Epidemiological Studies of *Streptococcus pneumoniae* in Infants: Development of Antibody to Phosphocholine

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The pneumococci possess, in addition to type-specific capsular polysaccharides, a number of antigens common to the species. Antibodies to phosphocholine (PC), a major determinant of the C-carbohydrate, have been shown to protect mice from experimental pneumococcal infection, but little is known of the role of anti-PC antibodies in humans or the extent to which anti-PC levels are affected by carriage or infection. We examined 115 sera from 30 infants, who were followed prospectively from birth through 4 years of age, for the presence of immunoglobulin M antibody to PC, using a solid-phase radioimmunoassay method. Infants were found to develop antibody to PC in response to pneumococcal carriage and infection, and nearly all infants developed some antibody. Antibody levels increased with age. By using a regression model including both age and nasopharyngeal carriage of pneumococci, anti-PC levels were found to be highest after exposure to two or three different types of pneumococci; levels were highest soon after acquisition of pneumococci and declined thereafter.

It has long been known that antibodies to pneumococcal polysaccharides are protective in mice and humans (13, 19), and more recent success with newer pneumococcal vaccines in preventing infection in adults is testimony to this fact (2). Infants are at high risk for pneumococcal infections (8), and young infants seldom make antibodies in response to pneumococcal carriage or immunization (10, 15). Pneumococci, however, possess other antigens, common to the species, which can elicit antibody protective against multiple pneumococcal serotypes (1, 7, 16, 17). One of these antigens is the phosphocholine (PC) determinant of pneumococcal C-carbohydrate, a cell wall component of pneumococcal strains of all capsular types (6, 18). In a previous study, we observed that naturally occurring antibodies binding PC are important in protecting mice from experimental infection with *Streptococcus pneumoniae* type 3 (4). The protective effects of anti-PC antibody had been unanticipated, since it was thought that much or all of the C-carbohydrate antigenic determinants would be masked by the thick type 3 capsule (2, 20). Upon further investigation, we found that capsular types 1, 3, 5, 6, and 19 had similar amounts of surface PC accessible to antibody, although quantities were much less than they were on an unencapsulated rough strain (21). We have also demonstrated that hybridoma anti-PC antibodies protect mice from experimental infection with types 1 and 3, and others have obtained

similar results with types 4 and 6A (J. B. Robins, personal communication). For unknown reasons, it has not been possible to protect mice from an exceptionally virulent type 5 strain. Types 3, 6, and 19 are among the most common types causing infections in children (8) and are frequently carried in the nasopharynx of healthy children (9, 12). In the study presented here, we have measured anti-PC immunoglobulin M (IgM) antibody in infants followed prospectively from birth to determine the relationship between the development of anti-PC antibody and known pneumococcal carriage and infection. Further study will be required to evaluate the possible protective role of anti-PC antibodies in humans.

MATERIALS AND METHODS

The patient population has been described in detail previously (9). Data are presented from 30 of the 82 infants in that prospective study. These 30 children were representative of the study population as a whole and were selected to show a complete range of exposure to pneumococci, from minimal to frequent nasopharyngeal carriage to overt infections. Ten infants without infection carried only one or two types, and 10 others also without infection carried three to six types. Of 10 infants who had pneumococcal infections documented by cultures of blood or middle ear fluid, 2 had bacteremia and all 10 had at least one episode of otitis media. All infants were cultured periodically for nasopharyngeal carriage of pneumococci, as previously described (9). Serum was obtained at the time of each culture, processed aseptically, and stored at 4°C until

used. Sera examined were from before and during carriage and before, at the onset of, and after infections. A total of 115 sera from the 30 children (two to eight samples per child) were tested.

