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Evaluation of Antibody Responses to Pneumococcal Vaccines with ELISA and Opsonophagocytic Assay

Antibodies to a capsular polysaccharide (PS) provide protection against *Streptococcus pneumoniae* which express the homologous capsular serotype, and pneumococcal vaccines are designed to induce antibodies in the capsular PS. Levels and opsonophagocytic capacity of antibodies to the capsular PS of *S. pneumoniae* serotype 19F were determined by sera from adults immunized with 23-valent *S. pneumoniae* capsular PS vaccines. Geometric means of IgG anti-19F antibody level and specific opsonic titer rise significantly after immunization. The level of anticapsular PS antibodies for *S. pneumoniae* 19F serotype is fairly well correlated (r^2=0.63) with the opsonophagocytic activities of sera. However, 3.7% (1/27) of serum samples display strikingly less opsonophagocytic activity than expected on the basis of their antibody level. Thus, antibody level may be of general use in predicting vaccine-induced protection among adults for 19F serotype. However, the opsonic activity data suggest that antibody levels are not always indicative of functional antibody.

Key Words : *Streptococcus pneumoniae; Antibodies; Enzyme-linked immunosorbent assay; Opsonins; Phagocytosis*

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INTRODUCTION

Streptococcus pneumoniae is a gram-positive bacteria encapsulated with type-specific polysaccharide (PS) capsule and the leading cause of pneumonia, otitis media, bacteremia, and meningitis. Even when treated with antibiotics, however, pneumococcal infections persist as major causes of morbidity and mortality among children and adults worldwide (1). Furthermore, emergence of drug-resistant pneumococcal infections will severely challenge clinicians treating patients with pneumococcal disease (2). For the above reasons, interests in preventing disease caused by *S. pneumoniae* have been rising again and the development of an effective vaccine against *S. pneumoniae* would be quite useful.

Antibodies to a capsular PS provide protection against *S. pneumoniae*, which express the homologous capsular serotype, and pneumococcal vaccines are designed to induce antibodies in the capsular PS. A 23-valent pneumococcal vaccine is currently available and used in adults and high-risk children over two years of age. It is composed of purified, capsular PS antigens of 23 serotypes of *S. pneumoniae*. Unfortunately, the vaccine is poorly immunogenic in children younger than two years old (3-5). To enhance the immunogenicity in these populations, pneumococcal capsular PS have been coupled to carrier proteins to form conjugate vaccines as in the case of conjugate vaccines for *Haemophilus influenzae* type b (Hib) which are now part of routine infant vaccination in many countries. Conjugate vaccines to *S. pneumoniae* contain proteins conjugated to five to nine different types of *S. pneumoniae* polysaccharide, and are being tested for their immunologic properties and their clinical efficacy (6-9).

Knowledge of the antibody response to vaccine in human is essential for predicting the efficacy of a vaccine formulation. Ideally, protective immunity including antibody response should be predictable by appropriate immunologic assays. To increase the assessment of pneumococcal vaccine, the concentration of anti-capsular polysaccharide should be associated with vaccine efficacy, also making the assessment of pneumococcal vaccine simpler. Previous studies have suggested that antibody levels correlate with in vivo animal protection (10) and in vitro opsonic activity (11-13). But a recent epidemiologic study suggested that antibody levels measured by ELISA may not predict vaccine efficacy against homologous serotypes whereas a functional assay, based opsonic activity of antibody offered more useful information (14).

Therefore in this study serum antibody levels to pneu-

mococcal PS type 19F was measured quantitatively by ELISA and opsonophagocytic activity was measured qualitatively by opsonophagocytic assay to evaluate antibody responses to the pneumococcal PS vaccine. And the correlation between antibody levels in serum with opsonization capacity on serotypes 19F was determined to see whether ELISA positive antibody translates into functional antibody or not.

