Modified Opsonization, Phagocytosis, and Killing Assays To Measure Potentially Protective Antibodies against Pneumococcal Surface Protein A

Calvin C. Daniels, Kyung-Hyo Kim, Robert L. Burton, Shaper Mirza, Melissa Walker, Janice King, Yvette Hale, Patricia Coan, Dong-Kwon Rhee, Moon H. Nahm, David E. Briles

Departments of Microbiology, Pathology, Pediatrics, and Genetics, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA; Department of Pediatrics, School of Medicine, Ewha Womans University, Seoul, South Korea; University of Texas Health Science Center, School of Public Health, Houston, Texas, USA; School of Pharmacy, Sungkyunkwan University, Suwon, South Korea

The standard opsonophagocytosis killing assay (OPKA) for antibodies to pneumococcal capsular polysaccharide was modified to permit an evaluation of the protection-mediating antibodies to pneumococcal surface protein A (PspA). We found that by increasing the incubation time with the complement and phagocytes from 45 min to 75 min, the protective activity was readily detected. In another modification, we used a capsule type 2 target strain that expressed PspA but not pneumococcal surface protein C (PspC). With these modifications separately or in combination, rabbit antisera to the recombinant α-helical or proline-rich domains of PspA mediated >50% killing of the target strain. The ability of normal human sera to mediate the killing of pneumococci in this modified OPKA correlated with their levels of antibodies to PspA and their ability to protect mice against fatal infection with a type 3 strain. Passive protection of mice against pneumococci and killing in the modified OPKA were lost when normal human sera were adsorbed with recombinant PspA (rPspA) on Sepharose, thus supporting the potential utility of the modified OPKA to detect protective antibodies to PspA. In the standard OPKA, monoclonal antibodies to PspA were strongly protective in the presence of subprotective amounts of anti-capsule. Thus, the currently established high-throughput OPKA for antibodies to capsule could be modified in one way to permit an evaluation of the opsonic efficacy of antibodies to PspA.

Pneumonia is the leading cause of death in children aged <5 years old worldwide, and Streptococcus pneumoniae is the leading etiology of serious pneumonia (1). Pneumococcal polysaccharide (PS) conjugate vaccines (PCVs) are highly efficacious against capsular types that are present in the vaccine (2–4). PCV7, which covered about 83% of invasive pneumococcal disease in children when it was introduced, gradually resulted in an almost complete absence of carriage and disease caused by the original 7 capsular types (5–7). One of the largest studies reported that after 7 to 8 years of PCV7 usage, the incidence of invasive pneumococcal disease was reduced by 77% among children aged <5 years. However, in the same age group, meningitis and invasive pneumococcal disease were reduced by only 64 and 49%, respectively, due to an increasing incidence of infections caused by non-PCV7 types (5). Thus, significant pneumococcal disease, especially pneumonia and meningitis, remained after the introduction of PCV7 (5, 6, 8–10).

To increase coverage, PCV7 was replaced in the United States in 2011 by PCV13, which includes 6 additional PSs. Unfortunately, PCV13 covered only 33 to 41% of the invasive pneumococcal disease (IPD) strains (>20 different capsular types) at the time of its approval (5, 6, 11). Total carriage was largely unaffected by PCV7, and 78% of carriage strains (30 different capsular types) in 2008 to 2009 were not covered by PCV13 (12). The diversity of carriage strains may be a harbinger of future invasive strains if even a minority of the nonvaccine serotypes develop genotypes that allow them to fill the niche created by PCV13.

These findings intensified interest in using protection-elicitting pneumococcal proteins as potential vaccine components (13–16). One of the leading candidates is pneumococcal surface protein A (PspA), which reduces complement deposition on pneumococci (17–19), minimizes complement-dependent phagocytosis (20), and protects pneumococci from being killed by cationic peptides released by apolactoferrin (21, 22). It is a surface-accessible (23, 24) choline-binding protein (25) expressed by virtually all pneumococci (26–28). Immunization with the α-helical region of PspA generates antibodies in humans, monkeys, rabbits, and mice that passively protect mice against infection (29–32). The proline-rich (PR) domain of PspA, as well as its nonproline block (NPB), elicits antibodies that passively protect mice against infection (33). Antibodies to PspA enhance complement deposition on pneumococci (34, 35) and phagocytosis of pneumococci in the presence of a complement (20). PspA-mediated cleavage of pneumococci from the blood of mice is dependent on the complement (36). These properties of PspA make it likely that the mediation of phagocytosis in vivo is a major protective mechanism of immunity to PspA. Antibodies to PspA also enhance the killing of pneumococci by the antibacterial peptides of apolactoferrin (22, 37).

To efficiently evaluate PspA and other noncapsular vaccine components in clinical trials, quantitative in vivo surrogates of protection are needed to both bridge between phase II (immuno-
genicity) and phase III (efficacy) trials and provide better insight into whether expensive phase III trials may succeed.

The ability of PS conjugate vaccines to elicit protection against pneumococci in humans is estimated using a quantitative opsonophagocytosis killing assay (OPKA). This assay, along with the anti-PS enzyme-linked immunosorbent assay (ELISA), has been the basis for licensing new PS-protein conjugates. The OPKA uses baby rabbit complement (BRC) and human HL-60 cells differentiated with dimethylformamide (DMF) to quantitate antibody-mediated complement-dependent opsonophagocytosis and killing of pneumococci. The assay quantitates the protective capacity by determining the dilution of each patient serum sample that facilitates the killing of 50% of the target bacteria. This endpoint is important for the OPKA to be quantitative and reproducible (38–40).

The standard OPKA does not generally detect 50% killing with antibodies to PspA, even though these antibodies are able to provide strong passive protection in mice against sepsis (29, 31). Although early attempts to quantitatively measure PspA-mediated killing of pneumococci by phagocytes were not successful (41), one report describes a qualitative OPKA for PspA (42).

