib strain used in this study was the Connaught production Eagan strain. Other type b strains (MinnA, Durst, 1613, 8358, DL41, and DL42) and nontypeable strains (PAK12085, SB30, SB32, and SB33) were used to map conserved epitopes. Bacteria were grown as described previously (21).

P1 purification. P1 was purified from Hib strain Eagan. Hib cells were pelleted with 0.1% (wt/vol) Cetavlon and then centrifuged at 8,000 × *g* for 10 min. A 100-g cell pellet was extracted with 2 liters of 0.4 M NaCl and then centrifuged at 8,000 × *g* for 30 min. The resulting pellet was resuspended in 1 liter of 50 mM Tris-HCl (pH 8.0)–0.5% Triton X-100–10 mM EDTA. The suspension was stirred at 4°C overnight and then centrifuged at 20,000 × *g* for 30 min. The supernatant was precipitated with ethanol (final concentration of 25%) at 4°C for 16 h and centrifuged at 20,000 × *g* for 30 min. The pellet was then resuspended in 100 ml of 50 mM Tris-HCl, pH 8.0, containing 0.15% sodium deoxycholate. The solution was centrifuged at 3,000 × *g* for 10 min to remove the insoluble material, and the supernatant was loaded onto a 50-ml DEAE-Sephacel column equilibrated in 50 mM Tris-HCl buffer, pH 8.0. The column was washed 10 times with 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.15% deoxycholate and 0.1% Triton X-100 and subsequently 5 times with 50 ml of 50 mM Tris-HCl buffer, pH 8.0. The P1 protein was then selectively eluted with 50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and further purified on a hydroxyapatite (HTP) column. The P1 eluate was diluted 10-fold with 50 mM Tris-HCl, pH 8.0, and then loaded onto a 25-ml HTP column equilibrated in 50 mM Tris-HCl buffer, pH 8.0. The column was washed 10 times with 25 ml of 50 mM Tris-HCl, pH 8.0, containing 0.5 M urea, 0.2% deoxycholate, and 0.1% Triton X-100 and then washed 5 times with 25 ml of 50 mM Tris-HCl buffer, pH 8.0. P1 was eluted with 50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and 10 mM EDTA. The purity of P1 was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amount of endotoxin lipooligosaccharide (LOS) present in the purified P1 solution was determined in a LOS-specific enzyme-linked immunosorbent assay (ELISA) (see below). Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) using bovine serum albumin as the standard. A detailed biochemical characterization of P1 will be published elsewhere.

Selection of peptides and peptide synthesis. To map the functional epitopes of

TABLE 1. Amino acid sequences of Hib OMP P1 peptides

Peptide	Residues	Sequence[a]
HIBP1-1	1–29	AAFQLAEVSTSGLGRAYAGEAAIADNASV(C)
HIBP1-2	60–88	GDVTSYAQIITNQIGMKAIKDGSASQRNV(C)
HIBP1-3	103–137	(C)VNDKFALGAGMNVNFGLKSEYDDSYDAGVFGGKTD
HIBP1-4	165–193	YAKAQVERNAGLIADSVKDNQITSALSTQ(C)
HIBP1-5	189–218	ALSTQQEFRDLKKYLPSKDKSVVSLQDRA(C)
HIBP1-6	226–253	(C)AGVMYQFNEANRIGLAYHSKVDIDFADR
HIBP1-7	248–283	IDFADRTATSLEANVIKEGKKGNLTFTLPDYLELSG(C)
HIBP1-8	279–312	LELSGFHQLTDKLAVHYSYKYTHWSRLTKLHASF(C)
HIBP1-9	307–331	KLHASFEDGKKAFDKELQYSNNSRV(C)
HIBP1-10	339–370	LYEKLTLRAGIAYDQAASRHHRSAAIPDTDRT(C)
HIBP1-11	384–412	LSVDLGYAYLKGKKVHFKEVKTIGDKRTL(C)
HIBP1-12	39–64	LFKTAQFSTGGVYIDSRINMNGDVTS(C)
HIBP1-13	400–437	(C)FKEVKTIGDKRTLTLNTTANYTSQAHANLYGLNLNYSF

[a] Underlined residues are predicted to be T-cell binding motifs by Rothbard-Taylor analysis (25).

P1, 13 peptides (25 to 30 residues long) (Table 1) covering 90% of the P1 sequence of the MinnA strain were synthesized with an Applied Biosystems model 430A automated peptide synthesizer as previously described (21).

Peptide-carrier conjugation. Individual peptides were conjugated to KLH by using sulfosuccinimyl(4-iodoacetyl)-amino-benzoate (Pierce) as a cross-linker at a 10:1 molar ratio of peptide to KLH according to the method of Panezutti et al. (21).

Purification of endotoxin from Hib strain Eagan. LOS was purified from the Hib Eagan strain by combining the methods previously described by Wu et al. (33) and Leive and Morrison (15). Briefly, LOS was extracted from bacteria by aqueous 1-butanol extraction. Following treatment with RNase, LOS was purified by Sephadex G-100 column chromatography. LOS fractions eluted from the Sephadex G-100 column were analyzed for the presence of ketodeoxyoctonate (29). The LOS fractions were analyzed by SDS-urea-PAGE followed by silver staining to assess the purity (29). The LOS concentration was determined by the ketodeoxyoctonate method (29). Purified LOS was used to generate antibodies in guinea pigs and rabbits. It was found that LOS induced better immune responses in the absence of adjuvants. Animals received a total of three injections of purified LOS (5 μg per dose) on days 1, 14, and 28. Blood samples were collected on day 42. The reactivities of anti-LOS antibodies in immune sera with Hib LOS were determined by Western blot (immunoblot) analysis.

