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

Chun-Zhen Hua,a Wei-Lin Hu,b,c Shi-Qiang Shang,d Jian-Ping Li,c Li-Quan Hong,e Jie Yan,b,c

Division of Infectious Diseases, Children’s Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People’s Republic of China; Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People’s Republic of China; Division of Basic Medical Microbiology, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People’s Republic of China; Department of Clinical Laboratory, Children’s Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People’s Republic of China; Department of Clinical Laboratory, The Affiliated Hospital of Hangzhou Normal University, Hangzhou, Zhejiang, People’s Republic of China

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 (GMs) 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 Bioresource 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 DNA samples 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′-GGCGCATATG [NdeI] GCAGG CAATGGTGCTGCT-3′), p6-R (5′-GGCGCTCGAG [XhoI] GTACAAGTA CACTACGACAG-3′), pD-F (5′-GGCGCATATG [NdeI] AATACCAAT GAAATCAACAG-3′), and pD-R (5′-GGCGCTCGAG [XhoI] TTATGTCCT TTTTAAATTCACT-3′) from NTHi strain 86-028. The total volume per PCR was 100 μl, which included 20 μl of each of the primers, 2.5 U of Ex-Taq DNA polymerase, and 100 ng of DNA template. The reaction mixture was initiated by incubation at 94°C for 5 min, amplification for 30 cycles, and then incubation at 72°C for 10 min. The products were detected in 1.5% ethidium bromide-prestained agarose gels after electrophoresis. The PCR products were purified using the PCR product purification kit (Qiagen) and then ligated into plasmid pMD18-T using the TA cloning kit (TaKaRa). The cloned target genes were sequenced by an instrument of 3730 type at Invitrogen Co., Ltd. (Shanghai, China). The two recombinant plasmids and pET42a vector (Novagen, USA) were digested with NdeI and XhoI endonucleases (TaKaRa). The DNA segments of the recombinant plasmids and pET42a vector (Novagen) were further studied. Twelve high-score combined T-cell and B-cell epitopes (combined T- and B-cell epitopes) for protein D. We predicted the B-cell epitopes with ANTIGENIC, and the epitopes with a score of >3 included. We predicted 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).

**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 titers (GMTs) of antibody levels rose almost 4-fold, from 2,605 to 16,199 for P6, and 8-fold, from 11,385 to 80,129 for protein D, in the children from birth to 3 years old. In comparison, the increase in the antibody level for P6 in children (>7 years) and adults was not significantly higher than the <1-month group. In contrast, the antibody level for protein D was slightly increased in adults >70 years old, while it was stable at a lower level in 7- to 70-year-old individuals (*P < 0.05*). Figure 2 shows the scatter distribution of antibodies against outer membrane proteins and antigenic epitope peptides. The GMTs of T- and B-cell combined antigenic epitopes in P6 and protein D were positively correlated with those of protein antigens (*P < 0.0001*). Similarly, their antibody titer distribution among age groups is the same as that in P6.
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). It should also be recognized that some other studies have demonstrated less robust protection: Prymula et al. (33) found that vaccinating children with a pneumo-

| Name          | Location (residues) | Amino acid sequencea (N terminus to C terminus) |
|---------------|---------------------|--------------------------------------------------|
| P6-2          | 2–25                | NKVKSLLVAGSVAAALACSSSN |
| P6-61         | 61–86               | TGEYVQILDHAAAYNAAPKVLVE |
| P6-95         | 95–122              | PEYNALGQRRADAVKGGLAKGVDAGK |
| P6-123        | 123–147             | KLGTVSGEGEPAVLHDDEEAYSKNR |
| Protein D-2   | 2–22                | KLKLALSSLAGVLAGCSSH |
| Protein D-105 | 105–136             | RYVVIDFTLKEIOSLMTENFETKDGKQAQY |
| Protein D-224 | 224–247             | TEYLQMGMDKLVQLIAYTDWKE |
| Protein D-332 | 332–351             | VQSMYDALLNKSGATGVFTD |
| Protein D-167 | 167–193             | GKKVGIYPEIKAPWHHQNGKDIAAES |
| Protein D-205 | 205–229             | KDMDVLYQTDFNLEKRIKTTELPQ |
| Protein D-255 | 255–280             | GYVNYYNSYDWFKGPGAMAEVKYADG |
| Protein D-294 | 294–323             | SKPDIVYTPYLKELAQYNEVHPYTVR KD |

a The potential T-cell epitopes are underlined, and the potential B-cell epitopes are in bold type.
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. 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 specific 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, a child-bearing age for women, which explained the low antibody levels to P6 and protein D in infants. In addition, a small increase in the levels of serum antibodies was observed in older groups. Our finding is somewhat contradictory with what was reported by Pichichero et al. (16), who suggested that adults age 18 to 60 years had anti-PD or anti-P6 levels that were higher than or similar to those in children. The discrepancy between these two studies might be due to different sample sizes or different NTHi exposure rates in these two study settings. Nonetheless, we also found that the anti-PD antibody concentration increased in the group >50 years old, while the anti-P6 antibody concentration was not increased, which is consistent with the finding by Pichichero et al. (16). Why anti-P6 antibody was not increased to the same extent as the anti-PD antibody in the same cohort may due to the different immunogenicities of the two proteins (29). Khan et al. (29) compared the serum IgG levels of protein D and P6 in paired acute- and convalescent-phase sera from children with acute otitis media. They found that the increased IgG titers to protein D were significantly higher than those to P6, indicating that the immunogenicity of P6, with a low molecular weight, was inferior to that of protein D (PD). It was reported that H. influenzae was one of the five most frequently occurring pathogens in community-acquired pneumonia, and the weakened immune systems in older people may increase their susceptibility to infections (36).

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+/HLa1001 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 QILDAA (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+/HLa1001 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
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