MAJOR ARTICLE

Antibody Responses to Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Adults: A Longitudinal Household Study

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Background. Natural immunity to *Streptococcus pneumoniae* is thought to be induced by exposure to *S. pneumoniae* or cross-reactive antigens. No longitudinal studies of carriage of and immune responses to *S. pneumoniae* have been conducted using sophisticated immunological laboratory techniques.

Methods. We enrolled 121 families with young children into this study. Nasopharyngeal (NP) swabs were collected monthly for 10 months from all family members and were cultured in a standard fashion. Cultured *S. pneumoniae* isolates were serotyped. At the beginning (month 0) and end (month 10) of the study, venous blood was collected from family members >18 years old. Serotype-specific antipolysaccharide immunoglobulin G (IgG) and functional antibody and antibodies to pneumolysin, pneumococcal surface protein A (PspA), and pneumococcal surface antigen A (PsaA) were measured in paired serum samples.

Results. Levels of anticapsular IgG increased significantly after carriage of serotypes 9V, 14, 18C, 19F, and 23F by an individual or family member. For serotype 14, a higher level of anticapsular IgG at the beginning of the study was associated with reduced odds of carriage ($P = .006$). There was a small (~20%) but significant increase in titers of antibodies to PsaA and pneumolysin but no change in titers of antibody to PspA.

Conclusions. Adults respond to NP carriage by mounting anticapsular and weak antiprotein antibody responses, and naturally induced anticapsular IgG can prevent carriage.

The marked reduction in the incidence of invasive pneumococcal disease after the first few years of life is related to several factors, including age-related changes in susceptibility to infection with *Streptococcus pneumoniae* [1]. The reason for this reduction in susceptibility is thought to relate to the maturation of the immune system and, at the same time, natural exposure to the pneumococcus. Despite the latter assumption, relatively few data exist on immune responses to the pneumococcus after natural exposure. Studies of the immune response to infection have revealed an increase in antibodies to various targets after both mucosal infection (such as otitis media and pneumonia) and invasive disease [2–4]. However, it is likely that the major stimulus of the natural development of protective antibodies is exposure to the pneumococcus via nasopharyngeal (NP) carriage, since this is common in the general population [5]. Although an anticapsular antibody response to carriage of serotype 7F or 8 among military recruits in an outbreak setting has been documented [6], a previous longitudinal study of healthy adults failed to identify an anticapsular antibody response to carriage or protection against carriage by serum antibody [7]. The latter study may well have suffered from the nonspecificity of the techniques used to measure titers of antibodies to the pneumococcal polysaccharides present in unvaccinated individuals [8–10]. Furthermore, ear-

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lier studies of the response to carriage were limited by their exclusive focus on the anticapsular antibody response, as measured by a binding assay.

Newer techniques now permit serotype-specific anticapsular antibodies to be measured with a sensitivity and specificity not previously possible [8], and new functional antibody assays and antipneumococcal protein assays permit a more comprehensive analysis of the immune response to *S. pneumoniae*. As part of a European Union–funded project, a longitudinal pneumococcal carriage study was performed in families with children. One objective of the study was to examine the antibodies induced by pneumococcal carriage.

SUBJECTS, MATERIALS, AND METHODS

The present study was a 10-month study of NP swabs from and basic epidemiological data on a large sample of preschool children and their families, who were living in an urban setting in the United Kingdom. The study started 1 October 2001, and the collection of swabs was completed by July 2002. The study protocol was approved by the North Hertfordshire ethics committee and the Public Health Laboratory Service ethics committee, and all study participants or their parents or guardians gave written, informed consent before participation. Human-experimentation guidelines of the authors' institutions were followed in the conduct of this research project. A total of 121 children from birth to 3 years of age were recruited, along with their entire families, from 4 general practices in Hertfordshire, through the primary health-care child registers. Individuals with the following conditions were excluded from the study: moderate to severe disability; cerebral palsy; syndromes and neurological disorders affecting swallowing; ear, nose, and throat disorders affecting the anatomy of the ear (i.e., malformed ears); confirmed or suspected immunodeficiency (congenital or acquired); immunosuppressive therapy; enrollment in a previous pneumococcal vaccine trial; or enrollment of a sibling in the present trial.