Production of P1-specific antisera. Anti-P1 antisera were prepared as follows. Five rabbits (Maple Lane Farm, Clifford, Ontario, Canada), two guinea pigs (Charles River, St. Constant, Quebec, Canada), and mice of five different haplotypes (A/J, BALB/c, C57BL/6, C3H, and SWR/J; Charles River) were immunized intramuscularly or subcutaneously with 5 to 100 μg of purified P1 protein emulsified in complete Freund's adjuvant (CFA). 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 postimmunization, and animals were exanguinated 4 weeks after the final immunization. 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-P1 antisera were further absorbed on a PRP affinity column and then on a Hib (Eagan) LOS affinity column. These affinity columns were prepared by coupling either PRP or Hib LOS to an amino-agarose gel (Pierce) by a periodate oxidation method previously described by Jennings and Lugowski (13). The specificity of anti-P1 antisera was assessed by P1-specific ELISA and immunoblots using *H. influenzae* cell extracts as antigens. Absorbed antisera were shown to be free of anti-LOS and anti-PRP antibodies by LOS-specific ELISA (2) and Farr-type radioassay, respectively. Three human convalescent-phase sera were generously provided by S. Halperin (Dalhousie University, Halifax, Nova Scotia, Canada).

Production of antipeptide antisera. Two rabbits were immunized intramuscularly either with 200 μg of peptides or with peptide-KLH conjugates containing 50 μg of peptide emulsified in CFA and then boosted with half the amount of the same immunogen in incomplete Freund's adjuvant 2 and 4 weeks after priming. Antisera were collected and stored as described above. A cocktail of peptides (HIBP1-3, -4, -5, -7, -9, -11, and -13) was either emulisified in CFA or adsorbed onto $AlPO_4$ (200 μg of each peptide per 3 mg of $AlPO_4$), and a 500-μl aliquot of adjuvanted peptides was used to immunize rabbits three times at 2-week intervals. Guinea pig antisera were collected and stored as described above.

Immunoassays. P1- and peptide-specific ELISAs were performed as described previously (21). Two pertussis toxin peptides, NAD-S1 (GALATYQSEYLAHR RIPP) and S3-P6 (FVRDGQSVIGACASPYEGRYRDMYDALRRLLY), were included as negative controls in all peptide-specific ELISAs. Assays were performed in triplicate, and the reactive titer of an antiserum was defined as the reciprocal of the dilution consistently giving a twofold increase in absorbance (optical density at 450 nm) value over that obtained with the preimmune serum. The specificity of each experimental antiserum was tested by immunoblotting as described by Panezutti et al. (21).

Bactericidal assay. The bactericidal assay was performed according to the method of B. Brodeur (1a) with minor modifications. Briefly, NTHi strain 12 was grown to a mid-log-phase concentration of 2×10^8 CFU/ml in brain heart infusion broth supplemented with NAD and heme, each at 4 μg/ml. The cells were diluted with saline to a final concentration of 4,000 CFU/ml and used immediately for the bactericidal assay. Bactericidal reaction mixtures (total, 250 μl) contained 100 μl of a dilution of either the test serum or the corresponding prebleed serum, 50 μl of complement (BioWhittaker, Walkersville, Md.), 50 μl of *H. influenzae* 12 suspension, and 50 μl of a medium solution containing 10% brain heart infusion broth and 50% Dulbecco's modified Eagle medium (Gibco BRL, Ontario, Canada). The complement (lyophilized powder) was reconstituted in a diluent provided by the supplier and then further diluted 1:1 in volume with the medium solution described above. The final concentration of complement in the bactericidal reaction mixture was 10% of the original reconstituted solution. $CaCl_2$ and $MgCl_2$ were added to the assay mixture to final concentration of 0.15 mM. After addition of all components, reaction mixtures were incubated for 60 min at room temperature in a shaker. After incubation, the reaction mixtures were placed in ice to stop the reaction. Counts of viable colonies were performed at 0 and 60 min by plating 125-μl samples of the mixtures onto chocolate agar plates. It was found that under these conditions, the bacteria remained alive but with limited multiplication as judged by the number of cell colonies grown on the agar plates. The bactericidal titer was defined as the highest dilution of serum that resulted in 50% killing of the test strain.

Protection studies. The protective ability of P1-specific and peptide-specific antisera was assessed by the infant rat model of bacteremia according to the method of Munson et al. (20) using the Hib MinnA strain as the challenge bacterium.

Lymphocyte proliferation assay. T-cell epitope mapping was performed by priming BALB/c, C57BL/6, and A/J mice with 5 to 10 μg of purified P1 emulisifed in CFA. Three weeks later, the spleens were removed and the splenocytes were cultured in RPMI 1640 (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Flow Laboratories), 100 U of penicillin (Flow Laboratories) per ml, 100 μg of streptomycin (Flow Laboratories) per ml, 10 U of murine recombinant interleukin-2 per ml, and 50 μM 2-mercaptoethanol (Sigma) for 5 to 7 days. Proliferative responses of the primed splenocytes to the panel of P1 peptides were determined in a standard in vitro assay (5).