At this time, the only quantitative surrogate assay available for measuring human antibodies to PspA is to use serial dilutions of pre- and postimmune sera to passively protect mice against intravenous (i.v.) sepsis (29). This assay works well and can provide confidence regarding the potential efficacy of vaccine formulations containing PspA. Unfortunately, a well-controlled quantitative study to examine a single pair of pre- and postimmunization sera at the necessary serum dilutions can require 70 or more mice (29) and cost about $3,000. A more rapid and much less expensive surrogate assay whose results strongly correlate with those of the passive protection assay is needed. It is considered likely that antibodies to PspA protect against i.v. sepsis primarily by enhancing the complement-dependent clearance of pneumococci by phagocytes (17, 20, 36, 43). If a standardized quantitative in vitro assay(s) for opsonic antibodies to PspA could be developed that used the existing OPKA platform, it would greatly facilitate the progress of PspA vaccine development, and possibly that of other noncapsular antigens that elicit opsonic antibodies, into phase II dose-response and phase III efficacy trials.

It seemed likely that although the levels of antibody to PspA that provide passive protection may be opsonic in vivo, they might not meet the threshold required to mediate 50% killing in the standard OPKA because of differences in the in vivo and in vitro environments. We expected that if the target pneumococci lacked pneumococcal surface protein C (PspC), antibodies to PspA might become more opsonic in vitro. This hypothesis was based on earlier observations that PspA and PspC (choline binding protein A [Cbpa]) decrease complement deposition on pneumococci and that mutants lacking the two molecules are even less virulent and more susceptible to complement deposition (18, 19, 44–47). By binding factor H, PspC inhibits the alternative (amplification) pathway of the complement (48, 49). In this study, we examined the possibility that increasing the incubation time with complement and phagocytes would cause antibodies to PspA to more readily mediate killing in the OPKA. We also examined the possibility that using a target strain with a mutation in the pspC gene would cause antibodies to PspA to more readily mediate killing in an OPKA and that the bacterial killing observed would correlate with passive protection. We also investigated the possibility that antibodies to PspA might exhibit a protective effect in the standard OPKA in the presence of a subprotective level of antibodies to capsule.
inactivated BRC to determine the percentage of CFU that survived. The percentage of bacteria killed was determined by subtracting the percent that survived from 100%. In the OPKA-1B, either D39 or TRE118 was used as the target bacterial strain, as indicated. This assay was always run in triplicate except for the data shown in Fig. 4B, for which the assay was run in sextuplicate.

**TABLE 1** OPKA protocols used in this study

| OPKA type | Type of assay | Colony enumeration method | No. of 3-fold serum dilutions tested | Assay buffer | E/T | Opsonization | Phagocytosis | Reference              |
|-----------|---------------|---------------------------|------------------------------------|--------------|-----|--------------|--------------|------------------------|
| 1A        | Endpoint dilution killing 50% of target bacteria | Automated counter | 8 | HBSS + 0.1% gelatin + 5% FBS | 400:1 | (Test serum + target bacteria) × 30 min, RT with shaking | (12.5% BRC + HL-60) × 45 min, 37°C, 5% CO₂ with shaking | Modified from Burton and Nahm (38) |
| 1B        | Endpoint dilution killing 50% of target bacteria | Automated counter | 8 | HBSS + 0.1% gelatin + 5% FBS | 400:1 | (Test serum + target bacteria) × 30 min, RT with shaking | (12.5% BRC + HL-60) × 30 min, 37°C, 5% CO₂ with shaking; HL-60, × 45 min, 37°C, 5% CO₂ with shaking | This study |
| 1C        | Endpoint dilution killing 50% of target bacteria | Automated counter | 8 | HBSS + 0.1% gelatin + 5% FBS | 400:1 | (Test serum + target bacteria) × 30 min, RT with shaking | (12.5% BRC + HL-60) × 75 min, 37°C, 5% CO₂ with shaking | This study |
| 2A        | % killing of bacteria at indicated serum dilutions | Manual | 1–4 | HBSS + 0.1% gelatin + 5% FBS | 200:1 | (Test serum + target bacteria) × 30 min, 37°C, 5% CO₂ with shaking | (7% BRC + HL-60) × 45 min, 37°C, 5% CO₂ with shaking | This study |
| 2B        | % killing of bacteria at indicated serum dilutions | Manual | 1–4 | HBSS + 0.1% gelatin + 5% FBS | 200:1 | (Test serum + target bacteria) × 30 min, 37°C, 5% CO₂ with shaking | (10% BRC + HL-60) × 30 min, 37°C, 5% CO₂ with shaking; HL-60, × 45 min, 37°C, 5% CO₂ with shaking | This study |

*The times of incubation with BRC and HL-60 cells are indicated. In OPKA-1B and OPKA-2B, bacteria are incubated with BRC and then incubated with HL-60 cells for the indicated time periods. In assays OPKA-1A and OPKA-1B, exactly the same total amount of BRC was used, but since the aliquot of phagocytes was present when the BRC was added in OPKA-1B, its concentration is less than in OPKA-1A, where the phagocytes were added after the initial incubation with BRC. OPKA-1B and OPKA-1C contain the indicated modifications of OPKA-1A, and OPKA-2B contains the indicated modifications of OPKA-2A.

b E/T is the ratio of HL-60 cells to target bacteria.

c RT, room temperature.

d The concentrations of BRC used in the different assay conditions were tested to ensure functional complement activity at the concentrations used.
of opsonized bacteria with BRC and phagocytes were separated into two incubations. The first (30-min incubation) was after the addition of the BRC to the opsonized bacteria, and the second (45-min incubation) followed the subsequent addition of HL-60 cells. As with OPKA-1A and OPKA-2A, all incubations with BRC and phagocytes were carried out at 37°C in 5% CO₂ with shaking at 700 rpm (Table 1). The target bacterial strain used was either D39 or TRE118, as indicated in the descriptions of individual experiments in Results.

