Anti-Pneumococcal surface protein A monoclonal antibodies: Correlation between in vitro complement deposition and passive mouse protection

Naeem Khan¹, ², $, Raies Ahmad Qadri² and Devinder Sehgal¹, #

¹Molecular Immunology Laboratory, National Institute of Immunology, New Delhi, India
and ²Department of Biotechnology, University of Kashmir, Srinagar, India

$Present address: Department for Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14424 Potsdam, Germany

Running title: In vitro complement deposition and passive protection

Key words: Anti-Pneumococcal surface protein A monoclonal antibodies; Surface binding; Passive mouse protection; bactericidal activity; Complement deposition; In vitro correlate

#Address correspondence to Devinder Sehgal, devinder@nii.ac.in.
ABSTRACT

Shortcomings of the licensed polysaccharide-based pneumococcal vaccine are driving efforts towards developing a protein-based vaccine that is serotype-independent and effective in all age groups. Opsonophagocytic killing assay (OPKA) is used to evaluate the antibody response against polysaccharide-based pneumococcal vaccines. However, OPKA is not reliable for noncapsular antigens. Thus, there is a need to develop an in vitro surrogate for protection for protein vaccine candidates like Pneumococcal surface antigen A (PspA). PspA is a serologically variable cell surface virulence factor. Based on its sequence PspA has been classified into families 1 (clade 1 and 2), 2 (clade 3, 4 and 5) and 3 (clade 6). Here, we report the characterization of 18 IgG anti-PspA monoclonal antibodies (anti-PspA<sub>hkR36A</sub> mAbs) generated from mice immunized with heat-killed strain R36A (clade 2). ELISA-based analysis of the reactivity of the mAbs with recombinant PspAs from the 6 clades indicated that they were family 1 specific. This was confirmed by flow cytometry using hyperimmune serum generated against PspA from R36A. Eight mAbs that bind at least one clade 1 and clade 2 expressing strain were evaluated for complement deposition, bactericidal activity and passive protection. Anti-PspA<sub>hkR36A</sub> mAb-dependent deposition of complement on pneumococci showed positive correlation with passive protection against strain WU2 (r = 0.8783, P = 0.0041). All our protective mAbs showed bactericidal activity; however not all mAbs that exhibited bactericidal activity conferred protection in vivo. The protective mAbs described here can be used to identify conserved protection eliciting B cell epitopes for engineering a superior PspA-based vaccine.
INTRODUCTION

The bacterial pathogen *Streptococcus pneumoniae* (pneumococcus) is responsible for causing pneumonia, septicemia, meningitis and otitis media in humans (1). According to the estimate made by the World Health Organization in 2005, 1.6 million individuals die of diseases caused by *S. pneumoniae* and most of these deaths occur in developing countries (2). In the year 2000, it was estimated that pneumococcal disease was responsible for about 8,00,000 deaths in children < 5 years of age (3). Currently available pneumococcal polysaccharide vaccine is not effective in children less than 2 years of age. Pneumococcal conjugate vaccines however overcome this limitation and are effective in children but have limited serotype coverage (4). The development of antibiotic resistance and emergence of non-vaccine serotypes poses difficulties in the management of pneumococcal infections. Efforts are on globally to develop a protein-based pneumococcal vaccine that confers serotype independent protection in all age groups (5-7).

A polysaccharide capsule envelopes *S. pneumoniae* and it serves as the major virulence factor by shielding pneumococci from immune attack. Unencapsulated strains of *S. pneumoniae* are known to be avirulent or highly attenuated. In addition to the capsule, several surface-associated proteins have been demonstrated to be involved in pneumococcal virulence and one such protein is Pneumococcal surface protein A (PspA) (8). A well-established opsonophagocytic killing assay (OPKA) is available for evaluating pneumococcal polysaccharide-based vaccines. The recent interest in protein-based pneumococcal vaccines has led to efforts towards developing an *in vitro* assay for noncapsular antigens that can help in predicting and quantitating the
protective activity of antibodies against protein vaccine candidates. Various investigators have tried to correlate anti-protein antibody titers, surface binding (by whole cell ELISA) and surface killing assay with *in vivo* protection (9-12). The notion of *in vitro* antibody mediated complement deposition as a possible surrogate for predicting *in vivo* protection has been proposed by Och *et al* and Goulart *et al* (13, 14). However, these investigators did not validate it with *in vivo* protection experiments. Availability of a robust *in vitro* assay would help in minimizing the use of animal models for testing protein vaccine candidates.

PspA is a polymorphic, surface-associated choline-binding protein (15). PspA has a predominantly α-helical coiled coil structure (16, 17). It is present in essentially all clinical isolates studied to date and is being pursued as a promising vaccine candidate (18). Based on the amino acid sequence, PspA has been classified into three families and six clades. Family 1 includes clade 1 and 2; family 2 includes clade 3, 4, and 5, and family 3 includes clade 6 (19). Studies have shown that 94–99% of the pneumococcal isolates analyzed belong to PspA family 1 and 2 (20, 21).

The complement mediated clearance of pneumococci is an important component of host defense mechanism (22). PspA deficient strain is cleared faster than wild type pneumococci and anti-PspA antibody facilitates complement-dependent phagocytosis of *S. pneumoniae* (23). Ren and coworkers have demonstrated that anti-PspA antibodies enhance complement activation and deposition on pneumococcal surface, and thus help in clearance (24).

Active immunization with PspA in animal models has proven protective against invasive disease and nasopharyngeal carriage (25). Mice immunized with DNA vaccine
expressing the extracellular domain of PspA were protected against intraperitoneal challenge with a pneumococcal strain bearing PspA from the same clade (26). The B cell epitopes recognized by protective mAbs have been mapped to the 192-260 amino acid region (27). Daniels et al recently demonstrated that the proline-rich region of PspA contains surface-accessible epitopes that are protective in both active and passive mouse protection experiments (28). PspA has been shown to elicit high antibody titers in humans and human anti-PspA sera can protect mice against pneumococcal challenge when transferred passively (18, 29).