RESULTS

P1 purification. Previous studies (16, 18) had shown that P1 could be purified from either stationary-phase or log-phase cells. In the present study, we designed a new purification scheme using cell pellets in which PRP had been extracted. P1 was found to be preferentially solubilized from cell pellet by using 50 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100 and 10 mM EDTA (Fig. 1A, lane 3). Ethanol precipitation was used to concentrate P1 from the crude extract (Fig. 1A, lane 4). P1 was then purified to homogeneity by DEAE-Sephacel and HTP chromatography (Fig. 1, lanes 5 and 6, respectively). The Tris-HCl buffer containing 0.5 M urea, 0.2% deoxycholate, and 0.1% Triton X-100 was found to be the most effective washing buffer to remove the last trace of LOS and protein contaminants during the HTP chromatography. The purified P1 protein had a heat-modified property (Fig. 1, lane

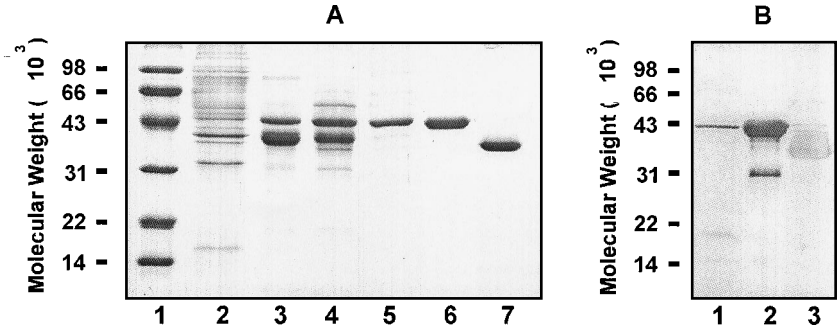

FIG. 1. Biochemical and immunological analyses of P1 using SDS-PAGE and immunoblots. (A) Coomassie blue-stained SDS–12.5% polyacrylamide gel showing the P1 purification process. Lane 1, molecular weight markers; lane 2, Hib cell pellet lysate (strain Eagan); lane 3, crude extract from 50 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100 and 10 mM EDTA; lane 4, pellet from 50% ethanol precipitation; lanes 5 and 6, P1 purified by DEAE-Sephacel and HTP chromatography, respectively; lane 7, nonheated purified P1. (B) Immunoblots of P1 against rabbit antiserum 250. Lane 1, Hib cell pellet lysate; lane 2, purified heat-treated P1; lane 3, purified P1 without heat treatment.

7) as previously described (8). Over 10 preparations of P1 had been tested for LOS contamination, and no detectable of LOS was observed as judged by LOS-specific ELISA.

Characterization of murine, guinea pig, and rabbit anti-P1 antisera. To identify the B-cell epitopes of P1, rabbits, guinea pigs, and five strains of mice of different haplotypes (*H-2*^{a}, *H-2*^{b}, *H-2*^{d}, *H-2*^{k}, and *H-2*^{q}) were immunized with P1 purified from Hib strain Eagan in the presence of Freund's adjuvant. All animals mounted a strong and specific anti-P1 antibody response as judged by P1-specific ELISA (Table 2). The specificities of rabbit anti-P1 antisera were determined by immunoblots. As shown in Fig. 1B, rabbit antiserum 250 reacted only with P1 in lane 1, which was blotted with Hib whole-cell lysate. The same antiserum recognized the chromatographically purified P1 (Fig. 1B, lanes 2 and 3). A second reactive band (about 30 kDa) in Fig. 1B, lane 2, was a degradation product of the P1 protein that was verified by the monoclonal antibody 7C8 (data not shown). At the present time, we cannot explain why the unheated P1 had less reactivity with rabbit antiserum 250. As shown in Table 3, passive administration of rabbit anti-P1 antiserum (rabbit 250) totally protected infant rats (10 of 10) against live Hib challenge, whereas rabbit anti-LOS antiserum was not protective (9 of 10 rats were bacteremic). Similar results were obtained with other rabbit anti-P1 antisera (rabbits 247 and 249). Guinea pig anti-P1 antisera did not protect infant rats against live Hib challenge (data not shown). These results indicate that purified P1 is immunogenic and capable of eliciting protective antibodies in rabbits.

Identification of the immunodominant linear B-cell epitopes of P1. The validation of the peptide-specific ELISAs used in this study has been reported previously (21). The reactivity of each solid-phase peptide with its corresponding antisera could be blocked specifically by preincubation of antisera with free peptide (21). The two pertussis toxin peptides, NAD-S1 and S3-P6, included as negative controls in the ELISAs were not recognized by anti-P1 antisera.

Anti-P1 antisera from five different mouse haplotypes were first screened for their ability to react with the panel of P1 peptides in peptide-specific ELISAs. The results are summarized in Table 4. Peptides HIBP1-3 (residues 103 to 137), HIBP1-7 (residues 248 to 283), HIBP1-9 (residues 307 to 331), and HIBP1-13 (residues 400 to 437) were recognized by all mouse anti-P1 antisera. In addition, antisera from four of the five mouse strains reacted strongly with HIBP1-5 (residues 189 to 218) and HIBP1-11 (residues 384 to 412). Therefore, the immunodominant linear B-cell epitopes recognized by mice reside within these six regions. None of the murine P1-specific

TABLE 2. Reactivities of antisera raised against native P1 in P1-specific ELISAs

Species and sample	Immunogen	Reactive titer[a]
Human[b]		
1	Hib	3,200
2	Hib	6,400
3	Hib	3,200
Guinea pig		
390	Native P1	204,800
392	Native P1	204,800
Mouse[c]		
A/J	Native P1	204,800
BALB/c	Native P1	102,400
C57BL/6	Native P1	102,400
C3H	Native P1	102,400
SWR/J	Native P1	102,400
Rabbit		
247[d]	Native P1	12,800
248	Native P1	6,400
249[d]	Native P1	6,400
250[d]	Native P1	6,400
251	Native P1	6,400

[a] Preimmunization titers (<400) were subtracted from postimmunization titers.
[b] Antisera were obtained from convalescent patients.
[c] Two mice per group were used for the immunogenicity studies, and reactive titers are expressed as the means of titers obtained from two mice.
[d] Anti-P1 antiserum was tested and shown to be protective in the infant rat model of bacteremia.

