Serum Concentrations of Antibodies against Outer Membrane Protein P6, Protein D, and T- and B-Cell Combined Antigenic Epitopes of Nontypeable *Haemophilus influenzae* in Children and Adults of Different Ages

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Nontypeable *Haemophilus influenzae* (NTHi) is one of the most common etiologies of acute otitis media, rhinosinusitis, and pneumonia. Outer membrane proteins (OMPs) are the main focus in new vaccine development against NTHi, as the *H. influenzae* type b (Hib) vaccine does not cover noncapsulated NTHi. The OMPs P6 and protein D are the most promising candidate antigens for an NTHi vaccine, and low antibody levels against them in serum may be correlated with infection caused by NTHi. In the current study, we measured the antibody titers against P6, protein D, and their T- and B-cell combined peptide epitopes in healthy individuals of different ages. We found that children <1 month old had the lowest antibody levels against NTHi P6, protein D, and their T- and B-cell combined antigenic epitopes. Antibody titers increased at ages 1 to 6 months, peaked at 7 months to 3 years, and remained high at 4 to 6 years. The antibody titers started to decrease after 6 years and were the lowest in the 21- to 30-year group. The geometric mean titers (GMTs) of T- and B-cell combined antigenic epitopes in P6 and protein D were positively correlated with those of the protein antigens. Among 12 peptides tested, P6-61, P6-123, and protein D-167 epitopes were better recognized than others in human serum. These findings might contribute to the development of an effective serotype-independent vaccine for *H. influenzae*.

*Haemophilus influenzae* is one of the normal inhabitants of the human nasopharynx and is responsible for pneumonia, acute otitis media (AOM), and acute rhinosinusitis (1–3). The presence or absence of a polysaccharide capsule segregates this bacterial species into two well-defined groups: one group of encapsulated strains and another group of noncapsulated strains, commonly referred to as nontypeable *H. influenzae* (NTHi) (3). Common infections caused by NTHi include otitis media in children and lower airway infections of chronic obstructive pulmonary disease in adults (4, 5). Vaccines composed of polysaccharide capsule conjugated to protein carriers have virtually eliminated infections caused by encapsulated *H. influenzae* type b, including meningitis and other systemic infections, in regions where the vaccines are widely administered (6, 7). However, these conjugate vaccines have no effect on infections caused by NTHi, and in regions with *H. influenzae* type b vaccination programs, nontypeable strains are now the most common cause of noninvasive *H. influenzae* infection, so that the development of the vaccine against NTHi is an urgent and challenging task (8–10).

Since NTHi organisms are noncapsulated bacteria, the outer membrane proteins (OMPs) are the main targets for vaccine designers. Several research groups have identified conserved surface proteins and tested them as putative vaccines, and the conserved NTHi antigens with demonstrated preclinical protective capacity have been identified, among which P6 and protein D are the most widely studied (11–14).

Experimental data derived from humans and animal models indicate that serum antibodies play a critical role in the host defense against NTHi infection (15). It has been reported that otitis-prone children develop a poor response following AOM and poor anamnestic responses to P6 protein (16, 17). Whether healthy individuals from newborns to the elderly are similarly hyporesponsive to P6 and protein D of NTHi has not been studied. The goal of this study was to evaluate and compare the serum antibody responses against outer membrane proteins P6, protein D, and their T- and B-cell combined antigenic epitopes in healthy children and adults of different ages.

**MATERIALS AND METHODS**

*H. influenzae* strains and culture. NTHi strain 86-028NP, which was used as the standard strain for diagnosis, was provided by the Global Biorepository Center of the ATCC and cultured in brain heart infusion.
broth (Becton Dickinson, BD, USA) supplemented with 10 mg/ml hemin (Sigma, USA) and 10 mg/ml NAD (Sigma) at 37°C in a humidified atmosphere with 5% CO₂ (18).

