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## Correlation of Opsonophagocytosis and Passive Protection Assays Using Human Anticapsular Antibodies in an Infant Mouse Model of Bacteremia for *Streptococcus pneumoniae*

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An infant mouse assay system for assessment of protective concentrations of human serum pneumococcal anticapsular antibodies is described. Passive immunization of anticapsular antibodies was evaluated for protection of infant mice challenged with *Streptococcus pneumoniae* serotypes 1, 4, 5, 6B, 18C, and 23A, with bacteremia as an end point. Protection was defined as no detectable bacteremia in 70% of mice 48 h after challenge. Type-specific anticapsular concentrations required for protection varied with serotype ( $\leq 0.05$  to  $>0.4$   $\mu\text{g/mL}$ ). Across serotypes, there was no significant correlation between human IgG concentration in mouse serum and protection from bacteremia or between IgG concentration and opsonophagocytic titer. Significant correlation ( $r = .84$ ,  $P < .001$ ) was observed between opsonophagocytic titer of human IgG antibody in mouse sera and protection from bacteremia. Thus, protective concentrations of anticapsular antibodies against bacteremia are serotype dependent. Opsonophagocytosis is a better predictor of in vivo protective capacity of pneumococcal anticapsular antibodies than are ELISA IgG antibody concentrations.

Infections caused by *Streptococcus pneumoniae* (pneumococcus) are a major cause of morbidity and mortality among children and adults worldwide. Pneumococcus is the leading cause of bacterial pneumonia and meningitis, accounting for >450,000 acute pneumonia hospitalizations and high rates of mortality in the United States each year [1–3]. In addition, pneumococcus is the leading cause of otitis media, the most common infection in children for which antibiotics are prescribed. In the developed world, pediatric serotypes commonly isolated are 4, 6B, 9V, 14, 18C, 19F, and 23F; for developing countries, in addition to these serotypes, others, such as 1, 5, and 7, are commonly isolated [1–3]. Serotype 23A is frequently isolated in Taiwan and Japan in both children and adults [4]

and has been shown, along with serotypes 3, 6, 14, 19, 23F, and 45, to have increased resistance to  $\beta$ -lactam antibiotics and azithromycin [5, 6].

Pneumococcal polysaccharide–protein conjugate vaccines are being developed for immunization of infants. Postvaccination antibody concentrations have been assayed by a recently validated and standardized ELISA that measures serotype-specific IgG (ss-IgG) [7]. Postvaccination rises in antibody concentration have been demonstrated; however, antibody levels that correlate with protection have not been determined. Furthermore, the protective antibody concentration may vary with serotype. Thus, it is difficult to estimate the likelihood of efficacy of individual polysaccharide–protein conjugates contained in candidate vaccines. The ELISA measures total binding IgG antibodies and does not distinguish between functional and nonfunctional antibodies. Therefore, the serum antibody concentration measured by this assay may vary with serotype and may or may not correlate with in vivo protection against invasive pneumococcal infection. Other in vitro or in vivo assays are being sought for evaluation of functional antibody activity. Recently, an in vitro opsonophagocytic assay that uses cultured phagocytic cells was described [8]. This assay seems to correlate with point estimates of vaccine efficacy and may be a better indicator of immune responses to vaccination than ELISA IgG determinations [9].

In this study, we evaluate the protective capacity of human serum anticapsular antibodies against pneumococcal bacteremia and fatal pneumococcal infection when passively administered to infant mice (suckling, 13–15 days old), using serotypes

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Informed consent was obtained from all subjects. The protocols conformed to the established guidelines for human experimentation of the US Department of Health and Human Services.

Studies were conducted after review and approval by the CDC Animal Care and Use Committee in accordance with Public Health Policy on Humane Care and Use of Laboratory Animals, 1986.

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1, 4, 5, 6B, 18C, and 23A, which were virulent in this model. We also describe an in vivo assay system in infant mice, which was used to estimate the minimum concentration of pneumococcal anticapsular antibody in human serum that provides protection. Finally, we examined our in vivo protection data for correlation with ELISA-measured ss-IgG antibody concentration and with opsonophagocytic (functional) antibody activity.

## Materials and Methods

**Human immune serum.** Serum samples were obtained from adults 6–8 weeks after they were vaccinated with a licensed 23-valent polysaccharide pneumococcal vaccine (Pneumovax 23; Merck Sharp & Dohme, West Point, PA). The samples, which were stored at  $-70^{\circ}\text{C}$ , were assayed for pneumococcal ss-IgG concentration by ELISA (described below) and were used in experiments to passively protect infant mice from challenge with 6 serotypes of *S. pneumoniae*. Serum samples used were Centers for Disease Control and Prevention (CDC, Atlanta) number 95016998 (individual serum), Schneider Children's Hospital (SCH) number 01001 (individual serum), 89SF (pooled sera supplied by Carl Frasch, Food and Drug Administration, Rockville, MD) [7], and BPIG lot 0490 (the hyperimmune globulin fraction of pooled serum specimens from adult humans immunized with 23-valent pneumococcal vaccine, 4-valent meningococcal vaccine, and *Haemophilus influenzae* type b vaccine; Massachusetts Biologics Laboratory, Jamaica Plain, MA).