The assay for human anti-PC IgM antibody is based upon that previously described for mice (4). The walls of vinyl microliter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight with 0.1 ml of PC (Sigma Chemical Co., St. Louis, Mo.) coupled to human serum albumin (PC-HSA; 1 μ g/ml in phosphate-buffered saline [PBS, 0.15 M, pH 7.4]). The wells were rinsed, filled with PBS containing 1% bovine serum albumin, and allowed to stand for 3 h at room temperature. A 1:30 dilution of each test serum in PBS with bovine serum albumin was added to a well and serially diluted for seven serial threefold dilutions in subsequent wells. Plates were incubated for 24 h at 4°C and rinsed with PBS. Goat anti-human IgM heavy chain (kindly provided by William Gathings and Max Cooper of our department) was labeled with 125 I by the chloramine T method and diluted in PBS with bovine serum albumin; 0.1 ml of the solution was added to each well and incubated overnight at 4°C. The wells were rinsed and cut from the plates, and the contents of the wells were counted in a gamma counter. To control for nonspecific antibody binding, each serum was also assayed in plates coated with HSA alone and in PC-HSA plates in the presence of 0.1% PC chloride added to the buffer. Counts in control HSA plates were subtracted from those in the PC-HSA test plates, and the final corrected anti-PC level was expressed relative to that of an adult control serum. The antibody content of this control serum was estimated by comparing it with mouse hybridoma anti-PC IgM antibody (22.1A4) obtained from L. Claflin, University of Michigan, Ann Arbor (21). Fixed volumes of control serum were assayed in the presence of serial dilutions of the hybridoma antibody to determine the percent inhibition of binding by the hybridoma antibody. The control serum was thus determined to contain the equivalent of 104 μ g of the mouse hybridoma IgM per ml. For comparison, sera from 28 normal adult volunteers (laboratory personnel and medical students) had a mean level of 76 μ g/ml (range, 0 to 283 μ g/ml; standard deviation = 72). We also assayed 25 sera from adults and prospective study infants for anti-PC IgG antibodies. IgG levels were about 25% of IgM levels and uniformly paralleled the IgM levels. We therefore elected to measure only IgM antibodies for the remainder of this study.

Each test result was examined with regard to the age of the patient and known pneumococcal carriage or infection. Simple correlations and regression plots of anti-PC levels versus age were done with the aid of Statistical Analysis System programs (SAS Institute, Inc., Cary, N.C.). To further explore the relationship between exposure to pneumococci and development of anti-PC antibody, a multiple regression model was constructed, using two parameters of exposure, the number of different pneumococcal types carried and the total duration of carriage of all types up to the age of testing. To facilitate the selection of a suitable model, a regression of the anti-PC level (in micrograms of antibody per milliliter $\div$ 100) on age, the number of types, and the total duration, along with various transformations of these variables, was done using the SAS STEPWISE MAXR procedure (Robert L. Good-

night, *SAS User's Guide*, 1979, SAS Institute). Variables that contributed little to the regression were eliminated, and a model was chosen which accounted for the most variation (in terms of R^2) while also having good probability (largest F values). The model used in this report is presented in Table 1 and will be discussed in further detail below. The equation for this model (given by the intercept and the regression coefficients of the variables in Table 1) was then used to generate predicted curves relating the anti-PC level to the number of types carried by age and the total duration of carriage by age, using the SAS General Linear Models program. In this model, no distinction was made between various degrees of exposure, such as carriage as opposed to infection. Similarly, the duration of carriage included all types, without distinction between sequential acquisition and carriage of different types.