**TABLE 2** Sensitivity of OPKA-1A, OPKA-2A, and OPKA-3 methods for the detection of antibody-dependent killing

| Expt | OPKA variant | Target S. pneumoniae strain | Phagocytosis assay incubation conditions (°C, % CO₂, time) | OPKA titers |
|------|--------------|-----------------------------|----------------------------------------------------------|-------------|
| A    | OPKA-1B     | TRE118                      | BRC (37°C, 5% CO₂, 30 min); HL-60 (37°C, 5% CO₂, 45 min) | 1,962       |
| A    | OPKA-1C     | TRE118                      | BRC + HL-60 (37°C, 5% CO₂, 75 min)                       | 2,621       |
| B    | OPKA-1A     | D39                         | BRC + HL-60 (37°C, 5% CO₂, 45 min)                       | 720         |
| B    | OPKA-1A     | TRE118                      | BRC + HL-60 (37°C, 5% CO₂, 45 min)                       | 2,261       |
| B    | OPKA-1C     | D39                         | BRC + HL-60 (37°C, 5% CO₂, 75 min)                       | 836         |
| B    | OPKA-1C     | TRE118                      | BRC + HL-60 (37°C, 5% CO₂, 75 min)                       | 2,785       |

* NT, not tested.

*Anti-clade 2 PspA domain*.

*Anti-PR domain*.

*NRS*, nonimmune normal rabbit serum.

*Pool 22 (38) is a pool of normal human serum that contains antibodies to most capsular polysaccharides, including capsular type 2.*

*In OPKA-2B, the opsonized target cells are incubated first with BRC for 30 min and then HL-60 cells are added and incubated for another 45 min.*

*In OPKA-2C, the OPKA protocol is the same as OPKA-2A, except that the incubation with complement and HL-60 cells was extended from 45 min to 75 min.*

*In OPKA-1C (Table 1) was identical to the OPKA-1A except that the combined incubation of the antibody-coated bacteria with BRC and HL-60 cells lasted for 75 min rather than 45 min.*

**RESULTS**

**Antibodies to PspA support the killing of PspC− pneumococci in a modified OPKA.** Using the OPKA-1A protocol, we observed >50% killing with human serum pool 22 (which contains Abs to type 2 PS) at dilutions of 1:4 and 1:12 but only 37% killing with the highest test concentration of the rabbit anti-PspA family 1 serum (1:4) (Fig. 1A). In contrast, when TRE118 (the pspC mutant of type 2 strain D39) was used as the target strain, a 1:4 dilution of rabbit antiserum to PspA gave 67% killing, and a 1:12 dilution gave 50% killing (Fig. 1B). This rabbit antiserum to PspA lacked a detectable Ab response to type 2 PS. Thus, the use of TRE118 as the target strain provided a significant improvement in the assay for detecting opsonic antibodies to PspA and permitted protective levels of antibodies to be expressed as the dilution, which killed 50% of the target strain. Most of the OPKA studies described in this paper used target strain TRE118. Exceptions are shown in the results depicted in Table 2, Fig. 1A, and Fig. 6.

**Longer total time of incubation increased assay sensitivity of protection mediated by antibodies to PspA.** This experiment compared the killing of TRE118 in assay protocols the OPKA-2A and OPKA-2B. In the OPKA-2A, the target bacteria were incubated with antibodies for 30 min followed by incubation with BRC and phagocytes for 45 min prior to plating to determine the CFU count (38, 39). In the OPKA-2B, the antibody-coated bacteria were incubated for 30 min after the addition of BRC and then were incubated for 45 additional min after the addition of HL-60 cells. We examined a rabbit antiserum to PspA family 1, which detects the α-helical domain of PspA (Fig. 2A), a rabbit antiserum to the proline-rich domain of PspA (Fig. 2B), and serum from nonim-
munized human J3, which appeared to lack Abs to the type 2 PS of the target strain but contained antibodies to PspA. Figure 2 shows that with each of these three sera, higher percentages of killing were observed with the sequential incubations with BRC and HL-60 cells for 45 min. In OPKA-2B, the antibody-coated bacteria had separate sequential incubations with BRC for 30 min and HL-60 cells for 30 min. Rabbit serum specific for the family 1 PspA α-helical domain (A) and rabbit antiserum specific for the proline-rich domain containing a nonproline block (B) were diluted 1:10, 1:30, or 1:90 and evaluated for killing in OPKA-2A and OPKA-2B. A human serum sample J3 from a nonimmunized adult (C) was diluted 1:30, 1:100, and 1:300 and tested in OPKA-2A and OPKA-2B. In each panel, the percentages of the target bacteria killed in OPKA-2A are shown in black; the results for OPKA-2B are shown in red. The greater killing in OPKA-2B than in OPKA-2A was statistically significant at P values of 0.0009, 0.0004, and 0.035 for panels A, B, and C, respectively. The means and standard errors are shown for each data set. To calculate statistical significance between the treatments with each serum, we first ranked all 6 data points (three from the OPKA-2A and three from the OPKA-2B) at each dilution. Next (A and B), the ranked data points all for all three dilutions were grouped into two groups of 9 according to assay type. Next, the 9 data points for OPKA-2A were compared with the 9 for OPKA-2B by the Mann-Whitney two-sample rank test. With the human serum (C), the highest dilution showed no killing with either treatment. For this serum, only data from the 1:30 and 1:100 dilutions were used for statistical analysis.

Passive protection of mice by human sera correlated with the level of antibodies to PspA. It is well established that most normal children ≥2 years of age and normal adults have readily detectable levels of antibodies to PspA in their blood serum (58–60). Normal human serum samples from 19 adult volunteers who were not previously immunized with any pneumococcal vaccine were evaluated for their ability to passively protect mice against i.v. challenge with the serotype 3 pneumococcal strain A66.1 expressing family 1 PspA. The ability of these human serum samples to passively protect CBA/N mice was determined by giving the serum intraperitoneally 1 h prior to i.v. injection of live A66.1. This protocol has been previously shown to provide a robust passive protection assay for evaluating human antibodies to family 1 PspA (29). The protection of mice correlated with the amount of IgG serum antibodies to PspA as determined by ELISA (r = 0.85, P = 0.0007) but not with the negligible levels of serum antibodies to the type 3 capsular PS (r = 0.39, P = 0.85) (Fig. 3). It is well established that anti-type 3 antibodies can passively protect mice from infection (41,61). Thus, the lack of a correlation between the level of anti-type 3 antibodies and protection was probably because of the virtual absence (Fig. 3) of antibodies to the type 3 PS in most of these serum samples.