**Animals.** Infant Swiss Webster mice (ND-4; suckling, 13–15 days old; 6–8 g) were obtained from Harlan Sprague Dawley (Indianapolis) or Taconic Farms (Germantown, NY). On arrival, infant mice were separated from their dams, weighed, and randomly redistributed among the dams in groups of 5 or 10 per dam. All animals were housed under standard conditions ( $25^{\circ}\text{C}$ , relative humidity  $\sim 40\%$ ), with food and water available ad libitum.

**Bacterial isolates and growth conditions.** Eleven isolates of *S. pneumoniae*, representing serotypes 1, 4, 5, 6B, 18C, and 23A, were supplied by various investigators (table 1). Passage of pneumococci was done in animals by intraperitoneal (ip) injection of mice; bacteria were recovered from blood culture and stored at  $-70^{\circ}\text{C}$  for future use. In brief, each isolate was passed four times through infant mice, with a single blood agar (BA; Trypticase soy agar supplemented with 5% defibrinated sheep blood; Becton Dickinson Microbiology Systems, Cockeysville, MD) plating between passages. Mice were challenged ip with  $10^7$  cfu, and 24 h later mouse blood was cultured for pneumococci isolation for the next passage. Optochin sensitivity and the Quellung test [10, 11] were conducted on each isolate for pneumococcal identification and typing.

To ensure that multiple experiments could be initiated from the same lot of cells, a standardized stock culture of each pneumococcal isolate was prepared. From a 15-h culture on BA, 5–10 colonies of the same morphology were transferred to 10 mL of brain heart infusion broth (BHI; Becton Dickinson) supplemented with 10% Levinthal's basal medium (BHI-10L; Becton Dickinson) and incubated for 18 h at  $37^{\circ}\text{C}$  in ambient air. The culture was diluted 10% in fresh BHI-10L and incubated until an optical density (at 492 nm [OD<sub>492</sub>]) of 0.9 (model B spectrophotometer; Beckman In-

**Table 1.** Serotype, virulence (bacteremic dose 100%) in infant mice and source of *Streptococcus pneumoniae*.

Serotype	Isolate	BD <sub>100</sub> <sup>a</sup> (cfu)	Isolation site	Source <sup>b</sup>
1	DS183-95	2	Blood	1
1	WRU294	$\leq 100$	Blood	2
1	2639207	$\leq 100$	Blood	2
4	DS2341-94	800	Cerebrospinal fluid	1
4	40113	$\leq 100$	Middle ear	2
5	SP84	10	Blood	3
6B	DS1756-94	5 <sup>c</sup>	Blood	1
6B	2759407	$\leq 100$	Cerebrospinal fluid	2
6B	20653	$\leq 100$	Blood	2
18C	DS07-95	200	Blood	1
23A	60156	$\sim 200$	Middle ear	2

<sup>a</sup> Lowest no. of colony forming units (cfu)/mouse that produces bacteremia in 100% of infant mice within 48 h after inoculation. Sensitivity of blood culture was detection of 10 cfu/mL (0.1 mL of blood cultured). Data represent results of duplicate experiments for each isolate.

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<sup>c</sup> Four mouse passages were required to achieve virulence.

struments, Fullerton, CA) was reached. This corresponds to a late logarithmic phase obtained in 5–6 h. The broth culture was aliquoted into 0.5-mL amounts and stored at  $-70^{\circ}\text{C}$  after being flash frozen in a bath of 95% ethanol in dry ice. Glycerol was added to some isolates at a final concentration of 10% prior to freezing.

On initiation of an experiment, thawed cells were subcultured on BA at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> atmosphere or in ambient air. Sufficient colonies from a 15-h culture were transferred to 5 mL of BHI-10L to give an OD<sub>492</sub> of 0.1. Cultures were incubated 3–5 h at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> atmosphere to mid-logarithmic phase (OD<sub>492</sub> = 0.40–0.60). Density was consistently determined to be 1 to  $3 \times 10^8$  cfu/mL. Cells were centrifuged at 15,600 g (Eppendorf Centrifuge 5414C; Brinkmann Instruments, Westbury, NY) for 1.5 min and resuspended in an equal volume of sterile, 0.01 M PBS, pH 7.2 (Life Technologies, Grand Island, NY). Cells were serially diluted 10-fold in PBS for animal inoculation.

**Bacteremic dose 100% (BD<sub>100</sub>).** The BD<sub>100</sub> was defined as the lowest inoculum that produced bacteremia in 100% of mice 48 h after challenge. Groups of 10 mice were challenged with doses varying 10-fold, from 10<sup>0</sup> to 10<sup>7</sup> cfu. Each mouse was inoculated ip with 0.1 mL of the appropriate dilution. For diluent control, 10 mice were injected with sterile PBS. Inocula were administered with a 1.0-mL tuberculin syringe fitted with a 27-gauge needle (Monoject; Sherwood Medical, St. Louis). Numbers of bacteria for challenge were estimated by OD<sub>492</sub> and verified by viability counts on BA. Forty-eight hours after challenge, 0.1 mL of blood was obtained by tail vein puncture and plated on BA. An animal was considered bacteremic if  $\geq 1$  cfu were isolated (lower limit of detection, 10 cfu/mL). BD<sub>100</sub> experiments were done in duplicate.