There is evidence to suggest that not all anti-PspA antibodies are protective. The goals of the present study were to identify anti-PspA<sub>hkR36A</sub> mAbs that recognize conserved cross-protective B cell epitopes and since in vitro surrogate of protection is not well established for non-capsular antigens we evaluated surface binding, complement deposition and bactericidal activity of anti-PspA<sub>hkR36A</sub> mAbs as potential in vitro correlates of protection. We found that all the 18 anti-PspA<sub>hkR36A</sub> mAbs recognized family 1 PspAs and did not bind PspAs representing families 2 and 3. We identified 4 anti-PspA<sub>hkR36A</sub> mAbs (P1E11, M4F4, P2A4 and P2B5) that augmented complement deposition on pneumococci, exhibited bactericidal activity and conferred protection against PspA clade 1 and 2 bearing S. pneumoniae strains in passive mouse protection experiments. Further, our data with strain WU2 suggested that anti-PspA<sub>hkR36A</sub> mAb dependent complement deposition on pneumococci strongly correlated with in vivo protection. We observed that all the protective anti-PspA<sub>hkR36A</sub> mAbs exhibited bactericidal activity, however not all anti-PspA<sub>hkR36A</sub> mAbs that showed bactericidal activity conferred in vivo protection.
MATERIALS AND METHODS

Mice. Six to eight wk old BALB/c (female) and CBA/N (male/female) inbred strains of mice were obtained from the Small Animal Facility of the National Institute of Immunology. Animals were rested and handled in accordance with the Institutional Animal Ethics Committee guidelines. Blood samples from healthy donors were taken with the approval and following the guidelines of the Institutional Human Ethics Committee. Experiments involving recombinant DNA and handling of S. pneumoniae were carried out in accordance with the Institutional Biosafety Committee guidelines.

Pneumococcal strains, plasmids and culture conditions. The pneumococcal strains and plasmids used in this study are listed in Table S1. Pneumococcal strains were maintained in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or tryptic soy agar supplemented with 5% (v/v) sheep blood (TSA) plate at 37°C in the presence of 5% CO₂. Mid-logarithmic phase pneumococcal culture was stored with 15% (v/v) glycerol or 17% fetal calf serum, aliquoted and stored at -70°C as described previously (30).

Escherichia coli were maintained in Luria-Bertani broth or Luria-Bertani agar plate with antibiotic(s) wherever required.

Molecular cloning, over-expression and purification of recombinant PspA. The sub-fragments encoding the N-terminal (surface exposed) region of PspA for clade 3 (PspATIGR4) and 5 (PspA ATCC 6303) were amplified by PCR using genomic DNA from the
pneumococcal strains TIGR4 and ATCC 6303 following the cloning strategy and PCR conditions described by Rohatgi et al. (30). The corresponding plasmid constructs for PspA clade 1 (pUAB069, strain L82016; PspA^{L82016}), clade 4 (pUAB100, strain JCP#56; PspA^{JCP#56}) and clade 6 (pUAB104, strain BG9300; PspA^{BG9300}) were kindly provided by Susan Hollingshead, University of Alabama, USA. Plasmid construct for PspA clade 2 (R36A; PspA^{R36A}) was published previously from our laboratory (30). For expression purposes pQE-30 Xa- and pET-20b-based constructs were transformed into *E. coli* expression strains SG13009 and BL-21 (DE3), respectively. Recombinant PspA was purified using Ni-nitriloacetic acid (Ni-NTA) affinity chromatography (Sigma-Aldrich, USA). The purity of the protein preparation was found to be >95% by SDS-PAGE and was of the expected molecular size.

**Enzyme linked immunosorbent assay.** The reactivities of the 18 IgG anti-PspA^{hkR36A} mAbs with recombinant PspAs representing the six clades of PspA were analyzed by ELISA. Briefly, 96-well polystyrene microtiter plates (Greiner Bio-one, Germany) were coated overnight at 4°C with recombinant PspA (50 µl of 2 µg/ml per well) in 100 mM carbonate-bicarbonate buffer, pH 9.5. The plates were washed with PBS containing 0.05% Tween-20 (PBST) and blocked with PBS containing 2% BSA at 37°C for 1 hr. After washing with PBST, the plates were incubated with the culture supernatant from the 18 anti-PspA^{hkR36A} hybridomas (in duplicate) at 37°C for 1 hr. The plates were washed with PBST and incubated with horseradish peroxidase conjugated goat anti-mouse Ig antibody (diluted 1 in 2500; Becton-Dickinson Bioscience, USA) followed by
incubation at 37°C for 1 hr. The colour was developed using 3, 3', 5, 5'-
tetramethylbenzidine/ H2O2 as substrate and absorbance recorded at 450 nm.

Generation of polyclonal sera against recombinant PspA<sup>R36A</sup>. Six to eight wk old
female BALB/c mice were immunized subcutaneously with 25 µg recombinant PspA<sup>R36A</sup>
emulsified with Imject alum [1:1 (w/w); Pierce, USA]. On day 14 and 28, mice were
given a booster injection with the same amount of antigen emulsified as described
above. Control mice received only Imject alum in PBS. One week after the second
booster, hyperimmune serum was isolated after bleeding mice retro-orbitally and ELISA
was performed to determine PspA-specific antibody titer.