**Serum specimens.** Six hundred five serum samples were received from 605 healthy donors from October 2013 to March 2014 when they visited the Children’s Hospital, Zhejiang University School of Medicine, or The Affiliated Hospital of Hangzhou Normal University, China (age range, 1 day to 103 years; mean ± standard deviation [SD], 35.7 ± 32.1 years; male-to-female ratio, 1:1.003). The samples were divided into 14 age groups, including <1 month, 26 cases; 1 month to 6 months, 27 cases; 7 months to 3 years, 76 cases; 4 to 6 years, 50 cases; 7 to 14 years, 49 cases; 15 to 20 years, 36 cases; 21 to 30 years, 48 cases; 31 to 40 years, 41 cases; 41 to 50 years, 47 cases; 51 to 60 years, 38 cases; 61 to 70 years, 42 cases; 71 to 80 years, 35 cases; 81 to 90 years, 44 cases; and >90 years, 46 cases. Informed consents for sample collection were obtained from all participants and individuals, with approval from the ethics committees of Children’s Hospital, Zhejiang University School of Medicine, and the Affiliated Hospital of Hangzhou Normal University. This research was conducted in accordance with the Declaration of Helsinki.

Amplification and prokaryotic expression of P6 and PD genes. Nontypeable *H. influenzae* DNA was extracted using the bacterial genomic DNA extraction kit (Qiagen, USA). The concentration and purity of the extracted DNAs were determined by a UV spectrophotometer. The Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to detect the signal peptide region. PCRs were performed to amplify the entire *p6* and *pd* genes using the primers *p6-F* (5’-GGGGCAATGATG [NdeI] GCGCATCGGCAATGCTGCTGCTGCG-3’), *p6-R* (5’-GGGGCCCTGAG [XhoI] GTACGCTA ACAGTGACGAC-3’), *pd-F* (5’-GGGGCAATGATG [NdeI] AAATCCAAATTACATGGACGAC-3’), and *pd-R* (5’-GGGGCCCTGAG [XhoI] TTATCTCC TTTGAAGACTCTT-3’) from *H. influenzae* strain 86-028NP (19, 20). The predicted T- and B-cell combined antigenic epitopes in different age groups of healthy individuals.

**RESULTS**

**Production of rP6 and rPD.** The rP6 and rPD were expressed at high levels after induction with IPTG and purified by Ni-NTA chromatography to a single band in an SDS gel (see the supplemental material).

**Prediction of combined T- and B-cell epitopes of P6 and protein D.** We predicted the T-cell epitopes with the SYFPEITHI software, and the predicted epitopes with a score of >25 were included. We predicted the B-cell epitopes with ANTIGENIC, and the epitopes with a score of >1 were included. Next, we selected the overlapping epitopes (combined T- and B-cell epitopes) for further study. Twelve high-score combined T-cell and B-cell epitopes, including 4 P6 epitopes and 8 protein D epitopes, were selected as candidates for ELISA analysis (Table 1).

**Antibody levels against P6, protein D, and their antigenic epitopes in different age groups of healthy individuals.** Figure 1 shows the antibody levels against P6 and protein D in children and adults of different ages. Children between 1 month and 6 years old had higher antibody levels for both P6 and protein D than those of other groups (*P < 0.01*), among which children between 7 months and 3 years old had the highest antibody levels. The geometric mean titer (GMT) of antibody (1:50 dilution in PBST–1% BSA); the serum samples were added to the plate at 100 μl per well and incubated at 37°C for 2 h. After the plates were washed with PBST, 100 μl of goat anti-human-horseradish peroxidase (HRP) (1:10,000 dilution) was used as the secondary antibody, and the mixture was incubated at 37°C for 1 h. After washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was allowed to react with the HRP for 15 min at 37°C. Finally, the color development reaction was terminated by adding 100 μl of 2 M H₂SO₄, and the absorbance at 450 nm was read. To provide quantitative results on antibody concentrations, the level of the specific antibody present in the unknown sample was determined by comparison to an internal reference serum (pool of recovered NTHi patient serum with high anti-P6 or protein D titters) (21, 22).