NP swabs were collected from all family members at the initial (home) visit and then every month for 10 months. Collection of samples from absent family members or family members with illness was performed at a later date but occurred within a 14-day period. NP swabs were collected by the study nurse using a flexible wire shaft with a calcium alginate tip and were transported, within 24 h of collection, to the Respiratory and Systemic Infection Laboratory at the Central Public Health Laboratories. All NP swabs were handled in accordance with the standard operating procedures of the World Health Organization (WHO) Pneumococcal Vaccine Trials Carriage Working Group [11] and were processed as described elsewhere [12]. Pneumococcal isolates from 1 or more (if morphologically distinct) colonies were serotyped by standard methods [13], by use of serum samples from Statens Serum Institut. After final identification, all pneumococcal isolates were stored in glycerol blood broth at −80°C.

At the beginning (month 0) and end (month 10) of the study, samples of venous blood (5–10 mL) were collected from each adult (>18 years of age). Pneumococcal conjugate vaccine was offered to all index children at the end of the study, and 5 mL of venous blood was collected 4 weeks after vaccination. Blood was allowed to clot at room temperature; after the blood clotted, the serum was separated, aliquoted, and stored at −80°C until assayed. At the WHO reference laboratory for pneumococcal serology in the Institute of Child Health (London), serum was assayed for antibodies to 9 individual pneumococcal capsular polysaccharides. Serum samples were assayed by use of an ELISA after adsorption with both cell-wall polysaccharide and 22F polysaccharide, as described elsewhere [14].

To assess whether the antibodies measured by ELISA were functional, serum samples were also assayed by use of an opsonophagocytic technique that detects functional anticapsular antibody. This assay was performed at the National Public Health Institute in Helsinki, Finland (KTL), as described elsewhere [10]. Antibodies to the pneumococcal proteins pneumolysin, pneumococcal surface protein A (PspA), and pneumococcal surface adhesin A (PsaA) were also measured at KTL, by use of an ELISA, as described in detail elsewhere [15].

Statistical analysis. For anticapsular antibodies, fold changes in antibody levels were calculated with 95% confidence intervals, and the log values of these fold changes were compared between exposure groups by use of *t* tests. The odds of carriage, according to initial log titer, was investigated by use of logistic regression; when significant, this value was further explored by stratifying the initial titer into 4 levels, to estimate whether there was evidence of a protective level. For this analysis, Fisher's exact test was used to compare carriage by initial titer level. For functional antibodies, Fisher's exact test was used to investigate the relationship between carriage and an opsonophagocytic assay (OPA) titer ≥ 8, as well as 4-fold changes in OPA titer from month 0 to month 10. For antiprotein, the fold change from month 0 to month 10 was compared between carriers and noncarriers by use of *t* tests. For postvaccine antibodies, normal error regression was used to investigate the relationship between postvaccination titers of antibodies and previous carriage, with adjustment for age.

RESULTS

A total of 121 families participated in the present study ($n =$ 489 subjects), of whom 106 families remained until the end of the study. A total of 3767 NP swabs were collected, of which 932 (25%) were culture positive for the presence of *S. pneumoniae*. Prevalence of NP carriage of *S. pneumoniae* was age related. The mean carriage was highest (52%) in individuals 0–2 years of age, was 45% in individuals 3–4 years of age, was

Table 1. Data on serotype-specific carriage in paired serum samples from adults enrolled in the study ($n = 57$).

Serotype	No. of isolates
6A	12
6B	5
14	11
19F	8
19A	1
23A	3
23F	5
9V	5
9N	2
11A	5
22F	5

NOTE. Additional isolates are as follows: 3 each of serogroup 3, serotype 18C, and nontypeable isolates and 1 each of serotypes 8, 16F, 20, 27, 31, and 35B.

21% in individuals 5–17 years of age, and was 8% in individuals >18 years of age. At the beginning and end of the study, paired serum samples were collected from 134 of the adults enrolled in the study and form the core of this analysis. Three of the adults were 18–24 years of age, 61 were 25–34 years of age, 67 were 35–44 years of age, and 3 were >45 years of age. Fifty-seven of the adults from whom paired serum samples were collected had carriage detected (77 carriage episodes) during the study period. In this subgroup, the most commonly carried serogroup was 6 ($n = 17$), followed by 14 ($n = 11$), 19 ($n = 9$), 23 ($n = 8$), and 9 ($n = 7$). Details of the serotypes carried are shown in table 1. Serum samples were collected from 42 children 4 weeks after administration of a conjugate vaccine.