These same human serum samples were also used to passively protect mice from fatal i.v. sepsis with strain BG7322 (type 6A PS, PspA family 1). As with the type 3 challenge strain, a statistically

FIG 2 Comparisons of combined 45-min versus sequential (30 plus 45 min) incubations on killing of the antibody-coated pspC mutant, TRE118, in OPKA-2A and OPKA-2B. In the OPKA-2A, antibody-coated bacteria were incubated with BRC (complement) and HL-60 cells for 45 min. In OPKA-2B, the antibody-coated bacteria had separate sequential incubations with BRC for 30 min and HL-60 cells for 30 min. Rabbit serum specific for the family 1 PspA α-helical domain (A) and rabbit antiserum specific for the proline-rich domain containing a nonproline block (B) were diluted 1:10, 1:30, or 1:90 and evaluated for killing in OPKA-2A and OPKA-2B. A human serum sample J3 from a nonimmunized adult (C) was diluted 1:30, 1:100, and 1:300 and tested in OPKA-2A and OPKA-2B. In each panel, the percentages of the target bacteria killed in OPKA-2A are shown in black; the results for OPKA-2B are shown in red. The greater killing in OPKA-2B than in OPKA-2A was statistically significant at P values of 0.0009, 0.0004, and 0.035 for panels A, B, and C, respectively. The means and standard errors are shown for each data set. To calculate statistical significance between the treatments with each serum, we first ranked all 6 data points (three from the OPKA-2A and three from the OPKA-2B) at each dilution. Next (A and B), the ranked data points all for all three dilutions were grouped into two groups of 9 according to assay type. Next, the 9 data points for OPKA-2A were compared with the 9 for OPKA-2B by the Mann-Whitney two-sample rank test. With the human serum (C), the highest dilution showed no killing with either treatment. For this serum, only data from the 1:30 and 1:100 dilutions were used for statistical analysis.
A significant correlation was observed between the anti-family 1 PspA levels in these serum samples and the time to death ($r = 0.67$, $P = 0.0016$; data not shown).

Killing of pspC mutant TRE118 in OPKA-2B correlates with the levels of antibodies to PspA in passive protection of mice by human serum. We next examined two of the 19 human serum samples, one that provided strong protection when given passively to mice and one that was not able to passively protect mice. These two serum samples were examined in the OPKA-2B at dilutions of 1:10, 1:30, 1:90, and 1:300 against the target strain TRE118. The highly protective serum demonstrated better killing in the OPKA-2B than the nonprotective serum at the 1:30 and 1:90 dilutions. At both dilutions, the differences were statistically significant ($P < 0.001$) (Fig. 4A), but the difference was the largest at the 1:90 dilution. The protective serum resulted in 87% killing, whereas the nonprotective serum resulted in only 14.6% killing. Two other human serum samples, which were highly protective in mice, were also examined in the OPKA-2B. Each showed the greatest differences versus the nonprotective serum at the 1:90 dilution (data not shown).

**FIG 3** Analysis of 19 normal human serum samples to look for correlations between levels of antibody to PspA or capsule (y axis) and the ability of the sera to protect mice from fatal pneumococcal infection with capsular serotype 3 strain A66.1 (x axis). The correlation between IgG antibody levels to PspA and passive protection was highly significant by Pearson’s linear regression correlation ($r = 0.71, P = 0.0007$). However, there was no correlation between IgG antibody levels to type 3 capsule and passive protection ($r = 0.39, P = 0.11$). Seven of these sera (open symbols) were also evaluated in the experiment shown in Fig. 4B.

**FIG 4** Correlation of killing activity in the OPKA-2B against the TRE118 target strain with passive protection against type 3 infection. (A) A human serum sample selected from the 19 examined in Fig. 3 that was highly protective in mice and a poorly protective human serum sample were examined in the OPKA-2B (at 6 replicates per data point) for their ability to kill strain TRE118. The poorly protective serum showed less killing of TRE118 at all dilutions than the highly protective serum. At 1:30 and 1:90, the two sera differed at a $P$ value of $<0.0001$ by Student’s test. (B) Seven serum samples were selected from the 19 sera in Fig. 3 (open symbols) that covered the range of activity in the passive protection and anti-PspA assays. All 7 sera were tested at a 1:90 dilution in the OPKA-2B against strain TRE118, with 6 replicates per data point; the mean percentage of OPKA killing is shown. The means and standard errors for each data set are shown. The mean percentage of TRE118 pneumococci killed at a 1:90 dilution showed a significant correlation with passive protection ($r = 0.85, P = 0.024$) by Pearson’s linear regression correlation.

**FIG 5** Adsorption of normal human serum with rPspA Sepharose removes its antipneumococcal activity in OPKA-2B against TRE118 target strains and in passive protection against fatal infection. The normal human serum sample J3, also depicted in Fig. 2C, was adsorbed with rPspA Sepharose 4B. The adsorption also resulted in a dilution that reduced the serum concentration to 61% of its original concentration. Correcting for this dilution, the adsorption removed 97.3% of the antibodies to PspA and none of the IgG antibodies to type 3 PS (data not shown). A mock adsorption with BSA-conjugated Sepharose 4B resulted in the same dilution but did not remove Abs to PspA (data not shown). Shown is the mean percent killing and standard error of TRE118 in the OPKA-2B for the no-serum control (Ringer’s diluent), the mock-adsorbed serum, and the PspA-adsorbed serum in the graph. A passive protection study was also conducted with Ringer’s lactate, mock-adsorbed serum, and PspA-adsorbed serum using 5 mice per group. Each mouse was given 100 μl of a 1:10 dilution of the indicated serum intraperitoneally (i.p.) 1 h prior to i.v. injection of CBA/N mice with 500 CFU of strain A66.1 type 3 S. pneumoniae. The time to moribund is depicted for each mouse as individual data points. The study was terminated at 510 h postchallenge, and all surviving mice are plotted at 510 h. The postadsorption sera showed significantly (*) less killing in the modified OPKA ($P = 0.019$) and in passive protection ($P = 0.02$) than did the serum adsorbed with BSA Sepharose.
We next used the OPKA-2B to examine 7 serum samples (Fig. 3, open squares) from the original 19 that represented the overall diversity of the 19 samples in terms of protective passive in mice and antibody levels to PspA. The ability of these 7 serum samples to kill TRE118 (a pspC mutant of D39) pneumococci at a 1:90 dilution in the OPKA-2B had a statistically significant correlation ($r = 0.85, P = 0.0238$) with the ability of the same samples to passively protect mice from type 3 pneumococci expressing clade 2, family 1 PspA (Fig. 4B). For the same 7 samples, the association of the percent killing in the OPKA-2B with antibody levels to PspA was almost significant ($r = 0.75, P = 0.05$; data not shown).