TABLE 3. Protective ability of rabbit anti-P1 antisera against live Hib challenge in the infant rat model of bacteremia[a]

Group no.	Antiserum	CFU/10 μl of blood (range) [*n*][b]	% Protection[c]
1	Anti-P1 (rabbit 250)	0 [0]	100
2	Anti-LOS	620 (50–2,000) [9]	25
3	Phosphate-buffered saline	830 (15–2,000) [10]	0

[a] Three groups of 10 five-day old infant rats were injected subcutaneously with 0.1 ml of rabbit antiserum or phosphate-buffered saline. After 24 h, the infant rats were challenged intraperitoneally with 260 CFU of Hib strain MinnA. Blood samples were collected 24 h after challenge.
[b] The level of bacteremia is expressed as the geometric mean of CFU values obtained for 10 infant rats tested individually. *n*, number of animals with detectable bacteria.
[c] 100 − [(CFU in each group/CFU in group 3) × 100].

TABLE 4. Reactivities of murine anti-P1 antisera with P1 peptides

Peptide	Reactive titer[a] in mouse strain:				
	BALB/c	C57BL/6	C3H	A/J	SWR/J
HIBP1-1	<200	<200	<200	1,600	<200
HIBP1-2	<200	<200	<200	<200	<200
HIBP1-3	6,400	3,200	3,200	12,800	12,800
HIBP1-4	6,400	<200	<200	3,200	<200
HIBP1-5	6,400	3,200	6,400	6,400	<200
HIBP1-6	<200	<200	<200	6,400	<200
HIBP1-7	51,000	12,800	51,200	51,200	800
HIBP1-8	<200	400	<200	800	<200
HIBP1-9	6,400	12,800	12,800	6,400	1,600
HIBP1-10	1,600	3,200	<200	6,400	3,200
HIBP1-11	<200	51,200	12,800	25,600	25,600
HIBP1-12	<200	<200	<200	3,200	<200
HIBP1-13	6,400	12,800	12,800	51,200	12,800

[a] Reactive titers are those obtained for antisera pooled from five mice.

antisera recognized peptide HIBP1-2 (residues 39 to 64), and only antisera from A/J mice reacted with peptides corresponding to residues 1 to 29 and 60 to 88, suggesting that the N-terminal region of P1 (residues 1 to 88) is immunorecessive in mice.

The reactivities of two different guinea pig anti-P1 antiserum specimens with the same panel of peptides are shown in Fig. 2. Five peptides, HIBP1-3, -5, -7, -9, and -11, corresponding to residues 103 to 137, 189 to 218, 248 to 288, 307 to 331, and 384 to 412, respectively, were recognized by both antisera. The P1 peptides were also tested for their reactivities with five different rabbit anti-P1 antiserum specimens (Fig. 3). Peptide HIBP1-9 (residues 307 to 331) strongly reacted with all five antiserum specimens, whereas HIBP1-3, -4, -5, -11, -12, and -13 were recognized by three of five rabbit antiserum specimens. All P1 peptides were recognized by the three human antiserum specimens tested (Fig. 4). However, the highest reactivities were observed for peptides HIBP1-6, -12, and -13, corresponding to residues 39 to 64, 226 to 253, and 400 to 437 of the mature P1 protein, respectively. Thus, these results suggest that there are three common human linear B-cell epitopes located within residues 39 to 64, 226 to 253, and 400 to 437 of P1.

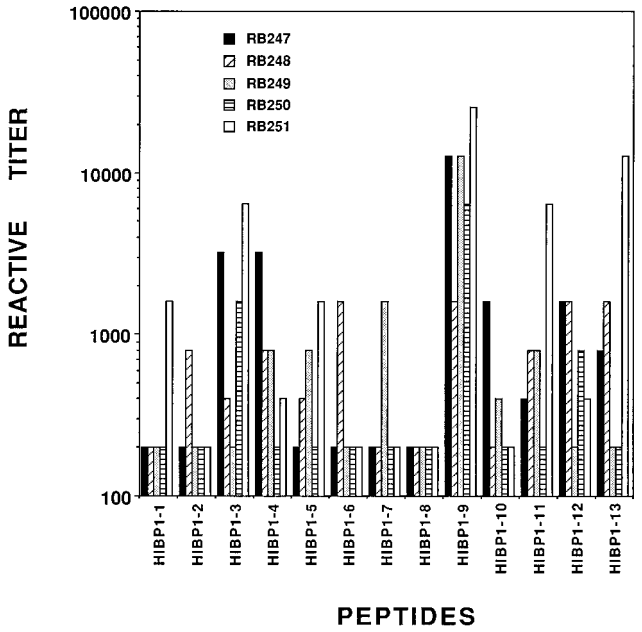

FIG. 3. ELISA reactivities of rabbit (RB) anti-P1 antisera 247, 248, 249, 250, and 251 with P1 synthetic peptides.