MATERIALS AND METHODS

Human sera

Twenty-seven healthy adult volunteers were immunized with a 23-valent pneumococcal vaccine (PNEUMO 23, Pasteur-Merieux Serums & Vaccines, France). Serum samples were collected before and a month after vaccination and were stored frozen at -20℃ until analysis.

ELISA

Sandwich type ELISA was used to determine levels of anti-type 19F pneumococcal PS (IgG) in sera (15). Wells of Immulon II microplates (Dynatech, Chantilly, VA, U.S.A.) were coated at 37℃ with 10 μg/mL 19F pneumococcal capsular PS overnight in phosphate-buffered saline (PBS). Serotype 19F pneumococcal capsular PS was purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). After being coated with the antigen, the plates were washed with PBS containing 0.1% Tween 20 and blocked with PBS containing 1% nonfat milk (Carnation, Los Angeles, CA, U.S.A.) for 30 min. A well-characterized serum pool (89-SF) from C. Frasch (Food and Drug Administration, Bethesda, MD, U.S.A.) was used as the standard in assays; 89-SF was assigned to contain 13 μg/mL 19F specific IgG antibody as published (16). All samples were preabsorbed with 3 μg of C-polysaccharide (C-PS; Statens Seruminstitut, Copenhagen, Denmark) per 20 μL of serum in a total volume of 1 mL of diluent for 30 min at room temperature (17, 18). The samples were then added to wells, titrated, and incubated for 2 hr. The wells in plates were washed four times and incubated with alkaline phosphatase-conjugated goat antibody against human IgG (Sigma, St. Louis, MO, U.S.A.). The amount of the enzyme immobilized to the well was determined with *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO, U.S.A.) in pH 9.8 diethanolamine buffer. Optical density at 405 nm was read with a microplate reader (Cambridge Technology, Watertown, MA, U.S.A.). The amount of antibody in the sample was determined by comparing the optical density of the samples with that of the standard curve generated with reference serum 89-SF. Detection limit for anti-19F IgG assay was 0.055 μg/mL.

Opsonophagocytosis assay

S. pneumoniae 19F (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in Todd-Hewett broth with 0.1% yeast extract and kept frozen in aliquots in Hanks' buffer with 15% glycerol. HL-60 cells (a human promyelocytic cell line from ATCC) were differentiated in a medium containing 0.8% dimethyl formamide for five days (13, 19) and used for this assay.

Opsonophagocytic activities of the samples were determined using the method of Gray (12) with minor modifications. Ten μL of bacterial suspension (~2,000 cfu) was incubated with 40 μL of serial 1:3 dilutions of heat-inactivated test sera (56℃ for 30 min) for 30 min at room temperature. The bacteria was then incubated with 40 μL of phagocytic cell (HL-60 cell line, differentiated to a granulocyte line using dimethyl formamide) suspension (~1,000,000 cells) and 10 μL of baby rabbit complement (Accurate Chemical, Westbury, NY, U.S.A.) for 1 hr at 37℃ with shaking. An aliquot (10 μL) of the reaction mixture was applied to a blood agar plate, which was incubated overnight at 37℃ in 5% CO_2, and the number of surviving colonies of bacteria was determined. Opsonization titer of a serum was determined as the reciprocal of the dilution of the serum that results in half as many viable bacteria were seen without antisera. Detection limit for opsonophagocytic assay was 1 opsonization titer.

Statistics

Two-tailed Student's t test and Pearson's correlation test were used to determine the statistical significance and a 95% confidence interval was determined using a computer statistics package, Version 7.5 SPSS.