Anticapsular antibodies. Antibodies to 9 pneumococcal serotypes were measured by ELISA, but data are shown only for the 6 serotypes of which significant carriage occurred (6B, 9V, 14, 18C, 19F, and 23F). For each serotype, individuals from whom paired serum samples were available were stratified according to whether neither the individual nor a family member carried the serotype of interest (exposure level 0), whether a family member carried the serotype of interest but the individual did not (exposure level 1), or whether the individual carried the serotype of interest (exposure level 2). Fold changes in titers of antibodies from month 0 to month 10 were then compared between these groups to determine whether individuals who carried the serotype of interest or had a family member who carried the serotype had larger increases in titer than those who did not carry the serotype (table 2). A graphical representation of this relationship is shown in figure 1, in which the month-0 titer is graphed against the month-10 titer and shown for individuals stratified by carriage status (exposure levels 0–2). For 4 of the 6 serotypes studied (9V, 14, 18C, and 23F), documented carriage of the serotype by the individual resulted in a significant increase in titer during the study period. In addition, for serotypes 14, 18C, and 19F, carriage by a family member but not by the individual also increased titers during the study period, presumably because of a short period of undocumented carriage. Only for serotype 6B was carriage not associated with an increase in serotype-specific IgG.

For a number of individuals in the present study, titers of serotype-specific IgG at month 0 were already high. It was therefore possible to analyze whether serum IgG might protect against carriage. An analysis of the odds of carriage, according to $\log_{10}$ titer, showed a significant protective effect for only serotype 14 (odds ratio, 0.29/$\log_{10}$ titer; $P = .006$). A clear relationship between prevaccination titers and carriage ($P = .04$) was demonstrated, with titers >5 μg/mL having a good correlation with protection against carriage. For the other serotypes, no protective effect was apparent, but the numbers of carriers were small.

Functional antibody responses. A total of 54 individuals were included in an analysis of functional antibody to 5 se-

Table 2. Comparison of fold changes in levels of serotype-specific anticapsular IgG between the beginning (month 0) and end (month 10) of the study, according to pneumococcal exposure level.

Serotype, exposure level (no. of individuals)	Mean fold change (95% CI)	P[a]
6B		
No carriage (71)	1.24 (1.06–1.46)	
Family (58)	1.22 (0.99–1.51)	.89
Individual (5)	1.15 (0.62–2.12)	.81
9V		
No carriage (116)	1.08 (0.99–1.17)	
Family (13)	1.11 (0.62–1.99)	.84
Individual (5)	2.55 (1.11–5.84)	.001
14		
No carriage (90)	1.25 (1.04–1.49)	
Family (33)	2.21 (1.42–3.46)	.006
Individual (11)	11.18 (4.16–30.08)	<.001
18C		
No carriage (118)	1.10 (0.98–1.24)	
Family (13)	1.66 (0.84–3.25)	.056
Individual (3)	2.66 (0.14–51.67)	.039
19F		
No carriage (92)	0.96 (0.87–1.05)	
Family (34)	1.34 (1.02–1.75)	.004
Individual (8)	0.94 (0.53–1.67)	.95
23F		
No carriage (81)	1.35 (1.11–1.65)	
Family (48)	1.12 (0.88–1.43)	.25
Individual (5)	3.65 (0.45–28.27)	.019

NOTE. Study subjects were grouped according to their carriage status: no carriage, no carriage in the individual or in a family member; family, carriage in a family member but not in the individual; and individual, carriage in the individual. CI, confidence interval.