**Passive protection and challenge.** In each trial, 5–20 mice were challenged. Mice were passively immunized with human serum at a dose calculated to achieve a final mouse serum antibody concentration of 0.05, 0.1, 0.2, 0.3, or 0.4  $\mu\text{g}/\text{mL}$  of ss-IgG as determined by ELISA. Mice were challenged 24 h later with the appropriate pneumococcal dose. Mouse average blood volume ( $\sim 1$  mL) was empirically determined to be  $\sim 14\%$  of body weight. Pla-

cebo control mice were given 5% human serum albumin (American Red Cross Blood, lot 0.270.084, 25%; supplied by Carl Frasch). All doses were delivered subcutaneously between the shoulders in 0.1-mL volumes with the syringe-needle combination previously described. Because the concentration of ss-IgG varied for each serotype, the dilution of human serum for each experiment varied such that, when injected into the mouse, the appropriate ss-IgG concentration was achieved. To verify the experimental ss-IgG dose and functional activity in most experiments, we euthanatized and collected blood from 3 mice from each dose group 24 h after immunization; the serum samples were then analyzed by ELISA and opsonophagocytic assay (described below).

The remaining mice were challenged ip, at 0.1 mL/mouse, with PBS containing the appropriate serotype and numbers of pneumococci ( $10^0$ – $10^3$  cfu). Challenge dose verification and bacteremia screening were conducted as described above. In addition, survival was recorded 7 days after challenge. The preferred end point for this model was bacteremia. Protection was defined as no detectable bacteremia (limit of detection, 10 cfu/mL) in 70% of mice 48 h after challenge with pneumococci. Passive protection experiments were run in duplicate.

**ELISA.** The ss-IgG antibody concentrations in human and mouse serum for the *S. pneumoniae* serotypes tested were measured by a modified ELISA [7]. The ss-IgG concentration in serum was calculated by measuring the absorbance at a wavelength of 490 nm ( $OD_{490}$ ) against a standard curve. The standard reference serum used on each plate was serum 89SF [7]. Since 89SF has no assignments for serotype 23A, the concentration measured against serotype 23F was used instead for animal dose concentrations. Data were analyzed by use of a 4-parameter logistic log curve fitting technique [12].

**Opsonophagocytosis assay.** Functional ss-IgG antibody titers for selected human and mouse serum samples were determined by use of a standardized opsonophagocytic assay that uses HL-60 granulocytes as effector cells [8]. Minor modifications were necessary to measure human functional antibodies in mouse serum. In brief, mouse serum samples were serially diluted (2-fold) in opsonophagocytic buffer composed of Hanks' buffer with  $Ca^{++}$  and  $Mg^{++}$  (Life Technologies) and supplemented with 1% gelatin and 0.1% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), with a starting dilution of 1:2. Final well volumes were maintained at 80  $\mu$ L and, thus, required a high volume (80  $\mu$ L/well) of serum in the initial duplicate wells. Because of this, serum samples from mice ( $n = 3$ ) within the same antibody-dose group were pooled to attain sufficient assay volume.

Viability counts were performed as previously described [8]. Opsonophagocytic titers were the reciprocal of the dilution with  $\geq 50\%$  killing, compared with control wells containing only bacteria, HL-60 cells, and complement (sterile baby rabbit [age, 3–4 weeks] serum; Pel-Freez, Brown Deer, WI). All plates included a quality control IgG preparation (Sandoglobulin at 6%; Sandoz, East Hanover, NJ) diluted in normal infant mouse serum. All samples with different concentrations of immune serum against a particular serotype were tested in the same microtiter plate.

**Statistical methods.** The  $BD_{100}$  was calculated by use of maximum likelihood techniques, which result in point estimates, standard errors, and associated confidence intervals. Since probit analysis [13] is well suited to the type of dose-response relationships

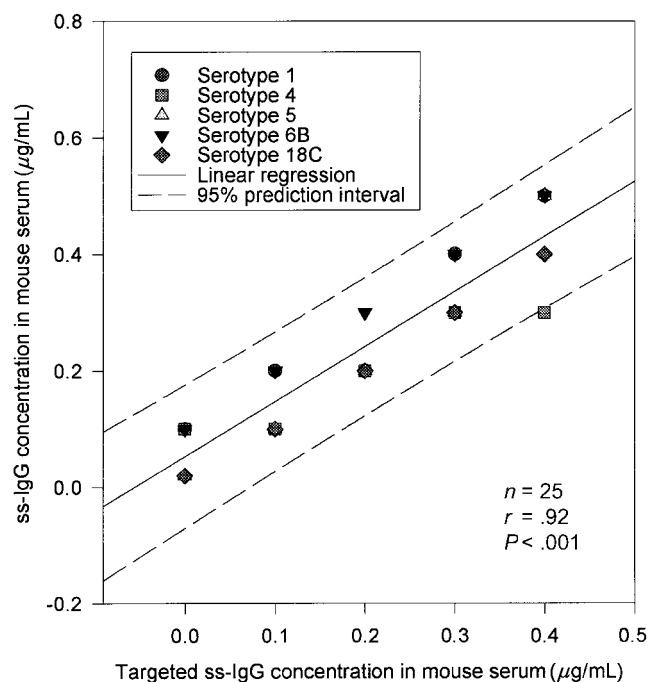
that were generated by the above animal challenge techniques, it was used with SAS software (CDC) to obtain these statistics. A combined  $BD_{100}$  for each experiment was generated by use of a weighted average with empirical weights (i.e., weights that are inversely proportional to the variances of the individual  $BD_{100}$  point estimates).

Because of serum volume limitations and the lack of apparent difference in the  $BD_{100}$  ( $< 2 \log_{10}$ ) among various isolates within a particular serotype, the results for a given antibody concentration obtained from all trials within a single serotype were pooled. This allowed us to increase the sample size per serotype and calculate a more accurate and statistically meaningful estimate of the minimum level of protection in our infant mouse model.