Protective human serum lost its ability to protect mice and mediate killing in the OPKA-2B when its antibodies to PspA were adsorbed. The normal human serum J3, which mediated strong killing activity in the OPKA-2B against strain TRE118 (Fig. 2C), was adsorbed with PspA-coated Sepharose beads to remove antibodies to PspA. Before adsorption, this serum contained 84 μg/ml of IgG antibodies to PspA. After adsorption, 97.3% of this antibody was removed compared to the control adsorption with BSA-conjugated Sepharose beads. The ability of this serum to mediate passive protection of mice against type 3 challenge and killing of TRE118 in the OPKA-2B were also largely removed (Fig. 5). This serum contained <20 ng/ml of antibodies to type 2 capsular PS and 2.3 μg/ml of anti-type 3 capsular PS. Since only 100 μl of a 1:10 dilution of the adsorbed serum was injected into the mice, the amount of passive antibodies to type 3 PS/ml in the recipient’s serum would have been about 15 ng/ml, well below the 350 ng/ml of antibody to PS shown to be required for protection against pneumococci in children (62). These data support the conclusion that the antibodies to PspA in this serum were required for the serum’s activity in the modified OPKA and for the passive protection of mice.

Further refinement of the modified OPKA. After the above studies were completed, we conducted additional studies with some of these sera in an effort to both improve and simplify the assay (Table 2). These studies were conducted using a 50% killing endpoint and compared the results obtained using the OPKA-1A, OPKA-1B, and OPKA-1C. The latter two assays used longer incubation times than did the OPKA-1A (Table 1). The most important result from this group of studies was that incubating the antibody-opsonized TRE118 bacteria simultaneously with BRC and phagocytes for 75 min (OPKA-1C) rather than separate 30- and 45-min incubations after the addition of BRC and HL-60 cells, respectively (OPKA-1B), yielded a 2- to 3-fold increase in the killing titer for antibodies to the α-helical and proline-rich domains of PspA (Table 2, experiment A). Thus, the killing by OPKA-1B that is mediated by antibodies to PspA (Fig. 2, 4, and 5) was probably an underestimation of the potential for antibodies to PspA to mediate the killing of pneumococci in the modified OPKA. The 75-min versus the 30- plus 45-min incubations resulted in only about 30% greater killing by antibodies to capsule. The failure of nonimmune rabbit sera to mediate killing in OPKA opsonic activity with nonimmune rabbit serum (Table 2, experiment A) further supported the conclusion that the protection seen with the two rabbit antisera was dependent on their antibodies to PspA.

In the studies in experiment B (Table 2), BRC and phagocytes were always mixed together prior to incubation with the antibody-opsonized bacteria at 37°C. The results from the incubations of antibody-coated D39 or TRE118 for 45 and 75 min with a mixture of BRC and phagocytes were compared. Regardless of the time of incubation or source of antibody, a severalfold-higher killing titer of TRE118 than D39 was observed (Table 2, experiment B) using protocol OPKA-1C than OPKA-1A. It was also observed, however, that the killing titers for antibodies to the α-helical and proline-rich domains were 2- to 5-fold higher when the incubation time was increased from 45 to 75 min. In contrast, the killing titer of the anti-capsular antibody (pool 22) was only slightly increased (Table 2, experiment B). Thus, the increase in killing titer resulting from the use of a pspC mutant target strain affected the titers with both antibodies to PspA and antibodies to capsule, whereas the increase in 50% killing titer due to increased incubation was quite specific for antibodies to PspA but not antibodies to capsule. In summary, using optimal conditions (TRE118 target strain and 75-min incubation with BRC and phagocytes) in experiments A and B, the OPKA titer of the antiserum to the α-helical PspA ranged from 1,000 to 1,500, and the titer of the antiserum to the proline-rich domain ranged from 250 to 400 (Table 2). However, by increasing the incubation time, it was possible to detect 50% killing of wild-type D39 at reciprocal dilutions of 106 and 64, respectively, for the rabbit antisera to the α-helical and proline-rich domains of PspA, respectively.

Synergy between MAbs to capsule and MAbs to PspA to type 3 PS. We also investigated the possibility that an *in vitro* assay to detect potentially protective antibodies to PspA might also be developed by using OPKA-1A, the standard UAB OPKA, in the presence of levels of anti-type 3 MAb that are too low to elicit protection on their own. As anticipated from the data with immune sera to PspA in Fig. 1, we observed that MAb XiR278 to PspA was unable to mediate significant levels of killing in the standard OPKA-1A even when undiluted XiR278 ascites fluid was used (data not shown). MAb to type 3 capsular PS was mixed 1:1 with MAb XiR278, and the mixture was diluted out in the OPKA-1A format using a capsular type 3 target (whose PspA is recognized by XiR278). The mixture (Fig. 6, red line) gave much more than 50% killing at a dilution of 1:900, while the same dilution of the anti-type 3 MAb by itself showed no killing (black line). Also depicted in Fig. 6 is a
control experiment, showing that mixing an anti-type 6B MAb with the anti-type 3 MAb failed to result in synergistic killing of the type 3 target strain.

**DISCUSSION**

With some modification, the standard OPKA may be used to quantitatively evaluate the protective efficacy of antibodies generated to PspA. Rabbit sera generated by immunization with rPspA fragments of the two major immunogenic regions of PspA, the α-helical (29, 31, 63) and the PR (33) domains, could be diluted to 1:200 or higher and kill the encapsulated target strains that expressed PspA and carried the pspC mutation. We also showed that the ability of normal human sera to passively protect mice from infection correlated with the level of antibody to PspA in the sera and with the ability of the sera to support killing in our modified OPKA. Using a human serum sample with a high antibody level to PspA and a negligible antibody level to capsular PS, we observed that adsorption with PspA-Sepharose removed antibodies to PspA and eliminated both the passive protection of the serum and killing in the modified OPKA. These observations indicate that (i) antibodies to PspA were able to mediate killing in the modified OPKA and (ii) the modified OPKA appeared to act as an in vitro surrogate for the ability of these sera to passively protect mice from infection. Thus, although the modified OPKA used a mutant target strain, it detected antibodies to PspA that might protect mice from otherwise fatal infection with very virulent pneumococci.

