Natural Development of Antibodies against *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* Protein Antigens during the First 13 Years of Life

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Conserved protein antigens have been investigated as vaccine candidates against respiratory pathogens. We evaluated the natural development of antibodies against *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* proteins during childhood. Serum samples were collected from 50 healthy children from their first months to age 13 years (median sampling interval, 6 months). We also analyzed serum samples from 24 adults. Serum IgG antibodies against eight pneumococcal proteins (Ply, CbpA, PspA 1 and 2, PcpA, PhtD, StkP-C, and PcsB-N), three *H. influenzae* proteins, and five *M. catarrhalis* proteins were measured using a multiplexed bead-based immunoassay. Antibody levels were analyzed using multilevel mixed-effects regression and Spearman’s correlation. Antibody levels against pneumococcal proteins peaked at 3 to 5 years of age and then reached a plateau. Antibody levels against *H. influenzae* proteins peaked during the second year and then stabilized. Antibody levels against *M. catarrhalis* proteins peaked during the first year and then slowly decreased. Peak antibody levels during childhood were higher than those of adults. Correlations among pneumococcal antibody levels were highest among anti-CbpA, anti-PcpA, and anti-PhtD antibodies (r = 0.71 to 0.75; P < 0.001). The children presented 854 symptomatic respiratory infections on 586 occasions. Symptomatic respiratory infections did not improve prediction of antibody levels in the regression model. The maturation of immune responses against the investigated pneumococcal proteins shares similarities, especially among CbpA, PcpA, and PhtD. Antibody production against *H. influenzae* and *M. catarrhalis* proteins starts early in life and reaches peak levels earlier than antibody production against the pneumococcal proteins. Basal antibody levels are not related to the occurrence of symptomatic respiratory infections.

Acute respiratory tract infections represent a substantial burden for childhood health care worldwide (1). Bacterial agents such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are important etiological agents of such infections (2–4). Elucidation of the characteristics of immune responses against these pathogens may enhance the development of vaccines against them. For instance, the identification of the best timing for antibody production following antigen exposure may optimize vaccine efficacy.

The prevention of infections caused by respiratory bacterial pathogens, especially *S. pneumoniae* and *H. influenzae*, has relied mostly on protein conjugate polysaccharide vaccines. However, such vaccines target specific capsular polysaccharides, restricting their effectiveness to a limited number of serotypes. The use of protein antigens may potentially provide serotype-independent protection and broader coverage of different strains of these bacteria (5–7). Recent trials have already shown promising results with protein antigens in the immunization of human subjects (8, 9) and in experimental animal models (10, 11) using the pneumococcal proteins Ply, CbpA, PspA, PcpA, and PhtD. Thus, the knowledge about the maturation of immune responses against protein antigens from respiratory pathogens might improve this new approach to vaccine development.

We aimed to evaluate the natural development during the first 13 years of life of antibodies against proteins from *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in a cohort of healthy children.

**MATERIALS AND METHODS**

**Study population.** Study participants comprised Finnish children from the ongoing population-based Type 1 Diabetes Prediction and Prevention (DIPP) study, a prospective investigation evaluating factors associated with the development of type 1 diabetes in children carrying predisposing HLA-DQ genotypes (12). In that study, children are observed from birth...
and have visits scheduled at 3- to 6-month intervals in the first 2 years of life and then at 6- to 12-month intervals until age 15 years. At each visit, parents are asked about symptoms and illnesses since the previous visit, and serum samples are drawn and stored at −70°C. For the present study, we selected 50 children with the highest number of serum samples available from a group of 109 participants randomly selected from the DIPP cohort for a previous study (13). The 50 children (30 girls) evaluated herein developed neither type 1 diabetes nor diabetes-associated antibodies by the end of their follow-up. We also analyzed a group of 24 Finnish adults randomly selected from a study evaluating patients who underwent elective tonsillectomy according to different clinical indications (14). From this group, we only analyzed serum samples collected immediately before surgery. None of the study participants had received any pneumococcal vaccines.

Written informed consent was obtained from all study participants or legal guardians before study enrollment. The study protocol was approved by the ethics committee of the Hospital District of Southwest Finland and of Sakatuka Central Hospital.