Linear correlations between groups were analyzed by use of Pearson's product moment correlation coefficient. Significant differences between paired data sets in  $2 \times 2$  tables were determined by use of Fisher's exact test (two-tailed). A significant level was set at  $P < .05$  for both tests. The opsonophagocytic titers and ELISA ss-IgG antibody concentrations ( $\mu$ g/mL) were converted to a  $\log_2$  base for statistical analysis. Statistical calculations were performed by use of computer software (SigmaStat, version 2.0; Jandel Scientific, San Rafael, CA, and Epi-Info, version 6.02; CDC).

## Results

**Serotype virulence.** To examine the virulence of various *S. pneumoniae* serotypes in infant mice, we challenged the mice



**Figure 1.** Correlation of measured vs. targeted pneumococcal serotype-specific human IgG anticapsular antibody (ss-IgG) in mice. Each data point represents average ss-IgG concentration of 6 mice; 30 mice were tested per serotype, and each serotype was tested at 5 dose levels. Linear correlation was analyzed by use of Pearson's product moment correlation coefficient, with level of significance at  $P < .05$ .

by ip injection with 10-fold dilutions of 11 isolates from 6 serotypes (table 1). These pneumococcal isolates were virulent, with  $BD_{100}$  levels  $\leq 800$  cfu (table 1).

**Passive protection.** The 11 virulent isolates described above were used to study the protective activity of passively administered human antibodies. All the isolates tested were of the opaque phenotype (determined by the methods of Weiser et al. [14]); therefore, no phenotype variability was observed among isolates within a particular serotype (data not shown). For each serotype studied,  $\leq 3$  human immune serum samples were tested for passive protection. The ss-IgG concentration in mouse serum specimens reached a maximum within 24 h after immunization and remained constant for at least 72 h, as demonstrated by ELISA (data not shown). A significant correlation ( $r = .92$ ,  $P < .001$ ) was demonstrated between the predicted and the actual mouse serum concentration (figure 1). This correlation was based on the quantity of ss-IgG injected into infant mice and the serum antibody quantity measured by ELISA 48 h after injection. All ss-IgG mouse serum levels were within the 95% prediction interval (figure 1).

Forty-eight hours after ip challenge, there was a significant ( $P < .05$ ) increase in the number of nonbacteremic mice (all serotypes tested), and at day 7 there was a significant increase in survival of mice receiving human immune serum, compared with mice receiving human serum albumin placebo (table 2). There was a statistically significant correlation ( $r = .95$ ,  $P < .001$ ) between survival and nonbacteremia in mice receiving ss-IgG, indicating that bacteremia at 48 h was a good predictor of mortality (table 2). The ss-IgG concentration in mouse serum required to protect against bacteremia varied with serotype, from  $0.05 \mu\text{g/mL}$  for serotype 4 to  $>0.4$  for serotypes 1 and 5 (table 2). Of the 6 serotypes tested, protective levels ( $\geq 70\%$  nonbacteremia) of ss-IgG in infant mouse serum were reached for 4:  $0.05 \mu\text{g/mL}$  for serotype 4,  $0.1 \mu\text{g/mL}$  for serotype 6B, and  $0.2 \mu\text{g/mL}$  for serotypes 18C and 23A (table 2). In addition, for all groups in which protection from bacteremia at 48 h was achieved, the survival rate at 7 days was  $\geq 72\%$  (table 2).

For 3 of the serotypes tested (serotypes 1, 4, and 6B), more than one antibody source was tested for protective activity, allowing us to compare the protective activities of similar con-

**Table 2.** Passive protection of human anticapsular antibodies to *S. pneumoniae* in infant mice.

Serotype (n)	Inoculum range (cfu)	No. of serum samples	No. of trials	Antibody dose ( $\mu\text{g/mL}$ )	Nonbacteremia at 48 h no./total (%)	Survival at 7 days no./total (%)
1 (3)	20–1500	3	5	0.00 <sup>a</sup>	1/56 (2)	6/56 (11)
				0.05	0/14 (0)	0/14 (0)
				0.1	2/44 (5)	8/44 (18)
				0.2	2/44 (5)	10/44 (33)
				0.3	2/41 (5)	10/41 (24)
				0.4	4/20 (20) <sup>b</sup>	6/20 (30)
4 (2)	25–1000	3	5	0.00	3/70 (4)	3/70 (4)
				0.05	11/15 (73) <sup>b</sup>	11/15 (73) <sup>b</sup>
				0.1	37/50 (74) <sup>b</sup>	36/50 (72) <sup>b</sup>
				0.2	34/35 (97) <sup>b</sup>	33/35 (94) <sup>b</sup>
				0.3	19/20 (95) <sup>b</sup>	19/20 (95) <sup>b</sup>
				0.4	20/20 (100) <sup>b</sup>	20/20 (100) <sup>b</sup>
5 (1)	25	1	2	0.00	0/20 (0)	0/20 (0)
				0.1	3/20 (15)	2/20 (10)
				0.2	1/20 (5)	1/20 (5)
				0.3	1/20 (5)	1/20 (5)
				0.4	7/20 (35) <sup>b</sup>	8/20 (40) <sup>b</sup>
6B (3)	25–300	3	9	0.00	7/125 (6)	10/116 (9)
				0.05	29/62 (47) <sup>b</sup>	37/55 (67) <sup>b</sup>
				0.1	60/84 (71) <sup>b</sup>	56/75 (75) <sup>b</sup>
				0.2	35/49 (71) <sup>b</sup>	40/49 (82) <sup>b</sup>
				0.3	7/20 (35) <sup>b</sup>	14/20 (70) <sup>b</sup>
				0.4	15/20 (75) <sup>b</sup>	19/20 (95) <sup>b</sup>
18C (1)	250	1	2	0.00	0/20 (0)	0/20 (0)
				0.1	12/20 (60) <sup>b</sup>	14/20 (70) <sup>b</sup>
				0.2	19/20 (95) <sup>b</sup>	18/20 (90) <sup>b</sup>
				0.3	18/20 (90) <sup>b</sup>	19/20 (95) <sup>b</sup>
				0.4	17/20 (85) <sup>b</sup>	16/20 (80) <sup>b</sup>
23A (1)	200	1	2	0.00	0/11 (0)	3/11 (27)
				0.05	2/11 (18)	5/11 (45)
				0.1	1/11 (9)	4/11 (36)
				0.2	8/11 (73) <sup>b</sup>	11/11 (100) <sup>b</sup>