Surface staining with anti-PspA<sup>hkR36A</sup> mAbs and anti-PspA<sup>R36A</sup> hyperimmune
serum. The surface binding of anti-PspA<sup>hkR36A</sup> mAbs with <i>S. pneumoniae</i> strains that
express clade 1 PspA (BG8838) and clade 2 PspA (WU2 and D39) was analyzed using
flow cytometry as described previously (31). Briefly, mid-logarithmic phase (OD<sub>600</sub> = 0.4)
pneumococci (10<sup>7</sup> cfu) were washed with PBS and incubated with 200 µl of culture
supernatant from the hybridomas at room temperature for 1 hr. After washing with PBS,
pneumococci were incubated with FITC-conjugated F(ab')<sub>2</sub> fragment goat anti-mouse
IgG + IgM (H + L) antibody (diluted 1 in 200; Jacksons ImmunoResearch Laboratory,
USA) followed by incubation at room temperature for 1 hr. After washing pneumococci
were fixed with 2% paraformaldehyde (PFA) for 10 min at 4°C and surface staining was
analyzed by flow cytometry (FACS caliber, Becton-Dickinson Bioscience).
The surface binding of anti-PspAR36A polyclonal sera (diluted 1 in 200) was analyzed with strains BG8838 (clade 1), D39 (clade 2), TIGR4 (clade 3), EF5668 (clade 4), ATCC 6303 (clade 5) and BG6803 (clade 6) using flow cytometry as described above. Geometric mean fluorescence intensity (GMFI) values equal to or greater than twice the value obtained with the preimmune control were considered significant.

**Purification of anti-PspA\textsuperscript{hkR36A} mAbs.** Hybridomas secreting anti-PspA\textsuperscript{hkR36A} mAbs were generated and cultured as previously described (30). Ascitic fluid was generated for the anti-PspA\textsuperscript{hkR36A} mAb secreting hybridomas. Briefly, BALB/c mice (3 mice per hybridoma) were injected intraperitoneally with 0.5 ml incomplete Freund's adjuvant (Sigma-Aldrich). Three days later 5 × 10\textsuperscript{6} hybridoma cells were injected intraperitoneally. After 7-10 days, ascitic fluid from the peritoneal cavity was centrifuged at 500 × g for 10 min at 4°C, the supernatant was collected and stored at -70°C. The mAbs were purified from ascitic fluid using Protein-G-Sepharose beads (GE Healthcare, USA) as described earlier (32). The protein concentration of the purified mAb was estimated using Micro BCA Protein Assay Kit (Thermo-Scientific, USA).

**Passive mouse protection assay.** Groups of eight 6-8 wk old CBA/N mice were injected intraperitoneally with purified anti-PspA\textsuperscript{hkR36A} mAb or matched isotype control. The amount of mAb administered for the high and low dose experiment was 5 and 1.25 mg/ kg body weight, respectively. An hour later, mice were challenged intravenously with 10\textsuperscript{7} cfu (100 times LD\textsubscript{50}) of strain BG8838 and 10\textsuperscript{3} cfu of WU2 (100 times LD\textsubscript{50})
(27). The survival of mice was monitored every 12 hr for the first 10 days and every 24 hr for the next 11 days.

**Blood bactericidal assay.** The anti-PspA<sub>hkR36A</sub> mAbs were analyzed by blood bactericidal assay as described previously (33). Briefly, human peripheral blood was collected using recombinant hirudin from yeast (100 U per ml blood) as anticoagulant. Pneumococci (500 cfu in 10 µl) were incubated with 235 µl of blood in the presence of either purified anti-PspA<sub>hkR36A</sub> mAb or corresponding isotype control (5 µl of 1 mg/ ml). Samples were incubated at 37ºC with rotation for 2 hr for D39 and 3 hr for BG8838. The surviving bacteria were enumerated by plating serial dilutions (in duplicate) on TSA plates and the mean values obtained were used to calculate bactericidal activity as described below. The data is presented as percent killing, which was arrived at using the formula: 

\[
\text{percent killing} = \left( \frac{\text{colony count with appropriate isotype control mAb} - \text{colony count with anti-PspA<sub>hkR36A</sub> mAb}}{\text{colony count with the appropriate isotype control mAb}} \right) \times 100.
\]

**Complement deposition assay.** The complement deposition assay was performed as described by Ochs et al with some modifications (14). Briefly, mid-logarithmic phase pneumococci (10<sup>7</sup> cfu) were washed with PBS and incubated with either anti-PspA<sub>hkR36A</sub> mAb or matched isotype control (100 µl of 20 µg/ ml) for 1 hr at room temperature. Treated pneumococci were incubated in 200 µl of normal human serum pooled from three healthy donors [diluted to 10% in Hank’s Balanced Salt Solution with Ca<sup>2+</sup> and Mg<sup>2+</sup> ions; Biological Industries, Israel] for 30 min at 37ºC. The mixture was incubated...
on ice for 10 min, washed with PBS and incubation continued with PBS-1% BSA containing mouse anti-human C3 mAb (diluted 1 in 50; Abcam, USA) for 45 min on ice. The bound anti-C3 antibody was detected using FITC-conjugate F(ab')2 fragment goat anti-mouse IgG + IgM (H + L) antibody (diluted 1 in 200). Pneumococci were fixed with 2% PFA for 10 min on ice and analyzed by flow cytometry.