**Statistical analysis.** Data from a minimum of three independent experiments were averaged and presented as the mean ± SD. A Mann-Whitney U test was used for statistical analysis. A Pearson product-moment correlation coefficient was used for correlation analysis. Statistical significance was defined as a *P* value of <0.05.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was carried out using a 96-well ELISA plate. The wells were coated with 1 μg/ml recombinant P6, protein D, or synthetic T- and B-cell combined antigenic epitopes (100 μl per well) in sodium bicarbonate (pH 9.4) coating buffer and incubated overnight at 4°C. After the plate was washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), blocking was performed at 37°C for about 1 h with 200 μl of 1% bovine serum albumin (BSA) in PBS, and the plate was then washed with PBST. Serum samples from healthy individuals were used as the primary antibody (1:50 dilution in PBST–1% BSA); the serum samples were added to the plate at 100 μl per well and incubated at 37°C for 2 h. After the plates were washed with PBST, 100 μl of goat anti-human-horseradish peroxidase (HRP) (1:10,000 dilution) was used as the secondary antibody, and the mixture was incubated at 37°C for 1 h. After the washing step, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was allowed to react with the HRP for 15 min at 37°C. Finally, the color development reaction was terminated by adding 100 μl of 2 M H₂SO₄, and the absorbance at 450 nm was read. To provide quantitative results on antibody concentrations, the level of the specific antibody present in the unknown sample was determined by comparison to an internal reference serum (pool of recovered NTHi patient serum with high anti-P6 or protein D titters) (21, 22).
Antibodies against P6, PD, and Epitopes of NTHi

and protein D, in that the children 7 months to 3 years old had the highest antibody levels for P6-2, P6-61, P6-95, P6-123, protein D-2, protein D-105, protein D-224, protein D-332, protein D-167, protein D-205, protein D-255, and protein D-294, compared with those of other groups (Fig. 3). Among the epitopes, the P6-61, P6-123, and protein D-167 were the predominant T- and B-cell combined epitopes (Fig. 3).

DISCUSSION

H. influenzae, one of the bacteria comprising the commensal flora of the human upper respiratory tract, is pathogenic and causes both localized and invasive (septicemic) infections (3, 23). The major focus of attention and research has been on infections caused by serotype b organisms, which cause several life-threatening illnesses in children. However, our previous studies showed that in China, most of the isolates responsible for H. influenzae-related infectious diseases are noncapsulated (nontypeable) strains (24, 25). Although NTHi isolates were initially associated with asymptomatic colonization, they are also pathogenic and frequently identified as the etiologic agent of otitis media, sinusitis, conjunctivitis, chronic bronchitis, and community-acquired pneumonia (1, 3, 23). Type b polysaccharide-protein conjugate vaccines are widely implemented; however, these vaccines will not protect against noncapsulated strains of H. influenzae (26). OMPs are the antigenic surface structures of NTHi, which are under active evaluation as vaccine antigens. Several OMPs of NTHi have been proposed as potential vaccine antigens on the basis of their sequence conservation, immunogenicity, and demonstration of significant protection in animal models following immunization (27). Two highly conserved proteins among NTHi strains have shown significant potential as vaccine candidates: P6 and protein D (13, 14, 22, 28–30). It was reported in a chinchilla model that immunization with P6 provides protection against AOM due to NTHi (31), and intranasal immunization with P6 has been shown to confer antigen-specific mucosal immunity and enhance mucosal clearance of NTHi (32). Protein D has shown protection against NTHi AOM in a chinchilla model as well. In addition, protein D has the potential to protect children against NTHi AOM, as shown in a randomized clinical vaccine trial in which protein D as a carrier protein was conjugated with pneumococcal capsular polysaccharides (14).