NOTE. There was statistically significant correlation ( $r = .95$ ,  $P < .001$ ) between survival and nonbacteremia in mice receiving serotype-specific IgG, indicating that bacteremia at 48 h was good predictor of mortality.

<sup>a</sup> Control mice injected with human serum albumin in absence of antibody.

<sup>b</sup> Significant difference,  $P < .05$ , compared with control mice. Fisher's exact test (two-tailed) was used for calculation of significant difference between groups.

**Table 3.** Comparison of the protective activity of different sources of anticapsular antibodies against bacteremia after challenge with serotype 6B isolate 20653.

ss-IgG level 48 h after immunization <sup>a</sup> ( $\mu\text{g}/\text{mL}$ )	Absence of bacteremia 48 h after challenge		<i>P</i> <sup>b</sup>
	Serum 89SF no./total (%)	Serum SCH no. 01001 no./total (%)	
0.0 <sup>c</sup>	2/37 (5.4)	5/55 (9.1)	0.7
0.05	6/16 (38)	19/35 (54)	0.4
0.1	23/29 (79)	24/24 (100)	0.03
0.2	17/17 (100)	9/9 (100)	NA

NOTE. ss-IgG, serotype-specific IgG; SCH, Schneider Children's Hospital; NA, not applicable.

<sup>a</sup> Mouse serum concentration at pneumococcal challenge.

<sup>b</sup> Level of significance ( $P < .05$ ) by Fischer's two-tailed exact test.

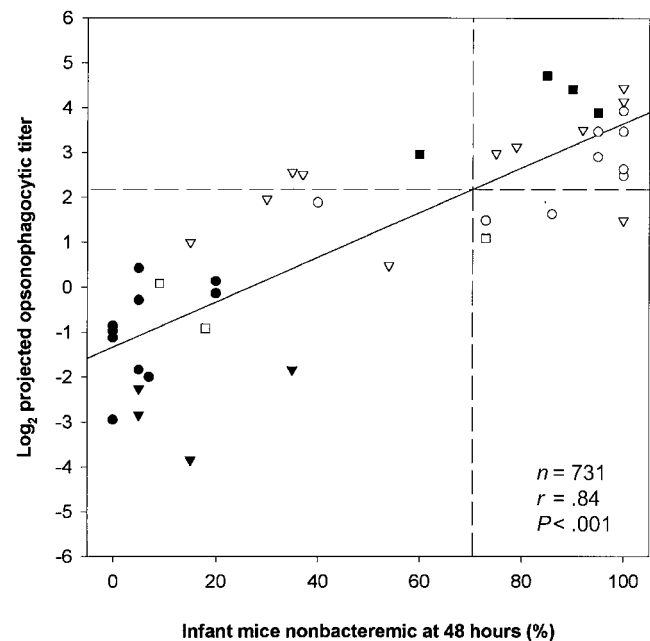
<sup>c</sup> Control mice injected with human serum albumin in absence of antibody.

centrations of antibodies against a particular serotype. For serotype 1, neither the CDC serum (no. 95016998, single donor) nor the BPIG antibodies (multiple donors) showed protective activity at a serum concentration of 0.3  $\mu\text{g}/\text{mL}$ . When single donor serum (SCH no. 01001) was compared with pooled serum (89SF) against a single 6B isolate (20653), the single donor serum had somewhat greater protective activity than 89SF (table 3). However, as seen in table 3, the protective level was between 0.05 and 0.1  $\mu\text{g}/\text{mL}$  for each antibody source. Similarly, these two antibody sources showed equivalent activity against serotype 4 isolate 40113 at equivalent antibody levels (data not shown). We did not measure antibody avidity for these sera; however, our data for a given serotype demonstrated a general similarity ( $\leq 2$  dilutions) of opsonophagocytic titers among the sera tested (data not shown). On the basis of a recent study [15], we inferred from our data that the antibody avidity of these sera were also similar.

The effect of varying the challenge isolate was also examined by comparison of the protective activity of antibody 89SF against two 6B isolates. There was no significant differences ( $P > .05$ ) in the number of nonbacteremic mice 48 h after challenge following passive immunization at several levels of antibody: 0.05  $\mu\text{g}/\text{mL}$ , 4 nonbacteremic of 10 tested versus 6 of 16; 0.1  $\mu\text{g}/\text{mL}$ , 19 of 20 versus 23 of 29; and 0.2  $\mu\text{g}/\text{mL}$ , 12 of 12 versus 12 of 12 for serotype 6B isolates 2759407 and 20653.