In our initial OPKA studies summarized above, pspC mutant pneumococci were opsonized with antibodies to PspA and then incubated for 30 min with BRC followed by a 45-min incubation with HL-60 phagocytes. Using this procedure, we observed that dilutions of about 1:100 to 1:300 of rabbit antiserum to PspA facilitated the killing of the pspC mutant pneumococci by HL-60 cells. In a further modification, the antibody-opsonized bacteria were incubated simultaneously with BRC and HL-60 cells for 75 min. With this procedure, immune rabbit serum to PspA supported the killing of >50% of the target pneumococci at dilutions of 1:1,000 to 1:1,500 (Table 2). The increased sensitivity of anti-PspA-mediated killing with the 75-min versus the 45-min incubation was probably because the longer incubation gave the HL-60 cells more time to phagocytize and kill the pneumococci opsonized by antibodies to PspA and BRC. Moreover, using the combined 75-min incubation rather than sequential 30- and 45-min incubations makes the assay amenable to the type of large-scale studies that would be needed in vaccine trials. The OPKA modifications we have described permit the ready detection of the protective effects of antibodies to PspA and also increase the OPKA titers of anti-capsular antibodies. However, such modifications are not recommended for the anti-capsule assay since they would be expected to reduce its specificity for antibodies to capsule by allowing antibodies to PspA and possibly other antigens to be more readily detected.

Passive protection of mice is the current standard for evaluating human immune responses to immunization with PspA (29). Our present findings suggest that it may be possible to use OPKAs similar to the ones described here as in vitro surrogates to detect protective immunity to PspA in serum from immunized humans. Such assays might greatly facilitate further human trials of vaccines containing portions of PspA.

It has been shown that antibodies to PspA can activate complement deposition (20, 35). Prior studies also indicated that the absence of PspC facilitates the alternative pathway following a classical pathway trigger (18, 19, 47, 48). As a result, we expected the antibodies to PspA to result in greater complement deposition and enhanced in vitro phagocytosis and killing of pneumococci if PspC were absent. The pspC mutant did turn out to be a much more sensitive target for detecting opsonization by antibodies to PspA, although we do not yet know if the mechanisms we proposed are the correct ones. Mutations in other genes that interfere with complement deposition might also enhance the protective effects of antibodies to PspA in the OPKA. Dalia and Weiser (64) and Dalia et al. (65) have recently shown that longer chain lengths of pneumococci and mutations in exoglycosidases can enhance complement deposition and phagocytosis by HL-60 cells.

In a different set of experiments, we observed that in the standard OPKA, the levels of monoclonal antibodies to capsule and to PspA that were too low to mediate detectable killing were able to synergize and mediate >50% killing. In view of this finding, it is possible that minute amounts of Abs to capsule or other pneumococcal antigens may have contributed to the killing in the PspA OPKAs described in this paper; also, the OPKA values for capsule in general may be affected in some cases by the presence of antibodies to PspA or other pneumococcal antigens in the immune sera examined. The synergy finding has the potential to be developed into a useful surrogate assay to detect protective antibodies to PspA in human or animal serum. Such an assay can be carried out using a constant nonprotective level of antibodies to capsular PS, to which decreasing amounts of antibodies to PspA would be added, and would be most applicable using target strains, such as type 3, for which anti-capsular antibodies can be rare.

As PspA and other surface proteins move closer to being used in human vaccines, the need for quantitative in vitro assays to measure the protective effects of anti-PspA in pre- and postimmunization sera becomes more critical. We have developed potential surrogate assays on the platform of the current very successful UAB OPKA, which measures antibodies to capsular PS. The modified OPKAs described in this paper should be able to form the basis for the development of validated assays to detect protective responses to PspA that are elicited by PspA-containing vaccines. Such assays can be run with the same equipment and computer software that are now used in the UAB OPKA for anti-capsular assays. The assays described here might also be applicable to other pneumococcal proteins (or antigens of other bacteria) that also elicit opsonizing antibody. To better understand whether these modified OPKAs can be used for the detection of protective antibodies, studies will be needed where human pre- and postimmunization sera are tested in these in vitro assays and also in passive protection in mice. To know if the assays are true surrogates of protection in people, they will need to be used in prospective studies of invasive infection or in vaccine efficacy trials.

**ACKNOWLEDGMENTS**

We acknowledge Susan K. Hollingshead for her interest and encouragement during this project and Christina M. Cronen and Kristopher R. Genschmer for their insights regarding the manuscript and its submission.

We acknowledge support from NIH grant no. AI021458 (to D.E.B.), the Epitope Recognition Core P30AR48311, contract no. AI-30021 (to M.H.N.), and KFDA no. 11172-360 (to K.H.K.).
REFERENCES

1. Wardlaw T, Johannson EW, Hodge M, World Health Organization. 2006. Pneumonia: the forgotten killer of children. The United Nations Children’s Fund (UNICEF)/World Health Organization (WHO), Geneva, Switzerland. http://whqlibdoc.who.int/publications/2006/9280640489_eng.pdf.

2. Haber M, Barskey A, Baughman W, Barker L, Whitney CG, Shaw KM, Orenstein W, Stephens DS. 2007. Herd immunity and pneumococcal conjugate vaccine: a quantitative model. Vaccine 25:5390–5398.

3. Hammit LL, Bruden DL, Butler JC, Baggett HC, Hurlburt DA, Rean-sonover A, Hennessy TW. 2006. Indirect effect of conjugate vaccine on adult carriage of Streptococcus pneumoniae: an explanation of trends in invasive pneumococcal disease. J. Infect. Dis. 193:1487–1494.