[a] Vs. no carriage

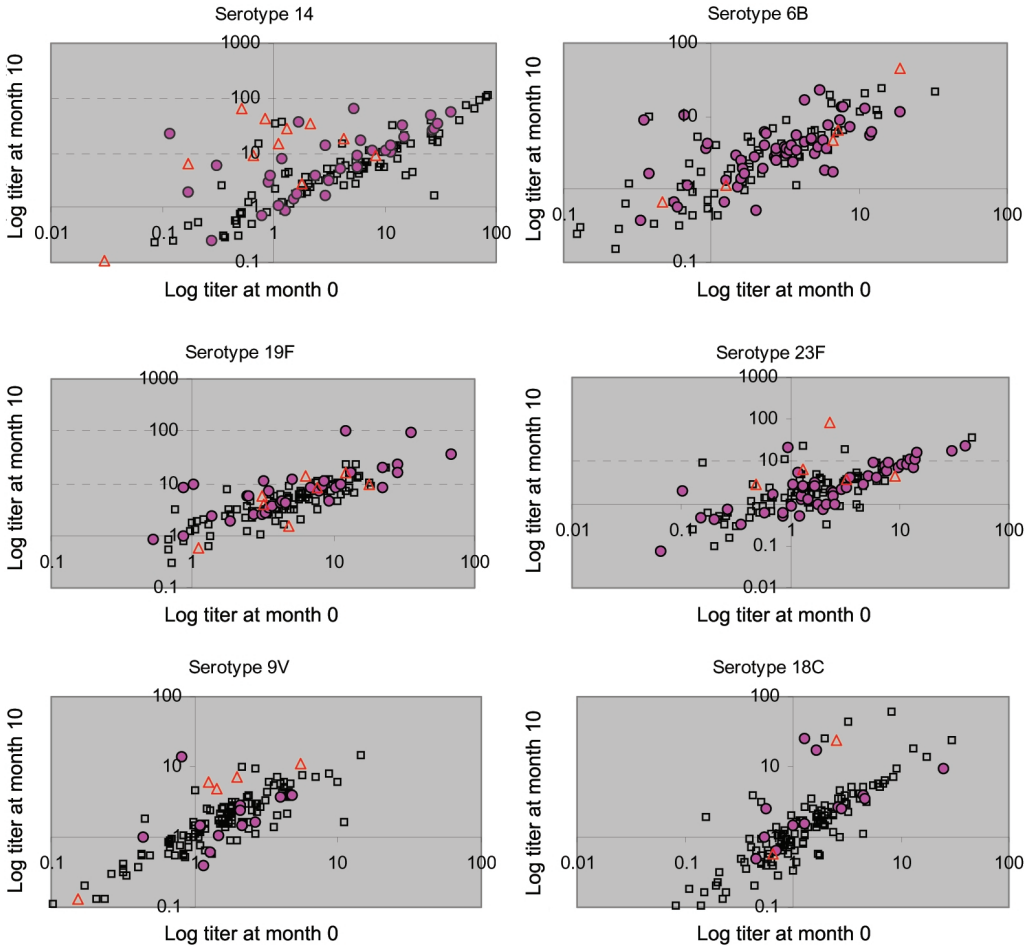

Figure 1. Serotype-specific antipneumococcal polysaccharide antibodies measured in adults at the beginning (month 0) and end (month 10) of the study period. Adults were stratified according to their carriage status: no carriage in the individual or in a family member *(open squares),* carriage in a family member but not in the individual *(purple circles),* and carriage in the individual *(red triangles).*

rotypes measured by use of an opsonophagocytic assay. They were chosen on the basis of having paired serum samples available and represented carriers and noncarriers of serotypes 6A, 6B, 14, 19F, and 23F. These serotypes were chosen because of the availability of a serotype-specific functional assay. Not all samples were tested for all 5 serotypes, just those from the carriers and a subset of noncarriers. In a few cases, the individual was not a carrier but was a household contact of a carrier. There was a positive correlation between OPA and serotype-specific IgG measured by ELISA (rank correlation for all data and the indicated serotype: 6B, 0.72; 14, 0.85; 19F, 0.28; and 23F, 0.68). The functional data were, however, not continuous, so the analysis of carriage in relation to OPA was performed by classifying OPA values <8 as negative and OPA values ⩾8 as positive. Using this stratification, we analyzed whether OPA positivity at month 10 was associated with carriage. Table 3 illustrates that, for serotypes 19F and 14, there was a significant association between carriage and OPA positivity at the end of the study, and the same trend was present for serotypes 6A and 6B. When we analyzed 4-fold changes between months 1 and 10, a highly significant association between carriage and an increase in functional antibody was noted for serotype 14 ($P<.001$) but not for the other serotypes studied. Unlike IgG measured by ELISA, the presence of functional antibody at the beginning of the study was not associated with subsequent protection against carriage of any of the serotypes.

Antiprotein responses. For PspA, no significant increase in titer was found in association with carriage. Titers of antibodies to PsaA and Ply had a weak relationship with carriage of any serotype. For PsaA and Ply, individuals who carried any serotype had 18% and 17% greater fold changes, respectively, between month 0 and month 10 than did those who did not carry any serotype ($P = .04$ and $P = .02$, respectively). Most individuals did not show large changes in titers from month 0 to month 10: only 7 had 4-fold increases. Titers of preexisting antibodies to any of the 3 proteins were not associated with protection against carriage.

Postconjugate vaccine analysis. Antibodies to 4 pneumo-

Table 3. Relationship between the functional antibody and carriage.