RESULTS

Inspection of the data revealed many instances in which a rise in the anti-PC level of an infant was clearly related to pneumococcal carriage or infection. Unequivocal evidence of an antibody response was best seen in certain patients with well-documented infections. Data from four of these patients are shown in Fig. 1. Patient no. 1, a previous carrier of pneumococcal types 35 and 6, developed otitis media with type 23 at 10 months of age, followed by a sharp rise in the anti-PC level to 332 μ g/ml. Although the patient subsequently carried various serotypes of pneumococci, his anti-PC level declined to near zero by 2 years of age. In patient no. 2, a gradual increase in the anti-PC level to 52 μ g/ml occurred by 15 months of age, in the absence of proven pneumococcal carriage. She then had bacteremia, followed by otitis media with type 6, and the anti-PC level rose to 145 μ g/ml. This level slowly declined over ensuing months, although she continued to be a carrier. Patient no. 3, who had no antibody at 2 months of age, developed an anti-PC level of 125 μ g/ml by the age of 15 months, after otitis media with type 23. Anti-PC levels fluctuated over the next 2 years, during which time several different pneumococcal types were carried. Patient no. 4, had a low anti-PC level (31 μ g/ml) at the age of 9 months, just before the onset of bacteremia and otitis media with type 14. The level of antibody promptly rose to 1,887 μ g/ml. This patient then experienced a series of recurrent infections, all otitis media, with type 14 or 6 pneumococci. Her anti-PC level rose to 333 μ g/ml on two occasions in response to infections with these two distinct types. The level remained high at 30 months of age, after her last episode of acute otitis media.

The experiences of the six remaining infants with infections, not illustrated, were as follows: four had modest anti-PC levels (38 to 68 μ g/ml), after single bouts of otitis media due to type 6, 9,

FIG. 1. Development of anti-PC antibodies in response to pneumococcal infections in four infants. The anti-PC level (micrograms of antibody protein per milliliter) are shown by solid vertical bars, the type of infection are depicted by symbols ($\circ$, otitis media; $\diamond$, bacteremia), and nasopharyngeal carriage is indicated by type below the time scale for age (months).

or 14 at the ages of 11 to 16 months; one had no response to infection (otitis media, type 19) or to subsequent carriage of types 7 and 33; and one could not be evaluated because of the time lapse (14 months) between infection and the next available serum. Two of the four with modest responses subsequently developed high anti-PC levels in association with carriage (one with type 19, 187 $\mu\text{g/ml}$ at 20 months of age, and one with type 6, 239 $\mu\text{g/ml}$ at 36 months of age). In addition to these 10 infants with documented infections, 3 others developed anti-PC antibody after clinical illnesses which were probably due to pneumococci; of these 3, 2 had otitis media associated with recent acquisition of a pneumo-

coccus and anti-PC levels of 93 and 96 $\mu\text{g/ml}$, respectively, and the third infant, suspected of having sinusitis due to type 23, developed a level of 139 $\mu\text{g/ml}$.

We next examined the development of anti-PC antibody with respect to age. In all, 25 of the 30 infants developed some measurable anti-PC level during the first 48 months of life. Antibody was observed in infants as young as 4 months, and there was a definite increase in the level with advancing age (Fig. 2). The correlation (Pearson) was fair ($r = 0.382$) but accurate ($P < 0.0001$) and accounted for about 15% of the variation in level in the regression model described below ($R^2 = 0.146$; $F = 19.28$ and $P < 0.0001$, with $\nu_1 = 1$ and $\nu_2 = 113$). The regression line in Fig. 2 gives the predicted mean level for age, and we considered this to be a rough indicator of an anti-PC response. By defining a responder as an individual whose rise in anti-PC level was above the mean for age, we identified 15 of the 30 infants as responders, irrespective of their clinical history. At the time of their first response, five infants were less than 12 months of age, five were between 12 and 24 months of age, and five were 24 months or older. These 15 responders included 5 of those with documented infections and the 3 with probable pneumococcal infections. In addition, there were five infants in whom a response was associated with the acquisition of a new carriage type. The two remaining infants had levels of 447 and 260 $\mu\text{g/ml}$ at 31 and 44 months, respectively; neither had cultures positive for pneumococci for over a year, although carriage of short duration could have escaped detection. Because of the wide range of experience with regard to carriage and infection over time, we used data from all 30 infants to construct a multiple regression model, relating the anti-PC level to age and exposure to pneumococci. As shown in Table 1, the key variables in the model proved to be the $\ln$ age, the number of types carried, the square root of the number of types, and the $\ln$ duration of carriage. Taken alone, as noted in regard to Fig. 2, age was by far the most important variable; together with other variables in the model, age was just a bit more important than the parameters of exposure. The model as a whole accounted for nearly a quarter of the variation in the anti-PC level ($R^2 = 0.234$) and fit the data very well ($F = 8.42$; $P < 0.0001$). Using the model to plot the anti-PC level as a function of the duration of carriage (Fig. 3), we can see that levels were highest during the first few months of carriage and declined steadily thereafter. The magnitude of this effect increased with age, as shown by lines indicating the levels of theoretical infants of 12, 24, and 48 months of age. Although this model does not take into account