*Association of ss-IgG antibody concentration (ELISA) and opsonophagocytic activity with protection.* One of the objectives of this study was to determine if serum IgG antibody concentration (ELISA) or opsonophagocytic (functional) antibody activity might be a better predictor of the in vivo protective capacity of human anticapsular antibodies against pneumococcal disease. The variability observed in ss-IgG concentration required for protection against bacteremia suggested that functional activity may differ among the various ss-IgG antibodies. We observed that opsonophagocytic activity was highly correlated with nonbacteremia at 48 h ( $r = .84$ ,  $P < .001$ ) and with survival at 7 days ( $r = .86$ ,  $P < .001$ ; figure 2). We also noted that protection was significantly more likely

( $P < .001$ ) when the opsonophagocytic titer of ss-IgG in mouse serum was  $\geq 8$  (i.e.,  $\log_2 3$ ; table 4 and figure 2). No correlation ( $r = .08$ ,  $P = .63$ ) was demonstrated between ELISA ss-IgG concentration in mouse serum samples and protection from bacteremia at 48 h across serotypes (figure 3). Likewise, no significant correlation ( $r = .20$ ,  $P = .22$ ) was observed between ELISA and opsonophagocytic activity for all serotypes combined in the serum samples tested for passive protection. However, when serotypes were examined individually, significant correlation between ELISA and opsonophagocytic activity was observed for serotypes 1 ( $r = .84$ ,  $P < .001$ ), 4 ( $r = .93$ ,  $P < .001$ ), 5 ( $r = 1.0$ ,  $P < .001$ ), 18C ( $r = 1.0$ ,  $P < .001$ ), and 23A ( $r = 1.0$ ,  $P < .001$ ) but not for serotype 6B ( $r = .47$ ,  $P = .14$ ). Similarly, significant correlation between ELISA and protection from bacteremia at 48 h was observed for serotypes 1 ( $r = .56$ ,  $P < .05$ ) and 4 ( $r = .75$ ,  $P < .01$ ) but not for serotypes 5 ( $r = .68$ ,  $P = .21$ ), 6B ( $r = .31$ ,  $P = .26$ ), 18C ( $r = .84$ ,  $P = .08$ ), and 23A ( $r = .91$ ,  $P = .09$ ).



**Figure 2.** Correlation between passive protection against bacteremia and projected opsonophagocytic titer ( $\log_2$ ) of pneumococcal serotype-specific human IgG anticapsular antibody in mouse serum. Individual serotypes: ● = 1, ○ = 4, ▼ = 5, ▽ = 6B, ■ = 18C, and □ = 23A. Protection against bacteremia was significant ( $P < .001$ ; Fisher's exact test) when opsonophagocytic titer ( $\log_2$ ) was  $\geq 3$ . Vertical and horizontal dashed lines denote 70% nonbacteremic level and corresponding opsonophagocytic titer, respectively. Correlation between survival of mice after passive immunization and projected opsonophagocytic titer ( $\log_2$ ) was also highly significant ( $r = .86$ ,  $P < .001$ ). Data were based on observation of 731 mice challenged with 1 of 6 serotypes: 1 ( $n = 163$ ), 4 ( $n = 140$ ), 5 ( $n = 80$ ), 6B ( $n = 235$ ), 18C ( $n = 80$ ), and 23A ( $n = 33$ ). Linear correlations were analyzed by use of Pearson's product moment correlation coefficient, with level of significance at  $P < .05$ .

**Table 4.** Relationship between human IgG concentration, opsonophagocytic titer, and protection against *Streptococcus pneumoniae* serotypes 5, 6B, and 18C in the infant mouse assay system of bacteremia using a postvaccination serum (Centers for Disease Control no. 95016998).

Targeted antibody concentration ( $\mu\text{g/mL}$ ) <sup>d</sup>	Measured antibody concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>			Opsonophagocytic titer <sup>b</sup>			Bacteremic mice (%) <sup>c</sup>		
	5	6B	18C	5	6B	18C	5	6B	18C
0.0	<0.1	0.1	<0.1	<2	<2	<2	100	100	100
0.1	0.1	0.2	0.1	<2	<2	2	85	85	40 <sup>e</sup>
0.2	0.2	0.3	0.2	<2	2	8	95	70 <sup>e</sup>	5 <sup>e</sup>
0.3	0.3	0.4	0.3	<2	4	16	95	65 <sup>e</sup>	10 <sup>e</sup>
0.4	0.5	0.5	0.4	<2	16	32	65 <sup>e</sup>	25 <sup>e</sup>	15 <sup>e</sup>

<sup>a</sup> Serum level of total pneumococcal serotype-specific human IgG anticapsular antibody (ss-IgG) in serum samples from infant mice just prior to injection of challenge dose as measured by ELISA. Each data point is based on average concentration from 6 mice.

<sup>b</sup> Opsonophagocytic titer (reciprocal of highest serum dilution showing  $\geq 50\%$  killing) in serum samples from infant mice just prior to injection of challenge dose. Each data point is based on pooled serum specimens from 6 mice.

<sup>c</sup> Bacteremia 48 h after inoculation. Twenty mice were tested for each antibody dose.