Statistical analysis. Statistical analysis was done using GraphPad Prism version 6 software (GraphPad Software Inc., USA). The passive mouse protection data was compared using Log rank test. We used parametric Pearson's linear regression correlation analysis to evaluate possible correlation between anti-PspA<sup>16R36A</sup> mAb dependent deposition of complement on pneumococci and passive mouse protection. A p value of < 0.05 was considered statistically significant. *, p < 0.05; **, p < 0.01 and ***, p < 0.001.
RESULTS

Cross-reactivity and surface staining of anti-PspA<sup>hkR36A</sup> mAbs is restricted to

**PspA family 1.** We previously reported that the relative avidity of the primary IgG anti-
PspA polyclonal antibody was higher in serum of mice immunized with heat-killed R36A
compared to recombinant PspA<sup>R36A</sup> (30). In this study, we analyzed 18 IgG anti-
PspA<sup>hkR36A</sup> mAbs for the extent of cross-reactivity with recombinant PspA belonging to
the 6 PspA clades (i.e. PspA<sup>L82016</sup>, PspA<sup>R36A</sup>, PspA<sup>TIGR4</sup>, PspA<sup>ICP#56</sup>, PspA<sup>ATCC 6303</sup> and
PspA<sup>BG9300</sup>) by ELISA (Table 1). All the 18 mAbs reacted with PspAs representing
clades 1 and 2 (family 1). None of the mAbs bound PspAs representing families 2
(clade 3, 4, and 5) and 3 (clade 6) suggesting that the anti-PspA<sup>hkR36A</sup> mAbs were family
1 specific.

Next, we assessed whether these 18 anti-PspA<sup>hkR36A</sup> mAbs bind to the surface of
pneumococci. For this purpose, we tested strains BG8838 (clade 1), WU2 and D39
(clade 2). Of the 18 mAbs, BG8838 and WU2 were recognized by 9 and 10 mAbs,
respectively (Table 2). As expected, all 18 anti-PspA mAbs stained D39. Nine mAbs
bound one clade 1 and two clade 2 PspA expressing strains. Eight (i.e. B3D12, B3H8,
L5C8, L5F10, M4F4, P1E11, P2A4 and P2B5) of these were selected at random for
further analysis.

To confirm our observation that anti-PspA antibody response elicited against
heat-killed R36A was family 1 specific, anti-PspA<sup>R36A</sup> hyperimmune serum was used to
test surface binding with strains expressing PspA belonging to families 1, 2, and 3 using
flow cytometry (Fig. 1). The data demonstrates that anti-PspA<sup>R36A</sup> hyperimmune serum
binds to family 1 PspA expressing strains BG8838 (clade 1) and D39 (clade 2). No binding was observed with PspA family 2 bearing strains TIGR4 (clade 3), EF5668 (clade 4) and ATCC 6303 (clade 5) (Fig. 2). Similarly, there was no surface staining with PspA family 3 expressing strain BG6803 (clade 6). Thus, our flow cytometry data demonstrates that binding of anti-PspA<sup>hkR36A</sup> hyperimmune serum to pneumococcal strains was family 1 specific and this corroborates the data obtained with anti-PspA<sup>hkR36A</sup> mAbs (Table 2).

**In vivo protective efficacy of anti-PspA<sup>hkR36A</sup> mAbs.** The relative protective activity of anti-PspA<sup>hkR36A</sup> mAbs B3D12, B3H8, L5C8, L5F10, M4F4, P1E11, P2A4 and P2B5 was assessed by passive mouse protection assay using BG8838 (clade 1) and WU2 (clade 2) as the challenge strains. CBA/N mice were given either purified anti-PspA<sup>hkR36A</sup> or the corresponding isotype control mAb (high dose; 5 mg per kg body weight) intraperitoneally. One-hour later mice were challenged intravenously with either BG8838 or WU2. The mice survival data with BG8838 as the challenge strain (Fig. 2A, C and E) suggested that anti-PspA<sup>hkR36A</sup> mAbs M4F4, P1E11 and L5C8 provided 87.5%, 75% and 50% protection, respectively compare to the corresponding isotype control (12.5%; Fig. 2A). The mAbs L5F10, B3H8 and B3D12 provided 75%, 62.5% and 37.5% protection, respectively when challenged with BG8838 (Fig. 2C). All mice in the set that received IgG1 isotype control mAb died within 7 days. The mAbs P2A4 and P2B5 protected 100% and 62.5% of the mice, respectively compare to 12.5% in the case of the isotype control when challenged with BG8838 (Fig. 2E). In experiments where mice were challenged with WU2 (Fig. 2B, D and F), M4F4 and P1E11 provided 100%
L5C8 failed to confer any protection like the IgG1 isotype control mAb (Fig. 2B). L5F10 conferred 62.5% protection while B3H8 and B3D12 provided 25% or less protection when challenge with WU2 (Fig. 2D). All the mice in the corresponding control group died within 3.5 days. P2A4 provided 100% while P2B5 provided 87.5% protection when challenged with WU2 whereas the corresponding IgG2a isotype control mAb protected only 12.5% of the mice (Fig. 2F).

On the basis of results of the passive mouse protection experiments described above mAbs M4F4, P1E11, L5F10, P2A4 and P2B5 were selected as they conferred more than 50% protection against the 2 challenge strains tested. We repeated the experiments with a lower dose (1.25 mg per kg body weight) of the purified anti-PspA<sup>hkR36A</sup> mAbs and the corresponding isotype control. The mice survival data following challenge with BG8838 suggested that P1E11, M4F4 and L5F10 provided 75, 62.5 and 37.5% protection, respectively whereas all mice died in the control group within 8 days (Fig. 3A) while P2A4 and P2B5 provided 62.5 and 50% protection, respectively compared to IgG2a isotype control where 12.5% survival was observed (Fig. 3C). The mouse survival data with WU2 as the challenge strain suggested that P1E11, M4F4 and L5F10 provided 87.5, 75, and 37.5% protection, respectively (Fig. 3B) while P2A4 and P2B5 provided 100 and 75% protection, respectively (Fig. 3D). In both the experiments all control mice died within 3.5 days. The consolidated data from the passive protection experiments demonstrates that 4 anti-PspA<sup>hkR36A</sup> mAb M4F4, P1E11, P2A4 and P2B5 confer 50% or more protection against intravenous challenge with PspA clade 1 and 2 expressing strains.
Correlation between *in vivo* protection, and *in vitro* complement deposition and bactericidal activity. We were interested in finding out the possible correlation between *in vivo* protection with *in vitro* bactericidal activity and complement deposition capability of anti-PspA\(^{hkR36A}\) mAbs. We employed blood bactericidal assay to evaluate the ability of the 8 anti-PspA\(^{hkR36A}\) mAbs (i.e., B3D12, B3H8, L5C8, L5F10, M4F4, P1E11, P2A4 and P2B5) to kill PspA family 1 expressing strains BG8838 (clade 1) and D39 (clade 2). Pneumococci were incubated with blood from healthy donors in the presence of either purified anti-PspA\(^{hkR36A}\) or matched isotype control mAb and surviving pneumococci were enumerated by plating. The results suggest that all 8 anti-PspA\(^{hkR36A}\) mAbs exhibited significant bactericidal activity (ranging from 25.7 to 80.3% compared to the matched isotype control) against BG8838 and D39 though the extent of bactericidal activity varied from one mAb to the other (Fig. 4).