Immunogenicity of P1 peptides. To determine the potential of synthetic P1 peptides as vaccine candidates, free peptides and peptide-KLH conjugates were individually assessed for their immunogenicity. Rabbit antipeptide antisera were first

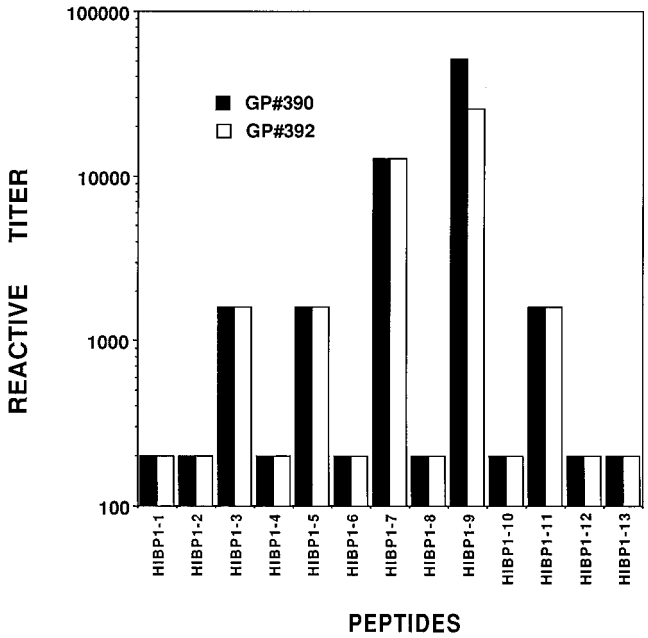

FIG. 2. ELISA reactivities of guinea pig (GP) anti-P1 antisera 390 and 392 with a panel of P1 synthetic peptides.

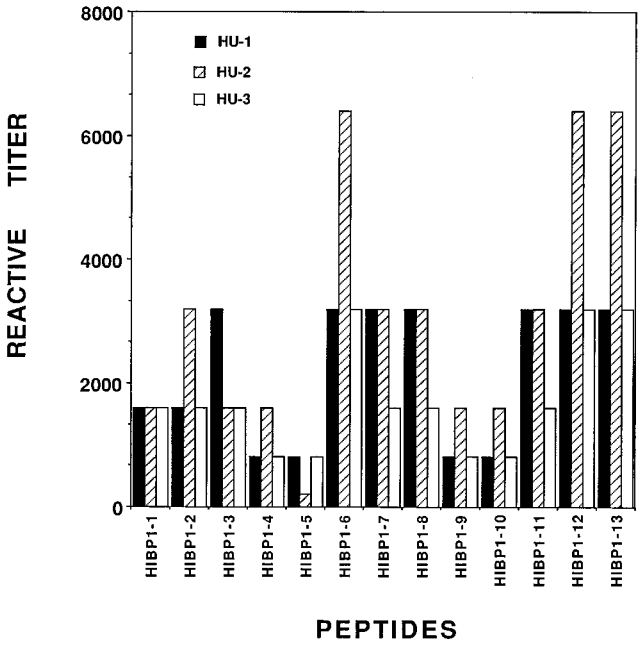

FIG. 4. ELISA reactivities of human (HU) convalescent-phase sera 1, 2, and 3 with P1 synthetic peptides.

TABLE 5. Immunological properties of rabbit antisera raised against P1 peptides and peptide-KLH conjugates

Immunogen	Antiserum reactivity in:			
	Specific ELISA against:		Immunoblot with:	
	Peptide	P1	Hib	NTHi
HIBP1-1	6,400	<200	Yes	Yes
HIBP1-1–KLH	204,800	<200	Yes	Yes
HIBP1-2	409,600	<200	Yes	No
HIBP1-2–KLH	1,638,400	<200	Yes	No
HIBP1-3	102,400	<200	Yes	Yes
HIBP1-3–KLH	102,400	<200	Yes	Yes
HIBP1-4	102,400	3,200	Yes	Yes[a]
HIBP1-4–KLH	25,600	<200	Yes	No
HIBP1-5	1,638,400	25,600	Yes	Yes
HIBP1-5–KLH	120,400	3,200	Yes	Yes
HIBP1-6	409,600	<200	Yes	Yes
HIBP1-6–KLH	12,800	<200	No	No
HIBP1-7	409,600	<200	Yes	Yes
HIBP1-7–KLH	409,600	6,400	Yes	Yes
HIBP1-8	<200	<200	No	No
HIBP1-8–KLH	<200	<200	No	No
HIBP1-9	819,200	51,200	Yes	Yes
HIBP1-9–KLH	102,400	<200	Yes	Yes
HIBP1-10	1,638,400	<200	Yes	No
HIBP1-10–KLH	51,200	800	Yes	Yes[a]
HIBP1-11	1,638,400	25,600	Yes	Yes[a]
HIBP1-11–KLH	51,200	25,600	Yes	No
HIBP1-12	409,600	<200	Yes	Yes
HIBP1-12–KLH	409,600	<200	Yes	Yes
HIBP1-13	102,400	<200	Yes	Yes
HIBP1-13–KLH	25,600	<200	Yes	Yes

[a] Rabbit antisera recognized one of the four NTHi isolates tested.

tested for their reactivities with the immunizing peptides. As shown in Table 5, all anti-P1 peptide antisera, except those raised against HIBP1-8 or the HIBP1-8–KLH conjugate, were shown to be strongly reactive with their respective immunizing peptides by ELISA. Interestingly, rabbit antisera obtained from free-peptide immunization show higher antipeptide titers than those obtained with peptide-KLH immunization. Also, the induction of high titers of peptide-specific immunoglobulin G antibodies in animals immunized with free peptides clearly indicates that the P1 peptides comprise both functional T-helper and B-cell determinants. Rabbit antipeptide antisera were tested further with native P1 by ELISA and immunoblotting. Most antipeptide antisera reacted with Hib P1 in immunoblots (Table 5). In addition, anti-HIBP1-4, anti-HIBP1-5, anti-HIBP1-7, anti-HIBP1-9, anti-HIBP1-10, and anti-HIBP1-11 antisera recognised solid-phase bound native P1 in the ELISA, indicating that these regions of P1 may be surface exposed. Further studies (data not shown) indicated that 30 to 80% of the antipeptide reactivities of these antisera could be absorbed by the intact Hib cells.