FIG. 2. Anti-PC (micrograms of antibody protein per milliliter) as a function of age.

the sequential carriage of different types, we can get an idea of the effect of exposure to multiple types by plotting the anti-PC level as a function of the total number of types carried before the time of testing. This is shown in Fig. 4 for infants aged 12, 24, and 48 months. The anti-PC level was low after the first type, peaked during carriage of the second or third type, and then declined.

Since repeated exposure to different pneumococcal types appeared to be an important factor in the development of anti-PC antibody, we next looked for any associations of level with particular types. The frequency of carriage of different pneumococcal types among the 30 study infants is given in Table 2. As we have previously reported for the study population as a whole (9), the four most common childhood pneumococcal types (types 6, 14, 19, and 23) accounted for about two-thirds of the total of 82 strains car-

ried. These types occurred frequently in carriage or infection, and 56 of 82 strains carried were associated with some detectable increase in the anti-PC level. The 15 infants who had antibody rises greater than the mean level for age had a total of 25 types possibly associated with a response, counting each type only once per patient. Type 6 appeared to be more frequent than other types in association with a rise in the anti-PC level, compared with its frequency of carriage in general.

DISCUSSION

The existence of anti-C-carbohydrate antibodies in human sera has been previously established (3), and PC is known to be a major determinant of this antigen (6, 11). Naturally occurring anti-PC antibodies are probably the result of immunostimulation with PC-containing

TABLE 1. Regression model for anti-PC level with respect to age and exposure to pneumococci^a

Parameter	Regression coefficient $\pm$ SE ^b	F value ^c	P value
Intercept	-4.056 ± 1.043		
ln age at testing	0.593 ± 0.154	14.86	0.0002
No. of types	-1.454 ± 0.410	12.57	0.0006
Square no. of types	4.878 ± 0.379	12.51	0.0006
ln duration of carriage	-0.339 ± 0.101	11.23	0.0011

^a The general form of the regression is $y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_k x_k + \epsilon$.

^b Units of the regression coefficients are the anti-PC level per unit of the variable. SE, of the estimate.

^c The F value, with $\nu_1 = 1$ and $\nu_2 = 110$, tests the hypothesis that the regression parameter equals zero, adjusted for all other variables in the model.

FIG. 3. Anti-PC (micrograms of antibody protein per milliliter) by duration of pneumococcal carriage for infants aged 12 (A), 24 (B), and 48 (C) months, drawn from the regression model described in Table 1 and in the text. Levels were highest early in carriage and subsequently declined.