We next tested whether these mAbs can enhance C3 deposition on the surface of clade 1 (BG8838) and clade 2 (WU2 and D39) expressing strains (Table 3). Monoclonal antibodies M4F4, P1E11, P2A4 and P2B5 showed significant enhancement in the complement deposition on pneumococci compared to the corresponding isotype control for the 3 strains analyzed. Seven of the 8 mAbs augmented complement deposition on D39 with B3H8 being the exception. B3H8, however, enhanced deposition of complement on WU2. It was observed that the extent of enhancement in C3 deposition depended on the anti-PspA\(^{hkR36A}\) mAb and the target strain.

We wanted to find out which aspect or feature of the antibody response correlated with *in vivo* protection. The surface staining, complement deposition, bactericidal activity and mouse protection data for mAbs B3D12, B3H8, L5C8, L5F10,
M4F4, P1E11, P2A4 and P2B5 is summarized in Table 4. The trend from the data appears to be that the higher the extent of complement deposition, the higher the bactericidal activity and *in vivo* protection. This is well illustrated by mAbs M4F4, P1E11, P2A4 and P2B5. While all the protective mAbs showed bactericidal activity, not all mAbs that exhibited bactericidal activity showed passive protection. For example, mAbs B3D12, B3H8 and L5C8 exhibited significant bactericidal activity, but showed no to poor ability to confer protection when given passively to mice.

To determine whether *in vitro* mAb dependent deposition of complement correlated with passive mouse protection parametric Pearson's linear regression correlation analysis was performed. The analysis indicated that there was a positive correlation between *in vitro* complement deposition and passive protection for strains WU2 (Fig. 5) and BG8838 (data not shown). The correlation was highly statistically significant for strain WU2 (*r* = 0.8783, *P* = 0.0041). Our data suggests that the antibody dependent deposition of complement on the pneumococcal surface can be a potential *in vitro* correlate of protection for strains like WU2.
DISCUSSION

PspA is a likely candidate for a protein-based vaccine against pneumococcal infections; however, its serological variability could restrict the coverage of a PspA-based vaccine. For this reason, gaining insight into the nature of PspA’s variability has been the subject of several studies that are directed at developing a protein-based pneumococcal vaccine. Studies aimed at investigating the level of cross-reactivity among PspAs in mice indicate that antibodies generated against PspA show higher cross-reactivity with strains expressing PspA of the same family compared to strains that bear PspA of a different PspA family. We screened our panel to identify anti-PspA<sup>hkR36A</sup> mAbs that exhibited the maximum reactivity across PspA clades. Our ELISA and flow cytometry based surface staining data revealed that all the 18 anti-PspA mAbs (raised against clade 2 expressing strain R36A) recognized family 1 PspA and not PspAs representing families 2 and 3. This is consistent with the observation that mice immunized with DNA vaccine expressing the extracellular domain of PspA were protected from strains bearing PspA of the same clade when tested in an intraperitoneal challenge mouse model. Briles and coworkers however reported that human anti-PspA antibodies, when administered to mice, conferred protection against strains expressing PspA belonging to families 1 and 2 (29).

Antibody-dependent complement mediated phagocytosis is a well-established mechanism of pneumococcal clearance (22). Antibodies directed at pneumococci help in clearance by augmenting opsonization. Antibodies to PspA enhance complement deposition on the pneumococcal surface thereby contributing to their protective effect.
A previous report suggested that polyclonal sera against PspA from families 1 and 2 help in enhancing complement deposition (34). Our anti-PspA<sup>hkR36A</sup> mAbs M4F4, P1E11, P2A4 and P2B5 augmented complement deposition on the 3 PspA family 1 expressing strains analyzed (Table 3). The observed variation in the degree to which various anti-PspA<sup>hkR36A</sup> mAbs augmented complement deposition across strains may have to do with the chemical nature of the capsule on the target strain, thickness of capsule and relative accessibility of the pneumococcal surface.