To determine whether P1 peptide-specific antisera would cross-react with P1 from NTHi, the rabbit antipeptide antisera were screened for their reactivities with P1 on immunoblots. Rabbit antisera raised against the synthetic peptides HIBP1-1, HIBP1-3, HIBP1-5, HIBP1-6, HIBP1-7, HIBP1-9, HIBP1-12, and HIBP1-13 recognized the P1 protein from the OMP extracts of seven Hib isolates (Eagan, MinnA, Durst, 1613, 8358, DL41, and DL42) and four nontypeable isolates (PAK12085, SB30, SB32, and SB33) tested (Table 5). These results suggest that residues 1 to 29, 39 to 64, 103 to 137, 189 to 218, 226 to 253, 248 to 283, 307 to 331, and 400 to 437 of the mature P1 protein contain epitopes that are highly conserved among typeable and nontypeable strains of *H. influenzae*. Rabbit anti-HIBP1-4, anti-HIBP1-10, and anti-HIBP1-11 antisera reacted with P1 from all seven Hib strains but only one nontypeable isolate (SB32) in immunoblots.

Protection studies. Since P1 peptides were shown to contain a potent T-helper determinant(s) and to induce strong immunoglobulin G antibody responses in rabbits, it was of interest to determine whether they were also protective immunogens. Cocktails of peptides from the potentially surface-exposed regions HIBP1-3, -4, -5, -7, -9, -11, and -13, either emulsified in CFA or absorbed onto alum, were used to immunize rabbits. Both formulations elicited strong antipeptide and anti-P1 immunoglobulin G antibody responses. The anti-P1 titers observed with rabbits immunized with peptides adjuvanted in CFA or alum were 102,400 and 12,800, respectively. The protective abilities of rabbit antipeptide antisera alone or in cocktail were determined in the infant rat model of bacteremia, using the Hib MinnA strain for challenge. None of the antisera showed protection against Hib infection in this model (data not shown). Furthermore, preliminary experiments revealed that the protective ability of rabbit anti-P1 antisera could not be inhibited by absorption with either a single peptide or a cocktail of the 13 peptides, even when concentrations of up to 200 μg of each peptide per ml of antiserum were used for absorption. In addition, none of the antipeptide antisera showed bactericidal activity against NTHi (data not shown).

Identification of immunodominant T-cell epitopes of P1. Little is known about the cellular immune response to *H. influenzae* OMPs. The lymphocyte proliferative responses of P1-specific T-cell lines to P1 peptides were determined in conventional proliferation assays. The results shown in Fig. 5 indicate that only certain peptides elicited proliferative responses and that the recognition of their T-cell epitopes was major histocompatibility complex restricted. Synthetic peptides corresponding to P1 residues 39 to 64 and 226 to 253 were shown to be highly stimulatory for C57BL/6 ($H\text{-}2^b$) T-cell lines. Similarly, peptides encompassing residues 339 to 370 and 400 to 437 of P1 were identified to be T-cell epitopes for A/J ($H\text{-}2^a$) and BALB/c ($H\text{-}2^d$) T-cell lines, respectively. Interestingly, three the four identified murine T-cell epitopes are located within conserved regions (residues 39 to 64, 226 to 253, and 339 to 370) of the mature P1 protein.

DISCUSSION

A promising strategy to develop a new generation of *H. influenzae* conjugate vaccines with T-cell-priming capability and enhanced protective ability against both Hib and NTHi is to use OMPs not only as additional immunogens but also as carriers for PRP. Such a vaccine may also have advantages over existing vaccines in which PRP is conjugated to either diphtheria toxoid, tetanus toxoid, CRM197, or OMP of *Neisseria meningitidis*. The inclusion of OMPs as antigens and autologous carriers in future multivalent diphtheria-tetanus-pertussis-Hib combined vaccines not only may help to reduce the problems of formulation with diphtheria or tetanus toxoid but also should be useful in protecting against otitis media, for which no vaccine currently exists. The biological function of the P1 protein of Hib has not been determined, but it has been regarded as a potentially important immunogen in future *H. influenzae* vaccines (9). P1 protein is found in all *H. influenzae* (typeable and nontypeable) strains, and anti-P1 antibodies have been shown to be protective in animal models (the present study and references 9, 11, and 16). Although a protective monoclonal antibody, 7C8, has been shown to recognize one of the variable regions (residues 184 to 193) of P1 (21),