<sup>d</sup> Target serum concentration of ss-IgG injected into infant mice. Prior to serum dilution, ss-IgG concentrations of serum against serotypes 5, 6B, and 18C were 12, 26, and 14  $\mu\text{g/mL}$ , respectively; opsonophagocytic titers were 8, 512, and 1024, respectively.

<sup>e</sup> Significant protection,  $P < .05$ , compared with control mice injected with human serum albumin in absence of antibody. Fisher's exact test (two-tailed) was used for calculation of significant difference between groups.

## Discussion

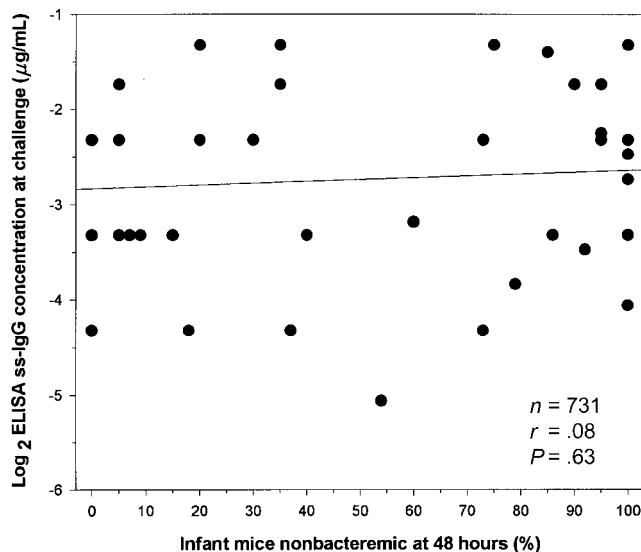
The protective concentration of anticapsular antibodies required for in vivo protection against various pneumococcal serotypes in humans has not been determined. Two analytical immunologic assays available for measuring anticapsular antibodies are RIA [16] and ELISA [7]. Although these assays yield data on the total concentration of serotype-specific antibodies, they do not reveal information about the in vivo antibody functionality. Therefore, the serum antibody concentration measured by these assays may not correlate with in vivo

protection against disease for a particular pneumococcal serotype.

Furthermore, the concentration of anticapsular antibody needed for protection may vary with serotype. Landesman and Schiffman [17] demonstrated that serotype-specific antibody concentrations, as measured by RIA, in serum samples collected prior to the development of bacteremia were  $<200$ – $300$  ng of antibody nitrogen/mL ( $\sim 2.0$   $\mu\text{g/mL}$  anticapsular antibody) and suggested that this may serve as an estimate of antibody levels that were not sufficient to protect high-risk populations. However, Musher et al. [18] found that as little as 50 ng of human anticapsular antibody was sufficient to protect mice (25–30 g) from fatal infection with serotype 4 pneumococci.

Recently, Romero-Steiner et al. [15] demonstrated that serum specimens from elderly people with elevated ELISA IgG concentrations ( $\geq 2$   $\mu\text{g/mL}$ ) do not necessarily have elevated functional antibody titers, as determined by opsonophagocytosis. In addition, discrepancies between human serum IgG antibody concentration and functional opsonophagocytic activity have been attributed to differences in antibody avidity [15]. Although we did not address antibody avidity in this study, we did explore whether in vivo protection can be correlated to potential in vitro correlates of protection. We established a model of infection in infant mice in which a relatively small inoculum of bacteria administered ip resulted in bacteremia and usually death. We have used this model to assay the efficacy of passively administered human antibodies to protect against bacteremia and death. The use of infant mice (6–7 g) allowed testing of multiple mice per antibody dose without requiring large amounts of human serum. We compared in vivo protection with serum ss-IgG concentration and opsonophagocytic titers.

A main objective of our study was to look at the protective capacity of human serum anticapsular antibodies against pneumococcal disease, by use of the 11 most common pediatric serotypes (serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, 23, 23A, and



**Figure 3.** Correlation between passive protection against bacteremia and pneumococcal serotype-specific human IgG anticapsular antibody (ss-IgG) concentration in mouse. Data were based on observation of 731 mice challenged with 1 of 6 serotypes: 1 ( $n = 163$ ), 4 ( $n = 140$ ), 5 ( $n = 80$ ), 6B ( $n = 235$ ), 18C ( $n = 80$ ), and 23A ( $n = 33$ ). Linear correlations were analyzed by use of Pearson's product moment correlation coefficient, with level of significance at  $P < .05$ .

23F) isolated in the world. However, preliminary virulence studies of our infant mouse model showed a wide range of virulence ( $BD_{100}$  levels of  $2-10^9$  cfu) among 23 isolates within these 11 serotypes (data not shown). Only 11 isolates, representing 6 serotypes (serotypes 1, 4, 5, 6B, 18C, and 23A), were virulent at  $BD_{100}$  levels  $\leq 800$  cfu. Numerous attempts were made without success to increase the virulence of those isolates that exhibited low virulence ( $BD_{100}$  levels  $>10^4$  cfu), by use of the agarose enhancement technique of Saladino et al. [19] or the multiple mouse passage technique (described above).