There is evidence to suggest that not all antibodies to PspA are protective indirectly implying that not all PspA epitopes elicit protective antibody responses. In our previous work we had observed that P1E11 and P2A4 compete for binding with a PspA<sup>R36A</sup> subfragment corresponding to 193-286 amino acid stretch (PspA<sup>R36A 193-286</sup>) indicating that these two mAbs recognize the same or overlapping epitope (30). Thus, M4F4, P1E11, P2A4 and P2B5 recognize at least 3 topologically distinct epitopes. The epitopes recognized by P1E11, P2A4 and P2B5 were localized to PspA<sup>R36A 193-286</sup> subfragment. The epitope recognized by M4F4 was mapped to the subfragment PspA<sup>R36A 98-192</sup> (30). Our data is consistent with that reported by Roche <i>et al</i> who localized the cross-protection eliciting region of PspA to the N-terminal 115 amino acid residues and ~104 C-terminal residues of the extracellular domain from strain EF3296 (12). Knowledge of the epitopes recognized by anti-PspA<sup>hkR36A</sup> mAbs that do and which do not elicit protective responses could be put to use to engineer a PspA vaccine that maximizes the proportion of protective antibodies in the antibodies generated. Fine mapping the conserved B cell epitopes recognized by protective anti-PspA<sup>hkR36A</sup> mAbs M4F4, P1E11, P2A4 and P2B5 can help in developing a superior PspA-based vaccine.
Our data indicate that complement deposition on pneumococci can be a used as a surrogate for *in vivo* protection for strains like WU2 (Fig. 5). Roche and workers had demonstrated that antibody titer and surface staining do not correlate with *in vivo* protection, and thus are not useful as a surrogate for protection (12). Cohen *et al* reported that whole cell ELISA is an inadequate predictor of *in vivo* protection (11). While the modified surface killing assay for PspA developed by Genschmer *et al* is likely to be significant (9), complement deposition as an *in vitro* correlate of protection is easier to perform and amenable to automation. The complement deposition assay could be potentially useful in quantitating the relative protective efficacy of antibodies against novel protein vaccine antigens (or their subfragments) to confer protection *in vivo.*
This work was supported in part by the intramural Research program of the National Institute of Immunology and a grant (BT/PR5037/MED/15/77/2012) from the Department of Biotechnology (DBT), Government of India. Naeem Khan was a recipient of Senior Research Fellowship from DBT.
REFERENCES


serologically highly variable and is expressed by all clinically important capsular

16. **Yother J, Briles DE.** 1992. Structural properties and evolutionary relationships of
PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence


18. **Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S,
Immunization of healthy adults with a single recombinant pneumococcal surface
protein A (PspA) variant stimulates broadly cross-reactive antibodies to
heterologous PspA molecules. Vaccine 18:1743-1754.

and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun
68:5889-5900.

20. **Hotomi M, Togawa A, Kono M, Ikeda Y, Takei S, Hollingshead SK, Briles DE,
Suzuki K, Yamanaka N.** 2013. PspA family distribution, antimicrobial resistance
and serotype of *Streptococcus pneumoniae* isolated from upper respiratory tract

Pneumococcal surface protein A (PspA) family distribution among clinical
isolates from adults over 50 years of age collected in seven countries. J Med


surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infect Immun 78:2163-2172.


34. **Ren B, Szalai AJ, Thomas O, Hollingshead SK, Briles DE.** 2003. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer
virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. Infect Immun 71:75-85.
**FIGURE LEGENDS**

**FIG 1** Surface binding of anti-PspA<sup>R36A</sup> polyclonal sera with pneumococcal strains representing the 6 clades of PspA. Surface binding with strains BG8838 (A), D39 (B), TIGR4 (C), EF5668 (D), ATCC 6303 (E) and BG6803 (F) was analyzed by flow cytometry using anti-PspA<sup>R36A</sup> polyclonal and pre-immune sera. Pre-immune serum (solid grey histogram) was used as the negative control. The GMFI values for the anti-PspA<sup>R36A</sup> polyclonal and pre-immune sera are shown. GMFI values ≥ 2 times the value obtained with the pre-immune sera were considered significant.

**FIG 2** Relative efficacy of anti-PspA<sup>hkR36A</sup> mAbs to protect mice against intravenous challenge. CBA/N mice were injected with purified anti-PspA<sup>hkR36A</sup> mAb M4F4, P1E11 or L5C8 (A and B), B3D12, B3H8 or L5F10 (C and D) and P2A4 or P2B5 (E and F) intraperitoneally at 5 mg per kg body weight (high dose). The corresponding isotype control mAb (IgG1 IC or IgG2a IC) was included in each set as the negative control. An hour later, mice were challenged with 10<sup>7</sup> CFU of BG8838 (A, C and E) or 10<sup>3</sup> CFU of WU2 (B, D and F), and mouse survival was recorded. The data for the group given anti-PspA<sup>hkR36A</sup> mAb was compared with the respective isotype control mAb using log-rank test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, statistically not significant.

**FIG 3** Anti-PspA<sup>hkR36A</sup> mAbs protect mice against pneumococcal infection even when given at a lower dose. CBA/N mice were injected intraperitoneally with 1.25 mg per kg body weight (low dose) of either anti-PspA<sup>hkR36A</sup> mAb M4F4, P1E11 or L5F10 (A and B),
and P2A4 or P2B5 (C and D). The control group was given the respective isotype control mAb (IgG1 IC or IgG2a IC). Mice were challenged with BG8838 (A and C) or WU2 (B and D) 1 hr later, and mouse survival was recorded. For other details refer to legend to Fig. 2.

**FIG 4** Anti-PspA<sup>hkR36A</sup> mAbs promote pneumococcal killing by human blood *in vitro*. *S. pneumoniae* strains BG8838 (A and B) and D39 (C and D) were incubated with blood from healthy donors and purified anti-PspA<sup>hkR36A</sup> or matched isotype control mAb. Matched IgG1 and IgG2a isotype control mAbs were used as the comparator in panels A and C, and B and D, respectively (not shown). The contents were rotated for (3 hrs for BG8838 and 2 hrs for D39) at 37°C and the surviving pneumococci were enumerated by plating. The identity of the anti-PspA<sup>hkR36A</sup> mAb and the percent killing are plotted on the X- and Y-axis, respectively. The percent bacterial killing was calculated as described in the Materials and Methods. The assay was performed at least four times and data from a single representative experiment is shown.