Serology. All serum samples were analyzed for IgG antibodies against eight recombinant pneumococcal protein antigens (Ply, CbpA, PspA 1, PspA 2, PcpA, PhtD, StkP-C, and PcsB-N), three H. influenzae recombinant protein antigens (protein D, NTHI0371-1, and NTHI0830), and five M. catarrhalis recombinant protein antigens (outer membrane protein CD, MC_RH4_2506, MC_RH4_1701, MC_RH4_3729-1, and MC_RH4_4730) using a multiplexed bead-based serological test. Ply, CbpA, PcpA, PhtD, StkP-C, and PcsB-N were conjugated in one bead region each. PspA 1 and PspA 2 were conjugated in the same bead region, and all H. influenzae and all M. catarrhalis antigens were conjugated onto one bead region per bacterium. We included the pneumococcal reference serum 007 on each plate as a standard (15), and it was assigned an arbitrary concentration of 1,000 U/ml for each anti-pneumococcal antibody. Pneumococcal antibody concentrations in the tested serum samples were determined in relation to the amount of antibodies assigned in the 007 serum. Levels of antibodies against H. influenzae and M. catarrhalis antigens were reported as median fluorescence intensity values. Control serum samples with high low antibody concentrations were analyzed on each plate and presented a coefficient of variation of <20% for all antibodies. We analyzed as many samples as possible from each study subject on the same plate, and all samples were assayed in duplicate. Further details of the serology protocol have been published elsewhere (16).

Statistics. Continuous variables were presented as medians (25th to 75th percentiles) as they showed a nonparametric distribution, except for geometric mean concentration (GMC) or log values. Longitudinal data on antibody levels were analyzed by assessing the linear regression equation. Angle coefficients that were not significantly different from zero were interpreted as segments with stable antibody levels, which were presented as geometric mean concentration (GMC) or log values. Longitudinal data on antibody levels were analyzed on an individual basis. Figure 2 depicts the antibody levels on an individual basis. Figure 2 depicts the antibody levels over time. The most frequent symptomatic respiratory infections, out of the 854 events, were the common cold (n = 386 [45.2%]), acute tonsillitis (n = 386 [45.2%]), acute sinusitis (n = 117 [13.6%]), and group A streptococcal pharyngitis (n = 30 [3.5%]). Some children had >1 serum sample analyzed in each age stratum.

TABLE 1 Distribution of serum samples, number of children evaluated, and number of time points when at least one symptomatic respiratory infection was reported by parents

| Age stratum (yr) | No. of samplesa (%) | No. of children (%) | No. of time points (%) |
|------------------|---------------------|---------------------|------------------------|
| 1st (1-2)        | 124 (12.1)          | 48 (96)             | 83 (14.2)              |
| 2nd (3-4)        | 154 (15.0)          | 50 (100)            | 105 (17.9)             |
| 3rd (5-6)        | 90 (8.8)            | 50 (100)            | 60 (10.2)              |
| 4th (7-8)        | 95 (9.3)            | 50 (100)            | 57 (9.7)               |
| 5th (9-10)       | 94 (9.2)            | 50 (100)            | 60 (10.2)              |
| 6th (11-12)      | 95 (9.3)            | 49 (98)             | 63 (10.8)              |
| 7th (13-14)      | 85 (8.3)            | 47 (94)             | 49 (8.4)               |
| 8th (15-16)      | 89 (8.7)            | 49 (98)             | 46 (7.9)               |
| 9th (17-18)      | 88 (8.6)            | 48 (96)             | 33 (5.6)               |
| 10th (19-20)     | 52 (5.1)            | 32 (64)             | 17 (2.9)               |
| 11th (21-22)     | 34 (3.3)            | 22 (44)             | 6 (1)                  |
| 12th (23-24)     | 19 (1.8)            | 11 (22)             | 6 (1)                  |
| 13th (>24)       | 5 (0.5)             | 4 (8)               | 1 (0.2)                |

a Some children had >1 serum sample analyzed in each age stratum.

RESULTS

The median follow-up period was 9 years (25th to 75th percentiles, 8 to 10 years; minimum, 7 years; maximum, 12.5 years). We analyzed a total of 1,024 samples and a median of 20 samples (25th to 75th percentiles, 19 to 22 samples; minimum, 15 samples; maximum, 27 samples) from each child. Table 1 shows the number of samples available in each age stratum. Most of the children had samples available up to their 10th year of age. The median sampling interval was 6 months (25th to 75th percentiles, 4.8 to 6.5 months; minimum, 2 months; maximum, 18.7 months). The median age of the subgroup of adults who underwent tonsillectomy was 35.5 years (25th to 75th percentiles, 25 to 44 years; minimum, 19 years; maximum, 65 years).