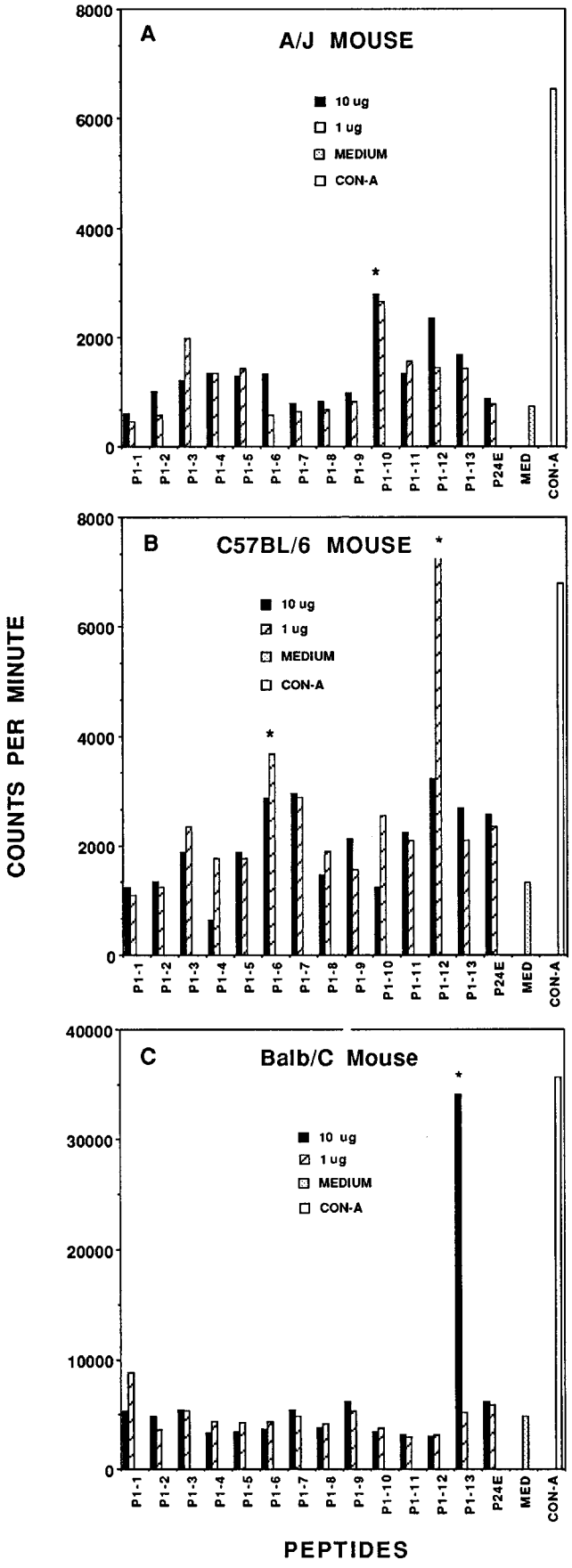

FIG. 5. Proliferative responses of P1-specific murine T cells to synthetic P1 peptides. T-cell proliferation assays were performed with primed splenocytes from mice of the indicated strains. Results are expressed as means from triplicate cultures. All standard deviations were less than 15% of the mean. Immunodominant T-cell epitopes are highlighted with asterisks. CON-A, concanavalin A.

protective antibodies to the conserved parts of P1 protein may be elicited by well-designed immunization strategies.

As a first step towards the design of an OMP-based *H. influenzae* vaccine, the antigenic determinants (B- and T-cell epitopes) of P1 were identified and their immunogenicity was carefully assessed. The antibodies raised against P1 in various mouse strains, rabbits, guinea pigs, and humans were screened for their reactivities with 13 P1 peptides representing 90% of P1. Six synthetic peptides, HIBP1-3, -5, -7, -9, -11, and -13, corresponding to residues 103 to 137, 189 to 218, 248 to 283, 307 to 331, 384 to 412, and 400 to 437 of mature P1, respectively, were recognized by most animal anti-P1 antisera (Fig. 6) and thus represent the immunodominant linear B-cell epitopes of P1. Three common human linear B-cell epitopes were mapped to residues 39 to 64, 226 to 253, and 400 to 437. These results are consistent with the hydrophilic regions identified by the computer-aided analysis of the secondary structure of the P1 protein (Fig. 7). The proposed model of P1 illustrated in Fig. 7 is based on the immunological studies described above and the secondary-structure analysis. The model suggests that there are 16 potential transmembrane sequences, 20 to 25 residues in length for amphiphilic α-helical structure and 14 to 20 residues for a potential transmembrane β-strand. The present immunogenicity studies also indicate that most immunodominant epitopes exhibit features of hydrophilic β-turns which are considered to be potential antigenic determinants (22). The C-terminal region of P1 (residues 400 to 412), previously identified as a surface-exposed epitope by using P1-specific monoclonal antibodies (21, 24), was recognized by all anti-P1 antisera. Although the protective epitope located within peptide HIBP1-4 (residues 165 to 193) (10, 21) was not identified as an immunodominant B-cell epitope, it was consistently recognized by rabbit anti-P1 antisera (Fig. 3). This variable region of P1 may not be immunogenic in some animal species. Alternatively, the lack of reactivity of the MinnA-derived HIBP1-4 peptide with anti-P1 (strain Eagan) antisera may be due to the fact that the Eagan strain has different protein sequence around this region (residues 165 to 193). Without the aid of three-dimensional structural data, it is not possible to predict the presence of conformational epitopes in this region of P1.

It has been hypothesized that expression of a strain-specific immunodominant B-cell epitope on the surface of the bacterium represents a mechanism of host evasion by NTHi and allows the bacterium to cause recurrent otitis media (28, 31). 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 in some cases neutralize the toxin's biological activities (1, 3, 11). Thus, synthetic peptides conceivably could be used as synthetic vaccines against diseases. To investigate whether P1 peptides induced antibodies functionally similar to those elicited by the native protein, rabbit antipeptide antisera were tested against typeable and NTHi isolates. As shown in Fig. 6, conserved regions among *H. influenzae* isolates were mapped to residues 1 to 29, 39 to 64, 103 to 137, 189 to 218, 226 to 253, 248 to 283, 307 to 331, and 416 to 437 of the mature P1 protein. However, P1 peptide-specific antisera either alone or in combination were not protective in the infant rat model studies. Further experiments