**FIG 5** Analysis of 8 anti-PspA<sup>R36A</sup> mAbs to assess possible correlation between mAb dependent complement deposition with their ability to passively protect mice from otherwise lethal challenge with strain WU2. Each dot represents an anti-PspA<sup>R36A</sup> mAb. The correlation between *in vitro* complement deposition and passive protection was highly significant by parametric Pearson’s linear regression correlation analysis ($r = 0.8783, P = 0.0041$).
Fluorescence intensity

Relative cell number

A B C
D E F
BG8838 (clade 1) D39 (clade 2) TIGR4 (clade 3)
EF5668 (clade 4) ATCC 6303 (clade 5) BG6803 (clade 6)

Pre-immune sera
Anti-PspA (R36A)
polyclonal sera

12.2
5.1
12.3
3.3
7.2
5.9
8.3
10.2
7.3
7.8
4.2
2.6

Fluorescence intensity
$r = 0.8783$
$P = 0.0041$
**TABLE 1** ELISA based analysis for reactivity of anti-PspA^{hkR36A} mAbs with recombinant PspAs representing the 6 PspA clades

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Pneumococcal strain (PspA family/ clade)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L82016 (1/1)</td>
</tr>
<tr>
<td>B3D12</td>
<td>16.5</td>
</tr>
<tr>
<td>B3H8</td>
<td>1.8</td>
</tr>
<tr>
<td>L5C8</td>
<td>17.1</td>
</tr>
<tr>
<td>L5F10</td>
<td>19.5</td>
</tr>
<tr>
<td>M4F4</td>
<td>30.0</td>
</tr>
<tr>
<td>P1E11</td>
<td>29.2</td>
</tr>
<tr>
<td>D1A5</td>
<td>10.6</td>
</tr>
<tr>
<td>K1B12</td>
<td>15.9</td>
</tr>
<tr>
<td>M6B2</td>
<td>13.5</td>
</tr>
<tr>
<td>P2F9</td>
<td>9.8</td>
</tr>
<tr>
<td>P2A4</td>
<td>21.6</td>
</tr>
<tr>
<td>P2B5</td>
<td>22.0</td>
</tr>
<tr>
<td>J4C1</td>
<td>28.2</td>
</tr>
<tr>
<td>P2C2</td>
<td>16.2</td>
</tr>
<tr>
<td>A1D9</td>
<td>2.0</td>
</tr>
<tr>
<td>C4B4</td>
<td>18.1</td>
</tr>
<tr>
<td>F4B6</td>
<td>24.7</td>
</tr>
<tr>
<td>D3H6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The culture supernatants from the 18 anti-PspA^{hkR36A} mAb secreting hybridomas were tested for reactivity with recombinant PspAs representing the 6 clades of PspA (extracellular domain with or without proline rich region) by ELISA. The numerical values represent fold change in absorbance relative to the control. Fold change values greater than twice the value obtained with the control were considered significant (columns shaded gray).
### TABLE 2  Surface binding of anti-PspA\textsuperscript{hkR36A} mAbs with strains expressing family 1 PspA

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Geometric mean fluorescence intensity (Fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BG8838 Clade 1</td>
</tr>
<tr>
<td>IgG1 IC</td>
<td>4.77 (1.0)</td>
</tr>
<tr>
<td>B3D12</td>
<td>13.10 (2.8)</td>
</tr>
<tr>
<td>B3H8</td>
<td>14.70 (3.1)</td>
</tr>
<tr>
<td>L5C8</td>
<td>18.70 (3.9)</td>
</tr>
<tr>
<td>L5F10</td>
<td>18.90 (4.0)</td>
</tr>
<tr>
<td>M4F4</td>
<td>59.50 (12.5)</td>
</tr>
<tr>
<td>P1E11</td>
<td>81.80 (17.2)</td>
</tr>
<tr>
<td>D1A5</td>
<td>4.04 (0.8)</td>
</tr>
<tr>
<td>K1B12</td>
<td>3.86 (0.8)</td>
</tr>
<tr>
<td>M6B2</td>
<td>5.64 (1.2)</td>
</tr>
<tr>
<td>P2F9</td>
<td>4.80 (1.0)</td>
</tr>
<tr>
<td>IgG2a IC</td>
<td>4.82 (1.0)</td>
</tr>
<tr>
<td>P2A4</td>
<td>23.60 (4.9)</td>
</tr>
<tr>
<td>P2B5</td>
<td>17.60 (3.7)</td>
</tr>
<tr>
<td>J4C1</td>
<td>3.88 (0.8)</td>
</tr>
<tr>
<td>P2C2</td>
<td>3.52 (0.7)</td>
</tr>
<tr>
<td>IgG2b IC</td>
<td>4.16 (1.0)</td>
</tr>
<tr>
<td>A1D9</td>
<td>4.35 (1.1)</td>
</tr>
<tr>
<td>C4B4</td>
<td>10.80 (2.6)</td>
</tr>
<tr>
<td>F4B6</td>
<td>3.70 (0.89)</td>
</tr>
<tr>
<td>IgG3 IC</td>
<td>4.26 (1.0)</td>
</tr>
<tr>
<td>D3H6</td>
<td>4.41 (1.0)</td>
</tr>
</tbody>
</table>
Surface binding of 18 anti-PspA\textsuperscript{hkR36A} mAbs with strains BG8838 (clade 1), and WU2 and D39 (clade 2) was analyzed by flow cytometry. Strain D39 was included in the analysis as the anti-PspA mAbs were raised against R36A, an unencapsulated derivative of D39. Pneumococci were incubated with 200 μl culture supernatant from the 18 anti-PspA\textsuperscript{hkR36A} mAb secreting hybridomas followed by staining with appropriate FITC-conjugated secondary antibody. Isotype matched control mAbs were included and samples were analyzed using a flow cytometer. The surface binding is expressed as geometric mean fluorescence intensity (GMFI) and the fold change relative to the corresponding isotype control mAb (assigned 1.0) is given in parentheses. A two or more fold increase in the surface staining relative to the corresponding isotype control was considered significant. IgG\textsubscript{1} IC, IgG\textsubscript{2a} IC, IgG\textsubscript{2b} IC and IgG\textsubscript{3} IC represent isotype control mAbs for the isotypes IgG\textsubscript{1}, IgG\textsubscript{2a}, IgG\textsubscript{2b} and IgG\textsubscript{3}, respectively.
The PspA family 1 expressing strains BG8838 (clade 1), and D39 and WU2 (clade 2) were incubated with either anti-PspA<sup>hkR36A</sup> or matched isotype control mAb. The bound complement C3 was detected by flow cytometry using anti-human C3 antibody followed by appropriate FITC-conjugated secondary antibody. Complement deposition is expressed as GMFI values.