4. Scott JR, Millar EV, Lipsitch M, Moulton LH, Weatherholtz R, Perilla MJ, Jackson DM, Beall B, Craig MJ, Reid R, Santomash M, O’Brien KL. 2012. Impact of more than a decade of pneumococcal conjugate vaccine use on carriage and invasive potential in Native American communities. J. Infect. Dis. 205:280–288.

5. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, adult carriage of Streptococcus pneumoniae. J. Infect. Dis. 201:32–41.

6. Croney CM, Coats MT, Nahm MH, Bries DE, Crain MJ. 2012. PspA family distribution, unlike capsule serotype, remained unaltered following introduction of the heptavalent-pneumococcal conjugate vaccine. Clin. Vaccine Immunol. 19:891–896.

7. Black S, Shinefield H, Cohen R, Floret D, Gaudelus J, Olivier C, Reinert VH, Brandileone MC, Andrade AL, Teles EM, Zanella RC, Yara TI, Di Fabio JL, Hollingshead SK. 2004. Systemic and mucosal protective immunity to pneumococcal surface protein A (PspA) in healthy adults with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 297:1784–1792.

8. Haas HE, Shutt KA, Moore MB, Beall BW, Bennett NM, Craig AS, Farley MM, Jorgensen JL, Lexau CA, Petit S, Reingold A, Schaffner W, Schiffer WR, Thomas A, Whitney CG, Harrison LH. 2009. Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. N. Engl. J. Med. 360:244–256.

9. Weinberger DM, Malley R, Lipsitch M. 2011. Serotype replacement in disease after pneumococcal vaccination. Lancet 378:1962–1973.

10. Ampolo K, Pavia AT, Chris S, Hersh AL, Bender JM, Blaschke AJ, Weisberg DM, Malley R, Lipsitch M. 2010. Pneumonia: the forgotten killer of children. The United Nations Children’s Fund (UNICEF)/World Health Organization (WHO), Geneva, Switzerland. http://whqlibdoc.who.int/publications/2006/9280640489_eng.pdf.

11. Mugarri R, Mirza S, Roche AM, Widener RW, Croney CM, Rheec DK, Weiser JS, Szalai AJ, Bries DE. 2012. Pneumococcal surface protein A inhibits complement deposition on the pneumococcal surface by compet-ition with the binding of C-reactive protein to cell-surface phosphocholine. J. Immunol. 189:5327–5335.

12. Ren B, Li J, Genschmer K, Hollingshead SK, Bries DE. 2012. The absence of PspA or presence of antibody to PspA facilitates the comple-ment-dependent phagocytosis of pneumococci in vitro. Clin. Vaccine Immunol. 19:1574–1582.

13. Mirza S, Wilson L, Benjamin WH, Jr, Novak J, Barnes S, Hollingshead SK, Bries DE. 2011. Serine protease PrA from Streptococcus pneumoniae plays a role in the killing of S. pneumoniae by allopneococci. Infect. Immun. 79:2440–2450.

14. Shaper M, Hollingshead SK, Benjamin WH, Jr, Bries DE. 2004. PspA protects Streptococcus pneumoniae from killing by allopneococci, and anti-body to PspA enhances killing of pneumococci by allopneococci. Infect. Immun. 72:5031–5040. (Erratum, 72:7379.)

15. Daniels GC, Bries TC, Mirza S, Hakansson AP, Bries DE. 2006. Capsule does not block antibody binding to PspA, a surface virulence factor of Streptococcus pneumoniae. Microb. Pathog. 40:228–233.

16. McDaniel LS, Scott G, Widenhofer K, Carroll J, Bries DE. 1986. Analysis of a surface protein of Streptococcus pneumoniae recognised by protective monoclonal antibodies. Microb. Pathog. 1:519–531.

17. Yother J, White JM. 1994. Novel surface attachment mechanism for the Streptococcus pneumoniae protein PspA. J. Bacteriol. 176:2976–2983.

18. Crain MJ, Waltman WD, Jr, Turner JS, Yother J, Talkington DF, McDaniel LS, Gray BM, Bries DE. 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae. Infect. Immun. 58:3293–3299.

19. Hollingshead SK, Baril L, Ferro S, King S, Coan P, Bries DE, Pneumo-coecal Proteins Epi Study Group. 2006. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. J. Med. Microbiol. 55:215–221.

20. Brandileone MC, Andrade AL, Teles EM, Zanella RC, Yara TI, Di Fabio JL, Hollingshead SK. 2004. Typing of pneumococcal surface protein A (PspA) in Streptococcus pneumoniae isolated during epidemiological surveil-lance in Brazil: towards novel pneumococcal protein vaccine. Vaccine 22:3890–3896.

21. Bries DE, Hollingshead SK, King S, Swift A, Braun PA, Park MK, Ferguson LM, Nahm MH, Nabor GS. 2000. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibi-dies that passively protect mice from fatal infection with Streptococcus pneumoniae bearing heterologous PspA. J. Infect. Dis. 182:1694–1701.

22. Bries DE, Hollingshead SK, Biers, DB, Dunnings-Walters S, S, Book MJ, Ferguson LS, Schilling M, Gravenstein S, Braun P, King S, Swift A. 2000. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. Vaccine 18:1707–1711.

23. McDaniel LS, Walsh BA, McDaniel DO, Bries DE. 1994. Localization of protection-eliciting epitopes on PspA of Streptococcus pneumoniae be-tween amino acid residues 192 and 260. Microb. Pathog. 17:323–337.

24. Nabor GS, Braun PA, Herrentman DJ, Heise ML, Pyle DJ, Gravenstein S, Schilling M, Ferguson LM, Hollingshead SK, Biers DE, Becker RS. 2000. Immunization of healthy adults with a single recombinant pneumo-coecal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine 18:1743–1754.

25. Daniels CC, Coan P, King J, Hales J, Benton KA, Bries DE, Hollingshead SK. 2010. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infect. Immun. 78:2163–2172.

26. Ochs MM, Bartlett W, Bries DE, Hicks B, Jurkuvnas A, Lau P, Ren B, Millar A. 2008. Vaccine-induced human antibodies to PspA augment complement C3 deposition on Streptococcus pneumoniae. Microb. Pathog. 44:204–214.

27. Ren B, Szalai AJ, Hollingshead SK, Bries DE. 2004. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect. Immun. 72:114–122.