The kinetics of antibody levels during the first 13 years of life and the antibody levels in adults are shown in Fig. 1. Antibody levels against pneumococcal antigens peaked at age 3 to 5 years and then reached a plateau for the remainder of the follow-up period. Antibody levels against H. influenzae and M. catarrhalis proteins peaked earlier, during the second and first year of life, respectively, and then antibodies against H. influenzae stabilized and antibodies against M. catarrhalis slowly decreased (angle coefficient, −0.006; P < 0.001) until the end of the follow-up. For all antibodies analyzed, the peak levels during childhood were higher than the peak levels of adults who underwent tonsillectomy. Although the fitted trajectory of antibody levels of the study group reached a plateau phase for most antibodies investigated, there were many ups and downs in antibody levels in this plateau phase on an individual basis. Figure 2 depicts the antibody levels throughout childhood of one of the subjects from the study group, as an example.

A total of 854 symptomatic respiratory infections were reported on 586 occasions. Table 1 shows the distribution of time points when at least one symptomatic respiratory infection was reported. More than half of the occasions with at least one symptomatic respiratory infection were reported during the first 4 years of life. The most frequent symptomatic respiratory infections, out of the 854 events, were the common cold (n = 386 [45.2%]), acute tonsillitis (n = 386 [45.2%]), acute sinusitis (n = 117 [13.6%]), and group A streptococcal pharyngitis (n = 30 [3.5%]).
otitis media \((n = 178 [20.8\%])\), cough \((n = 177 [20.7\%])\), pharyngitis \((n = 44 [5.2\%])\), bronchitis \((n = 21 [2.5\%])\), pneumonia \((n = 18 [2.1\%])\), sinusitis \((n = 16 [1.9\%])\), and influenza \((n = 14 [1.6\%])\). Adding the occurrence of symptomatic respiratory infection in general or the occurrence of each specific type of respiratory infection did not improve the prediction by the multilevel mixed-effects linear regression for any investigated antibody (data not shown).

There was a positive correlation between the levels of antibodies against all pneumococcal antigens, and this correlation was stronger among CbpA, PcpA, and PhtD than among other pneumococcal protein antigens (\(r = 0.71\) to 0.75 for correlations among CbpA, PcpA, and PhtD; \(r = 0.50\) to 0.67 for the other correlations; \(P < 0.001\) for all comparisons).

**DISCUSSION**

Previous studies have already evaluated the dynamics of antibody production against some pneumococcal proteins during the first few years of life. To our knowledge, this is the first study simultaneously evaluating antibody levels against eight pneumococcal proteins, along with antibodies against *H. influenzae* and *M. catarrhalis* proteins, in a cohort with long-term follow-up.

Similar to reports from previous studies evaluating a few antigens at a time, we demonstrated here that IgG production against several pneumococcal proteins starts early in life (17–19), reaching peak levels and stabilizing during the first years of life (20). Ren et al. (21) showed that antibody levels against PlyD1, PcpA, and PhtD rise in synchrony during the first months of life. We have confirmed and expanded that finding, showing that the kinetics of antibody levels against the eight pneumococcal proteins analyzed (including Ply, PcpA, and PhtD) is similar throughout childhood. This concordance is more pronounced among anti-CbpA, anti-PcpA, and anti-PhtD antibodies. We previously showed the absence of significant cross-reactivity between these antibodies using our immunoassay (16). These results suggest that the maturation of immune responses against the pneumococcal antigens studied is synchronous. Slight differences in the kinetics of these antibodies might be due to differences in antigen immunogenicity, epitope accessibility, and the half-lives of the antibodies. Our results also suggest that the concurrent exposure to the investi-
gated pneumococcal proteins does not interfere with antibody production against these antigens, which has important implications for vaccine development.

Antibody production against *H. influenzae* proteins also starts in the first months of life, reaching peak levels and then a plateau at a younger age than antibodies against pneumococcal proteins. This early and rapid increase in antibody levels during the first 2 years of life was previously demonstrated for antibodies against *H. influenzae* proteins, such as protein D and other outer membrane proteins (22, 23). To our knowledge, no previous study has shown the plateau phase in childhood after the antibodies against *H. influenzae* proteins reached peak levels. This may be explained by the short follow-up of previous studies (up to 2 or 3 years of age), the design of those studies (analysis of serum samples from different children in distinct age strata instead of collection of serial and systematic serum samples from the same child), or the use of different *H. influenzae* protein antigens.