RESULTS

19F-specific IgG antibody

Serum samples from 27 adults were analyzed for their 19F-specific IgG antibody levels (as shown in concentration) using 89-SF as the standard. IgG antibody levels of pre- and postimmune sera are summarized in Table 1. Although before immunization, 13 sera (48.2%) showed the mean antibody levels to 19F serotype higher than 1.9 μg/mL, the estimated 'protective level in humans' (20), after immunization, 26 sera (96.3%) showed anti-

Table 1. 19F specific IgG antibody (μg/mL) in 27 adults given one dose of 23-valent pneumococcal PS vaccine

	Antibody concentration (μg/mL)		
	Range	Mean	Standard deviation
Preimmunization sera	0.20-10.20	1.63*	3.23
Postimmunization sera	2.10-52.00	10.88*	2.78
Fold increase	1.90-64.00	19.02	-

*Geometric mean

Table 2. Opsonic titer of anti-19F antibody in 27 adults given one dose of 23-valent pneumococcal PS vaccine

	Opsonic titer		
	Range	Mean	Standard deviation
Preimmunization sera	0.50-121.40	3.59*	4.80
Postimmunization sera	1.00-285.30	28.62*	4.80
Fold increase	1.00-90.20	11.12	-

*Geometric mean

body levels higher than 1.9 μg/mL. Geometric mean (GM) preimmunization titer for 19F specific IgG antibodies was 1.63±3.23 μg/mL (range 0.20-10.20 μg/mL) and the GM titer following immunization was 10.88±2.78 μg/mL (range 2.10-52.00 μg/mL). After immunization, GM of 19F specific IgG antibodies rose significantly (p<0.01). Mean fold rises in 19F specific IgG antibodies were 19.02 (range 1.90-64.00). All subjects achieved at least a 2-fold increase in antibody levels. In 10 among 27 postimmune sera, there were more than 10-fold differences in fold increases of antibody levels.

Opsonophagocytic assay

To investigate the effectiveness of 19F specific IgG antibodies, the opsonic capacity of 27 sera from persons immunized with the 23-valent vaccine was determined. Similar to results by ELISA, a significant rise in GM opsonic titers after immunization was seen. The specific opsonic activity of serum appeared to increase after immunization. GM specific opsonic titer of serum increased significantly from 3.59±4.80 (range 0.50-121.40) in preimmunization to 28.62±4.80 (range 1.00-285.30) at one month after immunization (p<0.01). Mean fold rises in opsonic titer of IgG anti-19F PS antibodies were 11.12 (range 1.00-90.20) (Table 2). Among 27 subjects, 22 showed increase in opsonophagocytic titer and 5 showed no change.

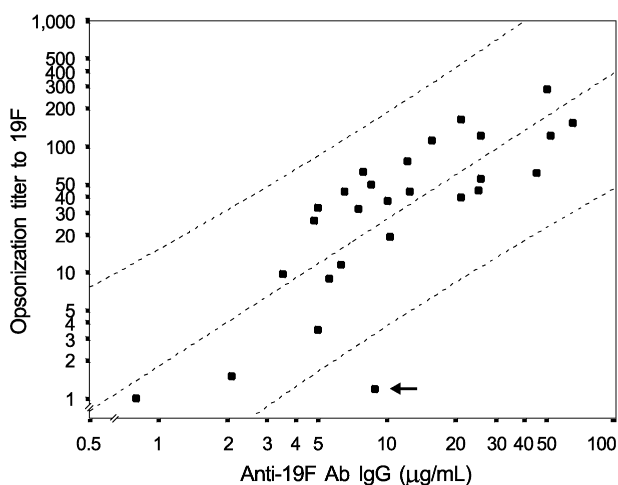

Fig. 1. Opsonization titer (y axis) vs. antibody concentration (x axis) for serotype 19F. The opsonic capacity against the 19F serotype was correlated with IgG anti-19F antibody concentrations (r^2=0.63) except for one sample (arrow). A 95% confidence interval for individual data points (dashed lines) was obtained with computer statistics package (Version 7.5; SPSS). Detection limits are indicated with wavy lines on axes.