Unlike some previous studies in which both the antibodies and the bacteria were administered ip [18, 20] or mixed in the same syringe prior to ip inoculation [21], we administered the antibodies subcutaneously 24 h prior to ip challenge. In addition, because not all pneumococcal serotypes were virulent for mice in previous studies [21–25], we focused our protection studies on serotypes that had demonstrable virulence for infant mice (table 1). We did not study serotypes that required ip inoculation with  $>10^4$  cfu because levels of such inocula are more likely to be much higher than those of the inoculum invading the bloodstream during infection in humans and therefore are not a valid test of the protective activity of serum antibodies. Several serotypes were not virulent at low inocula despite attempts to increase their virulence by multiple passages through mice.

Two recent papers described a model of fatal pneumococcal infection in infant rats after intrapulmonary (or ip) injection of 1–1500 bacteria [19, 26]. The researchers established bacteremia by using isolates of 10 different pneumococcal serotypes. However, this model required bacterial inoculum in 0.5% agarose to establish infection at low bacterial inocula. We were able to reproduce these findings in the infant rat but not in the infant mouse (data not shown). Furthermore, infection was not highly reproducible in their model; in 41% of control group experiments mortality was low ( $<40\%$ ), and in 21% of these same experiments bacteremia was not detected and mortality was not observed [26]. In addition, correlation between protection of human serum in this model and opsonophagocytic antibody activity in vitro was not studied [26].

Of the 6 serotypes used in our infant mouse assay model, we observed statistically significant increases ( $P < .05$ ) in the numbers of nonbacteremic and surviving mice at serum antibody concentrations of  $\leq 0.2$   $\mu\text{g/mL}$  for serotypes 4, 6B, 18C, and 23A (table 2). For serotypes 4 and 6B, antibody concentrations as low as 0.05  $\mu\text{g/mL}$  significantly ( $P < .05$ ) increased the number of nonbacteremic mice, compared with the number of controls. These results are consistent with those of Musher et al. [18], who observed similar protective serum antibody levels against serotype 4. However, Stack et al. [26] reported higher minimum protective serum concentrations for serotypes 4 and 18C (IgG  $\geq 0.5$   $\mu\text{g/mL}$ ). It is possible that estimates of antibody concentrations required for protection may have been elevated in the infant rat model. This model utilizes agarose, which might

artificially protect the bacterial inoculum by posing a diffusion barrier. Agarose could interfere with physiologic diffusion of proteins, including antibody and complement, and recruitment or activity of phagocytes.

In our study, antibodies to serotypes 1 and 5 provided significant increases in nonbacteremic mice at the highest antibody concentration tested (0.4  $\mu\text{g/mL}$ ); however, protection of  $\geq 70\%$  of mice was not attained. We did not test passive protection against serotypes 1 and 5 with higher antibody concentrations. Stack et al. [26] found that serum concentrations of  $\geq 0.55$   $\mu\text{g/mL}$  for serotype 1 and  $\geq 0.84$   $\mu\text{g/mL}$  for serotype 5 provided significant protection against fatal infection. They did not test intermediate serum concentrations ( $>0.11$  to  $<0.55$   $\mu\text{g/mL}$  for serotype 1 and  $<0.84$   $\mu\text{g/mL}$  for serotype 5) for protective efficacy.

Thus, our study indicates that the concentration of anticapsular antibodies required for protection against bacteremia is serotype dependent and may be as low as 0.05 or  $>0.4$   $\mu\text{g/mL}$ . However, postvaccination serum antibody concentrations that correlate with long-term protection may be significantly higher than the concentration required for shorter term protection. For example, a 0.15- $\mu\text{g/mL}$  concentration of serum anticapsular antibody correlates with protection of humans and animals against invasive *H. influenzae* type b infection, but a postvaccination concentration of 1  $\mu\text{g/mL}$  is required for long-term protection [27–29]. Therefore, it is possible that the postvaccination serum antibody concentrations required for protection in humans against pneumococcus may be many times higher than our point estimates of protective antibody levels in infant mice. In addition, all the antibodies used in this study were induced by vaccination of adults with polysaccharide vaccine. It is possible that the concentration of antibodies that protect may differ depending on the antibody source (e.g., “natural” antibodies or conjugate vaccine-induced antibodies in infants).

In our study, there was significant protection when the opsonophagocytic titer was  $\geq 8$ . The results in table 4 confirmed the minimum protective level extrapolated from figure 2. In addition, we observed a strong correlation between opsonophagocytic titer and protection from bacteremia. However, contrary to previous observations, there was no significant correlation across serotypes between IgG antibody concentration and opsonophagocytic activity for the serum samples tested [8, 30]. Also, we did not demonstrate a correlation between IgG antibody concentration and protection from bacteremia when all serotypes were combined. When individual serotypes were examined, a significant correlation between ELISA ss-IgG concentration and absence of bacteremia was observed for only 2 of 6 serotypes tested (serotypes 1 and 4). We speculate that the differences in correlation results may be due to variations in the avidity of the antibodies for polysaccharide. Studies correlating antibody avidity and protection are ongoing.

In summary, our data suggest that our infant mouse model is useful as an in vivo assay to evaluate and correlate the pro-

tective capacity of human pneumococcal anticapsular antibodies. Specifically, an opsonophagocytic titer of  $\geq 8$  correlates with protection in infant mice. Clinical investigators attempting to estimate the protective efficacy of conjugate vaccine-induced anticapsular antibodies, on the basis of serum antibody measurements by the current ELISA, must do so with caution. This study emphasizes the need for a functional antibody assay for determining minimum levels of protection of human serum. Additional studies are needed to further examine the interrelationship(s) among antibody concentration, opsonophagocytosis, antibody avidity, and protection in animals.

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