Antibody levels against *M. catarrhalis* proteins peaked very early in life and then slowly decreased for the remainder of the follow-up period. Previous studies showed different kinetics for antibodies against distinct *M. catarrhalis* proteins. For instance, Verhaegh et al. (24) and Samukawa et al. (25) demonstrated that antibodies against UspA increase early in childhood, and Verhaegh et al. (24) showed that antibodies against other *M. catarrhalis* outer membrane proteins did not increase during a follow-up period of 2 years. In contrast to our results, none of these studies showed a decrease in antibody levels against *M. catarrhalis* proteins after reaching peak levels during childhood, probably because of the short follow-up or the use of different antigens.

Interestingly, peak levels of all investigated antibodies during childhood were higher than the levels of adult patients who underwent tonsillectomy. This might be explained by the decrease in bacterial exposure (symptomatic infection or colonization) during adulthood compared to that in childhood (26, 27). The more frequent bacterial exposure during childhood might boost immune responses against these proteins, thus keeping up higher levels of antibodies against these antigens.

We demonstrated that the occurrence of symptomatic respiratory infections does not influence basal antibody levels. This finding is most likely explained by the facts that most symptomatic respiratory infections during childhood are viral, the elevation in antibody levels during symptomatic bacterial infection is brief and returns to basal antibody levels after the convalescence period, and basal antibody levels probably depend more on stimuli from nasopharyngeal colonization than on symptomatic bacterial infections. For instance, Turner et al. (28) showed that nasopharyngeal
carriage by *S. pneumoniae* was closely associated with basal antibody levels against 27 pneumococcal proteins in a follow-up study evaluating children from birth to age 2 years. The timing of antibody responses demonstrated herein also suggests that basal antibody levels are associated with nasopharyngeal bacterial colonization. In our study, antibodies against *M. catarrhalis* proteins peaked earlier than antibodies against *H. influenzae* and *S. pneumoniae* proteins, and it has been shown that *M. catarrhalis* nasopharyngeal colonization starts earlier in life than colonization of the other two bacteria (29, 30). It has also been shown that there is a progressive increase in the nasopharyngeal colonization rate in the first 2 years of life in Finnish children, concordant with the increase in antibody levels against pneumococcal proteins in this period (31). In addition, the study by Turner et al. (28) showed that acquisition of nasopharyngeal carriage by pneumococcus is not associated with a 2-fold or higher increase in antibody levels against protein antigens. In turn, Andrade et al. (32) demonstrated that a ≥2-fold rise in antibody levels against the pneumococcal antigens analyzed herein (≥1.5-fold for PcP) is a sensitive and specific marker for detection of pneumococcal infection in children with pneumonia. These results suggest that the immune response during bacterial colonization is different from the immune response during symptomatic bacterial infections.

Our study had some limitations. The sampling interval was quite wide at older ages, which might decrease the precision in the modeling of the kinetics of antibody levels compared to that at younger ages. However, antibody levels showed little variation at older ages, and the precision was probably not importantly affected. The longer time interval between visits of older children might also implicate less accuracy in data collection regarding symptoms of respiratory infections, because parents reported symptoms and illnesses since the last visit. Nevertheless, we emphasize that parents were highly motivated to participate in the study, and they sought to provide accurate data. In addition, antibodies against protein antigens from *H. influenzae* and *M. catarrhalis* were analyzed using one bead per bacterium. We suggest that future studies evaluate these antigens separately in order to obtain more detailed information regarding antibodies against these bacteria. Finally, we recognize that there might be issues regarding the generalizability of our results to settings with important socioeconomic and environmental differences. These factors are known to influence pneumococcal colonization (33), so regions with markedly different colonization patterns might show different dynamics of antibody production against pneumococcal proteins.

In conclusion, the natural developments of the antibody responses against the eight pneumococcal proteins analyzed in this study are similar and reach their peaks at 3 to 5 years of age. The concordance seems to be strongest between anti-CPaA, anti-PcpA, and anti-PhtD antibodies. Antibody production against *M. catarrhalis* and *H. influenzae* starts early in life and reaches peak levels during the first and second year of life, respectively, and then stabilizes or declines earlier than antibody production against the pneumococcal proteins. Basal antibody levels are not influenced by the occurrence of symptomatic respiratory infections. These findings suggest that the combination of protein antigens seems to be a viable option for the development of new vaccines against *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* infections in young children.

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