Serotype, carriage status	OPA+/total tested, no.	P[a]
19F		
No carriage	1/20	.02
Carriage	4/8	
23F		
No carriage	520	.37
Carriage	4/8	
6A		
No carriage	3/16	.66
Carriage	3/11	
6B		
No carriage	10/29	.06
Carriage	5/6	
6B		
No carriage	8/21	.09
Family carriage	2/8	
Carriage	5/6	
6B OPA for carriage of 6A		
No carriage	7/15	1.00
Carriage	4/10	
14		
No carriage	9/17	.05
Carriage	10/11	

NOTE. Subjects were classified according to carriage of the serotype for which functional antibody was measured and opsonophagocytic activity (OPA) (OPA positivity was defined as a titer ⩾8) at the end of the study was compared between the 2 groups. For serotypes 14 and 19F, OPA positivity was significantly associated with having carried these serotypes during the study period.

[a] Fisher's exact test.

coccal serogroups (6B, 14, 19F, and 23F) were also measured in 42 children who received a single dose of a licensed pneumococcal conjugate vaccine (Prevenar; Wyeth) at the end of the study. For each serotype, individuals were stratified according to their level of exposure to the relevant serotypes (6B, 14, 19F, and 23F) and their age at vaccination. Seventeen of the children were 1–2 years of age, 21 were 2–3 years of age, and 4 were 3–4 years of age at the time of vaccination. No evidence of a significant effect of prior exposure (carriage in the individual or contact with a family member who carried the serotype) on titers of antibodies after vaccination was found after adjustment for age (*P* values for trend per level of exposure for the indicated serotype: 6B, .14; 19F and 23F, .52, .18, .38, and .39)

DISCUSSION

The present study is, to our knowledge, the first to use highly specific immunological assays that measure both capsule and pneumococcal proteins to document the immune response to pneumococcal carriage in a longitudinal study of healthy adults. The unique data set obtained by monthly collection of NP swabs and the corresponding serological data allowed us to quantify the impact of both carriage as documented in the individual and possible carriage inferred by that documented in a family member. Carriage by an individual was shown to induce an increase in antibody to the homologous capsular polysaccharide of 5 of the 6 serotypes analyzed. Only exposure to serotype 6B failed to induce an immune response, which may be a result of the relatively poor immunogenicity of the serogroup 6 polysaccharide capsule [16]. By use of this data set, the duration of carriage has been estimated to be 19 days in adults [17]. Thus, even with monthly collection of swabs, episodes of carriage are likely to be missed. This is reinforced by the demonstration of an increase in titers of antibodies to serotypes 14, 18C, and 19F in individuals who did not carry the serotype but for whom carriage of the relevant serotype was documented in a family member. These data are critical for analysis of the relationship between carriage and immune response, since studies that do not take into account the likelihood of missed carriage episodes and classify only individuals with documented carriage as carriers may fail to see differences between study groups.

Although the immune system in young children is considered to be immature and although, classically, young children fail to respond to carbohydrate vaccine antigens, a recent study of the development of natural antibodies after carriage showed that, in children as young as 6 months old, homologous anticapsular antibody responses were present after carriage of serotypes 11A and 14 [18]. However, in children <2 years of age, titers of antibodies in those with documented colonization or acute otitis media with serotypes 6B, 19F, and 23F did not differ from titers of antibodies in those without such exposure. Although the latter finding may be due to the inability of young children to respond to those 3 serotypes, it may also be explained by unidentified periods of carriage in individuals labeled as not having been exposed; thus, the differences in antibody levels between the groups may be blurred.

Little is known about the effect of pneumococcal carriage on subsequent antibody responses to pneumococcal conjugate vaccine. However, a number of investigators have studied the effect of preexisting antibody on responses to *Haemophilus influenzae* type b (Hib) conjugate vaccines. Such studies have failed to find any effect of preexisting serum IgG specific for Hib capsule on the subsequent antibody response to Hib conjugate vaccine in adults [19] or infants [20, 21]. Although prevaccination samples were not available for analysis in the present study, carriage history did not influence the antibody responses of children to a single dose of pneumococcal conjugate vaccine, although the number of children studied was small.