Correlation between antibody levels and opsonophagocytic titer

When the relationship between opsonic activity and antibody levels for 27 postimmunization samples was examined, the opsonic capacity against the 19F serotype was fairly well correlated with 19F specific IgG antibody concentration (r^2=0.63) (p<0.01) except for one sample (arrow) (Fig. 1). This outlying sample showed reproducibly less opsonic capacity than expected on the basis of its antibody concentration.

DISCUSSION

This study confirms a correlation between antibody levels and opsonic activity for the *S. pneumoniae* 19F serotype included in the currently used 23-valent PS vaccine. Therefore antibody levels may be of general use in predicting vaccine-induced protection among adults for 19F serotype. However, the opsonic activity data of one outlier sample clearly demonstrate that antibody levels are not always indicative of specific functional antibody.

Because human sera contain relatively high levels of antibodies to cell wall PS (18) and commercial pneumococcal vaccines and reagents in the assays are known to contain varying and significant amounts of cell wall PS (17), anti-cell wall PS could be measured as anti-capsular PS antibodies (18, 21-23). By neutralizing antibody to cell wall PS, more accurate estimates for antibody to capsular PS can be obtained (15, 24). This study avoided

measuring anti-cell wall PS antibody, and measure IgG by ELISA.

Precise serologic measurement of pneumococcal antibody responses is critical in immunized children to determine the antibody response to vaccination. In ELISA, the binding functions of antibody for PS antigens are measured. However, it has been shown that antibody to some pneumococcal strains cross-reacts with other bacteria such as certain strains of *Escherichia coli* and *Klebsiella* species (25). Since cross-reactive antibodies may have little functional capacities (24, 26), these antibodies found in young children may not be effective. Therefore, it may be important to verify the biologic and protective functions of antibody by "functional" assays. The opsonophagocytic assay, which measures bacterial uptake and typical killing by human neutrophils in the presence of antibody and complement, is the only method that can be used for functional assays in pneumococci in vitro (20). The correlation between antibody levels in serum and opsonization capacity on serotypes 19F was determined to see whether ELISA positive antibody translates into specifically functional antibody or not.

Because the protective efficacy of pneumococcal vaccine against invasive disease entails production of serum antibodies that support opsonophagocytosis and killing of *S. pneumoniae*, the ability of sera to support opsonization of type 19F *S. pneumoniae* in vitro following immunization was determined. Similar to results by ELISA, a significant rise in mean opsonic titers after immunization was seen. The specific opsonic activity of serum appeared to increase after immunization.

The observed correlation in this study was slightly lower than those reported by others (11, 27), perhaps because the sera was examined without removing IgA and IgM antibodies, which have different opsonic capacities. However, the correlation was still high, and antibody levels may be of general use in predicting vaccine-induced protection among adults for 19F serotype. The opsonic activity data showed in one outlier sample, however, suggest that antibody levels are not always indicative of functional antibody.

In our study, the poor opsonic function may be due to their expression of different IgG subclasses or presence of IgA or IgM, since the IgA, IgM or IgG subclass compositions of the samples were not investigated. Ineffective antisera or antibodies have been reported previously (28-31). The ineffectiveness of certain antibacterial antibodies has been thought to be due to the presence of IgA and IgM which have little opsonic activity (26, 29), expression of different IgG subclass or antibody V regions (28, 30), the use of antibody C region defective in opsonization, inhibition of opsonization by an excess amount of IgA anti-capsular PS antibody (29), or alteration of the capsular PS by a nonimmunoglobulin molecule in sera (e.g., an enzyme) (31). Nevertheless, the most likely reason for the poor opsonic function of antibody is suggested to be due to the V region that binds the epitopes that are infrequent, inaccessible for the granulocytes, and with low avidity (27).

This study suggests that the concentration of antibody for 19F serotype may predict its in vivo protection value of a vaccine although antibody levels are not always indicative of functional antibody. The causes for the ineffectiveness of this outlying antibody should be elucidated. Evaluation of antibody responses to other pneumococcal serotypes will be necessary whether same assumption can be applied or not.

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