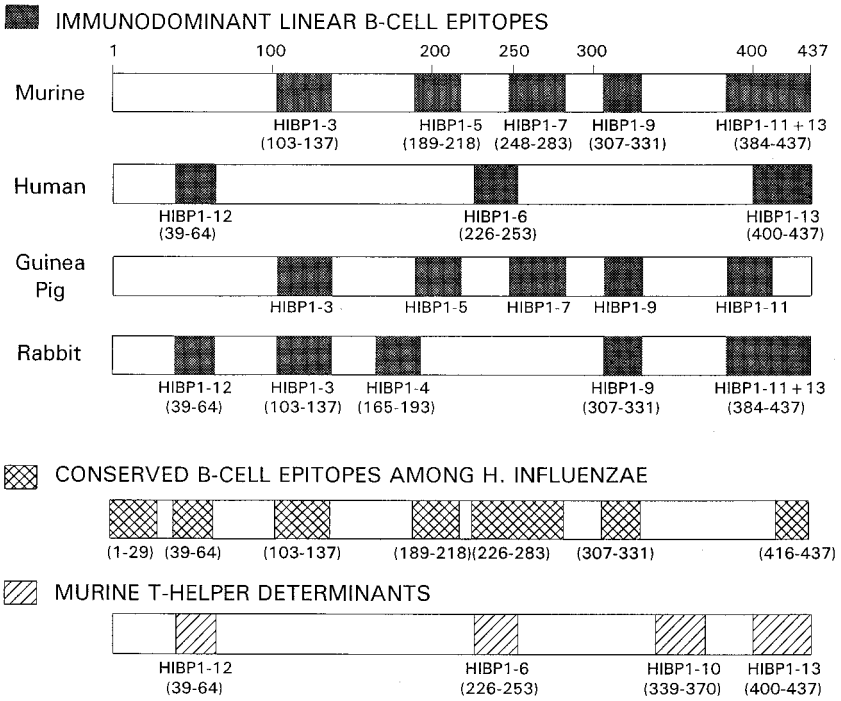

FIG. 6. Diagrammatic representation of the murine T-cell and immunodominant linear B-cell epitopes of P1.

were performed to determine whether P1 peptides could block the protective ability of rabbit anti-P1 antibodies in the infant rat model of bacteremia. Unfortunately, P1 peptide either alone or as a cocktail of 13 peptides could not inhibit the protective ability of rabbit anti-P1 antisera. These results strongly suggest that protective antibodies cannot be generated from P1 peptides and the protective epitope(s) recognized by rabbit anti-P1 antibodies is essentially conformational. These results are very similar to our previous report that the protective epitopes of the P2 protein are conformational (4).

Little is known about the cellular immune responses to Hib infection. Precise locations of T-cell epitopes in each of the OMPs have not been reported. All P1 peptides, with the exception of the peptide HIBP1-8 (residues 279 to 312), induced strong antipeptide immunoglobulin G antibody responses. These results indicate that functional T-helper cell epitopes are located throughout the P1 protein except between residues 279 and 312. However, murine T-cell epitope mapping studies show that P1 peptides corresponding to residues 39 to 64, 226 to 253, 339 to 370, and 400 to 437 were capable of stimulating the proliferation of P1-specific T-cell lines. Interestingly, three of the four identified murine T-cell epitopes are located within the conserved regions (residues 39 to 64, 226 to 253, and 339 to 370) of the mature P1 protein. These results support further

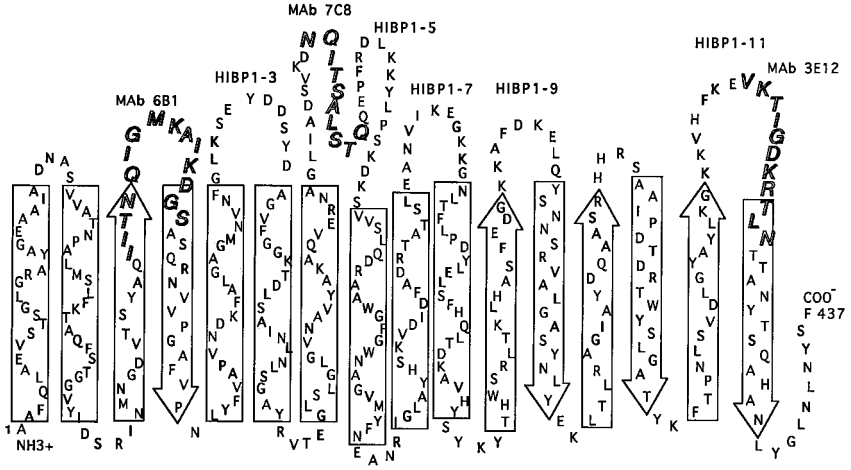

FIG. 7. Model of Hib P1, predicted from the secondary structure analysis (6), hydrophobicity (12), amphiphilicity, and reactivity with monoclonal antibodies (MAbs). There are 16 potential transmembrane domains: the potential amphiphilic α-helical structures are boxed, and the potential transmembrane β-strands are shown by arrows. The surface-exposed epitopes identified by MAbs 6B1, 7C8, and 3E12 in our previous study (21) are labelled in bigger fonts. In addition, these three regions are known variable domains of Hib P1.

the idea that P1 or its peptide fragments may be good carriers for PRP.

Two models have been proposed to identify regions of antigens involved in T-cell recognition. Delisi and Berzofsky found that a large proportion of T lymphocytes appear to react with α-helical amphipathic regions within the amino acid sequence of the antigen (7). On the other hand, the studies of Rothbard and Taylor point to the existence of a 4- to 5-amino-acid sequence motif in a large number of T-cell antigenic determinants (25). This motif is composed of either a glycine or a charged amino acid residue in the first position, followed by 2 or 3 hydrophobic residues, followed by either a glycine or a polar or a charged residue in the last position. Interestingly, none of the identified murine T-cell epitopes were predicted as potential T-cell determinants by the amphipathicity algorithm of Delisi and Berzofsky. As shown in Table 1, three of four murine T-cell epitopes contain one or more T-cell binding motifs as predicted by Rothbard and Taylor (25). Furthermore, these four P1 murine T-cell determinants do not exhibit specific major histocompatibility complex class II binding motifs predicted by other studies (26, 27).

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