Recent developments in the techniques used to measure pneumococcal antibodies have enabled us to apply the ELISA technique to serum samples from unimmunized adults. Previously,

analysis of such serum samples was complicated by the nonspecificity of the techniques used to measure serotype-specific IgG [22] and the consequent poor correlation with functional antibody. This may explain why Gwaltney et al.'s study of 15 families from whom swabs were collected every 2 weeks and blood samples were collected every 3 months, over the course of 1 year, failed to detect serotype-specific antibodies induced by carriage of *S. pneumoniae* [7]. The recent modification of an ELISA to incorporate the adsorption of serum samples with both cell-wall polysaccharide and 22F polysaccharide has improved the specificity of the assay [8], and it was an assay with this modification that we used. The increased specificity of the assay is reflected in the correlation between serotype-specific ELISA titers and OPA, which was strong for serotypes 14 and 23F but weak for serotype 19F (data not shown). Interestingly, OPA increased in relation to the carriage of serotype 19F, but IgG did not, as measured by ELISA, suggesting that the ELISA for serotype 19F is less sensitive. In contrast, for serotype 14, we have shown an increase in both IgG (as measured by ELISA) and functional antibody after carriage. These tools now allow us to explore natural immunity to the pneumococcus in adults with a much improved degree of confidence.

Pneumococcal conjugate vaccines have been shown to induce indirect protection through reduction in NP carriage [23]. It is of some interest, therefore, to understand what level of antibody might be required to protect against NP carriage, since this might be used as an indirect correlate when assessing the immunogenicity of new formulations of pneumococcal conjugate vaccines. A titer of 5 μg/mL at the beginning of the study was found to correlate with protection against carriage of serotype 14. This titer is much higher than the putative serum level of protection against invasive disease that has been estimated by a WHO working group (in the general range of 0.2–0.4 μg/mL) [1]. It is possible that absolute levels required to prevent carriage may differ between the serotypes, but it is interesting that this putative protective titer (5 μg/mL) is identical to the serum concentration of anti-Hib capsular antibody that was shown to correlate with protection against colonization after administration of a Hib conjugate vaccine [24] and is also identical to the titer required to prevent vaginal or rectal colonization by type III group B *Streptococcus* (C. Baker, personal communication). It is likely that high serum levels of IgG are required for protection against carriage, since they need to leave the serum compartment and enter mucosal secretions. It is also likely, therefore, that, in an infant immunization program, a booster dose of vaccine will be important for optimizing and prolonging the indirect effect of the vaccine, since only a small proportion of infants will achieve titers >5 μg/mL after priming but many more will achieve titers above this threshold after a booster dose of conjugate vaccine.

The role that pneumococcal proteins play as targets for protective immunity has received much attention during recent years as interest in proteins as alternative antigens for inclusion in pneumococcal vaccines has intensified [25, 26] and as better markers of pneumococcal infection have been sought [27]. Antibody responses to pneumococcal proteins (PsaA, PspA, and Ply) have been documented to increase with age in a Finnish cohort; these increases were strongly associated with pneumococcal exposure by carriage or infection (acute otitis media) [15]. In a developing-country setting (Kenya), antibodies to PsaA, PspA, and Ply were present in serum samples from all 220 individuals studied (2 weeks–84 years of age) [28], although the relationship to contact with the pneumococcus was not defined for this cohort. In the present study, colonization with the pneumococcus was only a weak stimulus for the development of antibody to PsaA and Ply and failed to stimulate anti-PspA antibodies. In contrast to titers of anticapsular IgG to serotype 14, existing titers of antibodies to any of the 3 proteins failed to provide protection against carriage. These data contrast with those published by McCool et al., who have studied the immune response to colonization and the role that preexisting antibodies to pneumococcal surface structures play in an experimental human-colonization model [29, 30]. In their studies of 12 adults successfully colonized with pneumococcal serotype 6B or 23F in an experimental setting, levels of serum IgG specific for PspA, but not PsaA, increased after colonization [30]. Antibody to homologous capsular polysaccharide was not described, probably because an earlier study used the same experimental colonization technique and was able to show an immune response to PspA but not to capsule in 6 subjects successfully colonized with a serotype 23F isolate [29]. The discrepant findings between the present study and previous studies are likely due to the very different study designs used and highlight the difficulty of extrapolating from experimental studies of selected strains in a relatively small number of carriers to the natural situation in vivo. The role that antiprotein responses, compared with anticapsular responses, play in the overall protection against the pneumococcus thus remains unclear.

The present study has improved our understanding of the development of natural immunity and the role that pneumococcal carriage plays. The use of vaccines that reduce NP carriage has resulted in dramatic indirect effects on disease in age groups outside of those targeted by the vaccine. We need to maintain a careful watch on the epidemiological factors of disease to understand the consequences of this reduced carriage on the maintenance of natural immunity.

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