### TABLE 1 Predicted T- and B-cell combined antigenic epitopes of outer membrane proteins in NTHi

<table>
<thead>
<tr>
<th>Name</th>
<th>Location (residues)</th>
<th>Amino acid sequencea (N terminus to C terminus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6-2</td>
<td>2–25</td>
<td>NKFKVSLLVAGSYAALACSSSNN</td>
</tr>
<tr>
<td>P6-61</td>
<td>61–86</td>
<td>TGEYVQILDHAAAYLNTAAPAIVLVE</td>
</tr>
<tr>
<td>P6-95</td>
<td>95–122</td>
<td>PETNHAGQRADAVKYLAGKVDAGK</td>
</tr>
<tr>
<td>P6-123</td>
<td>123–147</td>
<td>KLGTVSVMEEPAPAVLDHEEAYASKNR</td>
</tr>
<tr>
<td>Protein D-2</td>
<td>2–22</td>
<td>KLKLTLAISLAAQVLAGCSSH</td>
</tr>
<tr>
<td>Protein D-105</td>
<td>105–136</td>
<td>RYYVIFTLKQESLEMTFENFETKDGGKQQVY</td>
</tr>
<tr>
<td>Protein D-224</td>
<td>224–247</td>
<td>TELLPMGDMLKLVQLIAYTDWKE</td>
</tr>
<tr>
<td>Protein D-332</td>
<td>332–351</td>
<td>VNQMYDALLNSKATGVTID</td>
</tr>
<tr>
<td>Protein D-167</td>
<td>167–193</td>
<td>GKKVGYPEIKAPWHFHQNGKDIAAET</td>
</tr>
<tr>
<td>Protein D-205</td>
<td>205–229</td>
<td>KTDMVYLQTFDNLKRIKTEELLPOQ</td>
</tr>
<tr>
<td>Protein D-255</td>
<td>255–280</td>
<td>GYWVNYYDWMFKPGAMAEVVKYADG</td>
</tr>
<tr>
<td>Protein D-294</td>
<td>294–323</td>
<td>SKEPDVYTPVLKELAVYNYVEHVPTVRKD</td>
</tr>
</tbody>
</table>

a The potential T-cell epitopes are underlined, and the potential B-cell epitopes are in bold type.

![FIG 1](http://cvi.asm.org) Box plots of the levels of antibodies against P6 and protein D of NTHi in different groups. Antibody titers were obtained using ELISA from plates coated with purified rP6 and rPD. A reference standard from a pool of patient serum was used to calculate an arbitrary unit of IgG antibody. The horizontal line in each box shows the median, and the top and bottom lines of the boxes show the interquartile range (IQR). * and o were discrete cases. m, month; y, year.
Coccal protein D conjugate vaccine reduces nasopharyngeal carriage of NTHi following the booster dose; however, this transient effect on carriage does not appear to be directly involved in the protective effect of vaccination against AOM. van den Bergh et al. (34) found that a pneumococcal protein D conjugate vaccine had no differential effect on nasopharyngeal NTHi colonization or 

H. influenzae density in healthy Dutch children up to 2 years of age, implying that further study on herd effects for NTHi are still needed.

The focus of this study was to examine the antibody responses in healthy individuals of different ages to the vaccine candidates P6 and protein D and their T- and B-cell antigenic epitopes of NTHi. We observed that both anti-P6 and anti-protein D levels were low in children when they were <1 month old, and children generally mounted serum antibody responses over time against these two antigens, with a peak at age of 7 months to 3 years. Several studies have reported that nasopharyngeal (NP) colonization causes children to develop antibodies to homologous and heterologous NTHi strains. The NP is considered a reservoir for NTHi, which becomes established during the first year of life (35). During that period, children with NP colonization by NTHi develop an immune response to this pathogen. Therefore, in the youngest group in our study, lower levels of anti-P6 or anti-protein D were observed in infants, while a significant increase in antibody levels was found in the 1-month to 3-year group, suggesting that NP colonization by NTHi was an immunizing event.

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Pichichero et al. (16) found a gradually increasing trend of antibodies to P6 and protein D in children age 6 to 30 months (16), and the increase in the levels of serum antibodies in our study is consistent with these earlier results. Individuals had a decrease in serum antibody to P6 and protein D after 3 years old, and the antibody levels were held at a low and stable level. The lowest antibody levels were found in 21- to 40-year-old groups, and the antibody levels were increased in 50 years old, while the antibody levels were held at a low and stable level. The lowest antibody levels were found in 21- to 40-year-old groups, and the antibody levels were increased in 50 years old, while the antibody levels were held at a low and stable level. The lowest antibody levels were found in 21- to 40-year-old groups, and the antibody levels were increased in 50 years old, while the antibody levels were held at a low and stable level. The lowest antibody levels were found in 21- to 40-year-old groups, and the antibody levels were increased in 50 years old, while the antibody levels were held at a low and stable level. The lowest antibody levels were found in 21- to 40-year-old groups, and the antibody levels were increased in 50 years old, while the antibody levels were held at a low and stable level. The lowest antibody levels were found in 21- to 40-year-old groups, and the antibody levels were increased in 50 years old, while the antibody levels were held at a low and stable level.