FIG. 4. Anti-PC level (micrograms of antibody protein per milliliter) by total number of types carried up to the time of testing for infants 12 (A), 24 (B), and 48 (C) months of age, drawn from the regression model described in Table 1 and in the text. Highest levels were seen after acquisition of the second or third pneumococcal type.

antigens in the environment, including those found on the surface of fungi, nematodes, and bacteria other than pneumococci (5, 14). In this study, we observed several infants who developed high anti-PC levels in the absence of known pneumococcal carriage, although short periods of carriage could have been missed. However, the largest and most unequivocal responses were seen in a direct temporal relationship to documented pneumococcal infection. Only 4 of 30 infants studied failed to develop some antibody to PC. Between these infants with no antibody and those with an obvious rise in the antibody level, it remains difficult to set criteria for an antibody response. Since levels generally increased as infants grew older, it seemed reasonable to define a level greater than the expected mean for age as constituting a response. With this criterion, responses were identified in half of the 30 infants studied. The pneumococcal experience of the 15 responders revealed a history of carriage or infection corresponding to the rise in the antibody level in all but two infants, both of whom had high levels in the absence of known recent exposure.

The model predicted highest levels early in the course of carriage, declining steadily with time, despite continued exposure (Fig. 3). This effect was particularly evident in infants described in Fig. 1 and might have several explanations, including tolerance, negative feedback of anti-PC antibody, or perhaps antibody-mediated elimination of pneumococcal antigens. Carriage could not be quantitated in terms of inoculum size or antigenic load, and light, but detectable, carriage of small numbers of pneumococci is quite conceivable. Similarly, antibiotic therapy may greatly reduce numbers of organisms present early on in infections, such as otitis media,

thus aborting an antibody response. Low anti-PC levels may also simply reflect the time at which sera were obtained. The peak response may be missed by measuring sera obtained too early or too late. The organism itself may be a factor. Despite our finding that different types had similar amounts of PC determinants on their surface (21), some types or strains may differ in the configuration and immunogenicity of their determinants. Among the 15 responder infants, type 6 accounted for 11 of the 25 pneumococcal strains associated with a response (Table 2); this was well out of proportion to its frequency of carriage and to its frequency in infections (12%) in our population (8). It is interesting in this regard that type 6 capsular polysaccharide, along with other common types 14, 19, and 23, is a rather poor immunogen in infants and young children (15). The failure to detect anti-PC antibodies in some infants, especially those with carriage of multiple pneumococcal types (Fig. 4), could be the result of a late maturation of their ability to make anti-PC antibody. Such a lag could be part of a slow maturation of the ability of these infants to make antibody responses to carbohydrate antigens in general, as the ability to make antibody to most carbohydrates is known to develop in infants from 6 months to 2 years of age (15).

This paper has not attempted to address the question of whether anti-PC antibodies can protect humans from pneumococcal infections, as seems to be the case for mice. Rather, we have tried to describe some of the characteristics of

TABLE 2. Frequency of carriage of different pneumococcal types and their association with increased anti-PC levels

Type	Frequency of carriage		
	In 30 infants	Associated with any rise in level (26 infants)	Associated with rise in level > mean for age (15 infants)
	No. (%) ^a	No. (%) ^b	No. (%) ^b
6	22 (27)	16 (73)	11 (69) ^c
19	13 (13)	7 (92)	1 (8)
23	12 (15)	7 (58)	5 (41)
14	6 (7)	5 (83)	2 (33)
Others ^d	29 (35)	21 (72)	6 (21)

^a Percentage of carriage isolates from the 30 infants; 100% of the infants carried pneumococci

^b Percentage of infants who carried indicated type. Of the 26 infants, 86% carried pneumococci associated with a rise in level. Of the 15 infants, 30% carried pneumococci associated with a rise in level > mean for age.

^c Significantly more frequent than for all other types ($P > 0.025$ by the chi-square test).

^d In order of frequency from highest to lowest: 11, 10, 18, 3, 4, 7, 9, 15, 22, 33, 38, 8, 13, and 34.

anti-PC antibody responses and their relationship to pneumococcal carriage and infection and the age of infants. The observation that brisk rises in anti-PC levels occurred most often in infants with well-documented pneumococcal infection suggests that the potential value of the anti-PC assay as a serodiagnostic test should be further explored. The finding that infants under 2 years of age often show an anti-PC response may have implications for approaches to protection which are based on immunity to surface components other than the capsular polysaccharides.

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