28. Bries DE, Tart RC, Wu HY, Walsh RA, Russell MW, McDaniel LS. 1996. Systemic and mucosal protective immunity to pneumococcal surface protein A. Ann. N. Y. Acad. Sci. 797:118–126.
37. Bitsaktsis C, Iglesias BV, Li Y, Colino J, Snapper CM, Hollingshead SK, Pham G, Gosselin DR, Gosselin EJ. 2012. Mucosal immunization with an unadjuvanted vaccine that targets Streptococcus pneumoniae PspA to human Fc receptor type I protects against pneumococcal infection through complement- and lactoferrin-mediated bactericidal activity. Infect. Immun. 80:1166–1180.

38. Burton RL, Nahm MH. 2006. Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. Clin. Vaccine Immunol. 13:1004–1009.

39. Burton RL, Nahm MH. 2012. Development of a fourfold multiplexed opsonophagocytosis assay for pneumococcal antibodies against additional serotypes and discovery of serological subtypes in Streptococcus pneumoniae serotype 20. Clin. Vaccine Immunol. 19:835–841.

40. Romero-Steiner S, Libbitti D, Pais LB, Dykes J, Anderson P, Whitin JC, Keyserling HL, Carlone GM. 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against Streptococcus pneumoniae using differentiated HL-60 cells. Clin. Diagn. Lab. Immunol. 4:413–422.

41. Briles DE, Forman C, Horowitz JC, Volanakis JE, Benjamin WH, Jr, McDaniel LS, Eldridge J, Brooks J. 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. Infect. Immun. 57:1457–1464.

42. Giefing C, Meinke AL, Hanner M, Henics T, Bui MD, Gelbmann D, Lundberg U, Senn BM, Schunn M, Habel A, Henriques-Normark B, Ortvist A, Kalin M, von Gabain A, Nagy E. 2008. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. J. Exp. Med. 205:117–131.

43. Ren B, Mccrory MA, Pass C, Bullard DC, Ballantyne CM, Xu Y, Briles DE, Szalai AJ. 2004. The virulence function of Streptococcus pneumoniae surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. J. Immunol. 173: 7506–7512.

44. Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. 2002. The role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae. Infect. Immun. 70:2526–2534.

45. Ogunniiyi AD, Grabovicz M, Briles DE, Cook J, Paton JC. 2007. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of Streptococcus pneumoniae. Infect. Immun. 75:350–357.

46. Ogunniiyi AD, LeMessurier KS, Graham RM, Watt JM, Briles DE, Stroehrer UH, Paton JC. 2007. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. Infect. Immun. 75:1843–1851.

47. Quin LR, Moore QC, III, McDaniel LS. 2007. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. Infect. Immun. 75:2067–2070.

48. Jarva H, Janulczyk R, Hellwage J, Zipfel PF, Björck L, Meri S. 2010. Three surface exoglycosidases from Streptococcus pneumoniae, NanA, BgaA, and StrH, promote resistance to opsonophagocytic killing by human neutrophils. Infect. Immun. 78:2108–2116.

49. Coral MC, Fonseca N, Castañoa E, Di Fabio JL, Hollingshead SK, Briles DE. 2001. Pneumococcal surface protein A of invasive Streptococcus pneumoniae isolates from Colombian children. Emerg. Infect. Dis. 7:832–836.

50. Hollingshead SK, Becker RS, Briles DE. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in Streptococcus pneumoniae. J. Bacteriol. 182:1979–1984.

51. Yu J, Lin J, Benjamin WH, Jr, Waites KB, Lee CH, Nahm MH. 2005. Rapid multiplex assay for serotyping pneumococci with monoclonal and polyclonal antibodies. J. Clin. Microbiol. 43:156–162.

52. Nahm MH, Olander JV, Magyaraki M. 1997. Identification of cross-reactive antibodies with low opsonophagocytic activity for Streptococcus pneumoniae. J. Infect. Dis. 176:698–703.

53. Avery OT, MacLeod CM, McCarty M. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. 79:137–158.

54. Wofsy L, Burr B. 1969. The use of affinity chromatography for the specific purification of antibodies and antigens. J. Immunol. 103:380–382.

55. Sun Y, Hwang Y, Nahm MH. 2001. Avidity, potency, and cross-reactivity of monoclonal antibodies to pneumococcal capsular polysaccharide serotype 6B. Infect. Immun. 69:336–344.

56. Baril L, Briles DE, Crozier P, King J, Punar M, Hollingshead SK, McCormick JB. 2004. Characterization of antibodies to PspA and PsmA in adults over 50 years of age with invasive pneumococcal disease. Vaccine 22:787–793.

57. Rapola S, Jantti V, Haikala R, Syrjanen R, Carlone GM, Sampson JS, Briles DE, Paton JC, Takala AK, Kilpi TM, Kayhty H. 2000. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. J. Infect. Dis. 182:1146–1152.

58. Virolainen A, Russell W, Crain MJ, Rapola S, Kayhty H, Briles DE. 2000. Human antibodies to pneumococcal surface protein A in health and disease. Pediatr. Infect. Dis. J. 19:134–138.

59. Austrian R. 1979. Pneumococcal vaccine: development and prospects. Am. J. Med. 67:547–549.

60. Siber GR, Chang I, Baker S, Fernsten P, O’Brien KL, Santosham M, Klugman KP, Madhi SA, Paradiso P, Kohberger R. 2007. Estimating the protective concentration of anti-pneumococcal capsular polysaccharide antibodies. Vaccine 25:3816–3826.

61. Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes AP, Ren B, Briles DE, Hollingshead SK, Leite LC. 2007. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against Streptococcus pneumoniae that correlates with antibody-mediated enhancement of complement deposition. Infect. Immun. 75:5930–5938.

62. Dalia AB, Weiser JN. 2011. Minimization of bacterial size for complement evasion and is overcome by the agglutinating effect of antibody. Cell Host Microbe 10:486–496.

63. Dalia AB, Standish AJ, Weiser JN. 2010. Three surface exoglycosidases from Streptococcus pneumoniae, NanA, BgaA, and StrH, promote resistance to opsonophagocytic killing by human neutrophils. Infect. Immun. 78:2108–2116.