The results of anti-P6 and protein D in different age groups obtained in our study suggested that exposure to H. influenzae leads to increased antibody responses to P6 and protein D, which indicates that they might be effective antigens for the development of a vaccine to prevent infectious disease caused by NTHi. How-
ever, the immune responses evoked by a subunit vaccine are often not optimal, and tandem-expressed antigens are neither easily obtained nor mass developed. When attacked by a pathogenic microorganism, the protection against infection mainly depends on the stimulation of an appropriate antibody; highly potent neutralizing antibodies can intercept a pathogen before it attaches to its target cell. This ability is based on the antibodies’ specific recognition of epitopes, the sites on the antigen. In addition, cellular immunity plays a crucial role in the production of high antibody titers. The peptide contains both T- and B-cell epitopes and is advantageous for eliciting an effective immune response (37). Recent studies have shown that recombinant proteins corresponding to strings of universal CD4+/H11001 T-cell epitopes as carriers induced an immune response against H. influenzae, which was as good as or better than that with the licensed vaccines (38). Thus, it is essential to study T- and B-cell combined epitopes for the development of novel vaccines. To develop an epitope-based vaccine, the identification of potential effective immunodominant epitopes is an initial and critical step. In this study, we characterized T- and B-cell combined epitopes of the outer membrane proteins P6 and protein D. In silico epitope prediction is a useful tool in the development of new vaccine formulations. By using the ANTIGENIC and Epitope prediction softwares, we identified four combined T- and B-cell epitopes of P6 and eight epitopes of protein D. We also used ELISA analysis to evaluate the efficiency of the epitopes. Our results showed that these selected epitopes were specifically recognized by the antibodies in the sera, and the distribution regularities of antibody levels in different age groups were positively correlated with those in P6 and protein D antigens, suggesting that T- and B-cell combined epitopes possessed satisfactory immunogenicity and can be used in the development of a vaccine against NTHi. Additionally, the reactivity of each epitope to the antibodies was different, in which P6-61, P6-123, and protein D-167 showed better immunogenicity. Earlier studies on epitopes of H. influenzae outer membrane proteins focused on P6. In a study by Beck-Sickinger et al. (39), the epitopes of P6 were identified and localized within residues 31 to 46 and 59 to 70 and in the C-terminal part of P6, which partly overlapped the predicted T- and B-cell combined epitope P6-61 in our study; however, the lipopeptides containing the sequence pattern QILDAHAA (P6 residues 47 to 54) and the mouse B-cell epitope GEYV (P6 residues 43 to 46) induced high titers of anti-P6 antibodies (39), which were in line with that of P6-61 in the study. Ishida et al. (40) established P6-specific CD4+/H11001 T-cell lines restricted by the human histocompatibility leukocyte antigen (HLA)-DR9 molecule and revealed a human T-cell epitope on P6 and its core peptide sequence (p77 to 85; EYNIALGQR). Nomura et al. (41) studied promiscuous peptides on the nontypeable H. influenzae outer membrane protein P6, identified that human B-cell epitope was located on p71 to 85, which could be recognized by T-cell lines (TCL) in the restriction of HLA-DR9, and induced a proliferative response. The epitope peptide sequences on P6 in these two studies were successfully
predicted (named P6-95) in the study as well. One limitation of our study is that we could not find a negative-control peptide using bioinformatic tools; however, future in vivo study can shed more light on the functions of these peptides.

In conclusion, we have analyzed the levels of antibody against P6, protein D, and their antigenic epitopes in healthy individuals of different ages. We found that children aged 1 month to 6 years old had the highest levels of antibody compared to other age groups, which may be related to the establishment of asymptomatic NP colonization of NTHi. In this study, T- and B-cell combined epitopes in the outer membrane proteins P6 and protein D from NTHi, which possessed immunogenicity similar to that of protein antigens, were identified and characterized, and they may be important epitopes to be included in a vaccine against NTHi. Nonetheless, further analysis and evaluation of the function of the antibodies to these epitopes in NTHi-infected patients still need to be carried out, which is important for both the understanding of immunological events and the development of more effective vaccines and diagnostic tools for NTHi-related diseases.

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