<sup>a</sup> Pneumococci incubated with anti-human complement C3 antibody followed by FITC-conjugated secondary antibody.

<sup>b</sup> Pneumococci incubated with 10% pooled normal human serum in Hank’s Balanced Salts Solution followed by anti-human C3 antibody and FITC-conjugated secondary antibody.

<sup>c</sup> These anti-PspA<sup>hkR36A</sup> mAbs are of IgG<sub>1</sub> isotype.

<sup>d</sup> These anti-PspA<sup>hkR36A</sup> mAbs are of IgG<sub>2a</sub> isotype.

<sup>e</sup> GMFI values greater than or equal to twice the value obtained with the corresponding isotype control were considered to be significant.
TABLE 4  In vivo protective efficacies of anti-PspA<sup>hkR36A</sup> mAbs correlates with the extent of complement deposition

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain</th>
<th>Anti-PspA&lt;sup&gt;hkR36A&lt;/sup&gt; mAb</th>
<th>B3D12</th>
<th>B3H8</th>
<th>L5C8</th>
<th>L5F10</th>
<th>M4F4</th>
<th>P1E11</th>
<th>P2A4</th>
<th>P2B5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface staining (x)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG8838</td>
<td>2.8</td>
<td>3.1</td>
<td>3.9</td>
<td>4.0</td>
<td></td>
<td></td>
<td>12.5</td>
<td>17.2</td>
<td>4.9</td>
<td>3.7</td>
</tr>
<tr>
<td>WU2</td>
<td>1.4</td>
<td>1.5</td>
<td>1.0</td>
<td>1.9</td>
<td></td>
<td></td>
<td>3.2</td>
<td>3.8</td>
<td>3.9</td>
<td>5.7</td>
</tr>
<tr>
<td>D39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.7</td>
<td>2.4</td>
<td>37.3</td>
<td>18.1</td>
<td></td>
<td></td>
<td>38.2</td>
<td>39.8</td>
<td>12.8</td>
<td>7.3</td>
</tr>
<tr>
<td>Complement deposition (x)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.7</td>
<td>15.2</td>
<td>3.2</td>
<td>8.6</td>
</tr>
<tr>
<td>BG8838</td>
<td>1.9</td>
<td>2.0</td>
<td>1.6</td>
<td>0.9</td>
<td></td>
<td></td>
<td>150.6</td>
<td>138.1</td>
<td>88.9</td>
<td>108.6</td>
</tr>
<tr>
<td>WU2</td>
<td>1.2</td>
<td>6.4</td>
<td>1.1</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D39</td>
<td>59.7</td>
<td>1.8</td>
<td>73.7</td>
<td>73.0</td>
<td></td>
<td></td>
<td>81.1</td>
<td>74.7</td>
<td>70.3</td>
<td>62.9</td>
</tr>
<tr>
<td>Bactericidal activity (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG8838</td>
<td>25.7</td>
<td>71.2</td>
<td>68.6</td>
<td>53.5</td>
<td></td>
<td></td>
<td>71.7</td>
<td>58.2</td>
<td>34.3</td>
<td>60.8</td>
</tr>
<tr>
<td>D39</td>
<td>55.4</td>
<td>39.6</td>
<td>66.8</td>
<td>54.8</td>
<td></td>
<td></td>
<td>80.3</td>
<td>60.4</td>
<td>66.5</td>
<td>51.9</td>
</tr>
<tr>
<td>Mouse passive protection assay (5 mg/kg) (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>87.5</td>
<td>75.0</td>
<td>100.0</td>
<td>62.5</td>
</tr>
<tr>
<td>BG8838</td>
<td>37.5</td>
<td>62.5</td>
<td>50.0</td>
<td>75.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WU2</td>
<td>12.5</td>
<td>22.5</td>
<td>0.0</td>
<td>62.5</td>
<td></td>
<td></td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>87.5</td>
</tr>
<tr>
<td>Mouse passive protection assay (1.25 mg/kg) (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>62.5</td>
<td>75.0</td>
<td>62.5</td>
<td>50.0</td>
</tr>
<tr>
<td>BG8838</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>37.5</td>
<td></td>
<td></td>
<td>62.5</td>
<td>75.0</td>
<td>100</td>
<td>75.0</td>
</tr>
<tr>
<td>WU2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>37.5</td>
<td></td>
<td></td>
<td>75.0</td>
<td>87.5</td>
<td>100</td>
<td>75.0</td>
</tr>
</tbody>
</table>

BG8838 expresses clade 1 PspA while WU2 and D39 bear PspA of clade 2. The 4 anti-PspA<sup>hkR36A</sup> mAbs that were the most protective are highlighted (columns shaded gray). ND, Not determined.
Surface staining and complement deposition are expressed as fold change (x) in GMFI value relative to the corresponding isotype control.

Bactericidal activity of the anti-PspA^{R36A} mAb is represented as percent killing. A higher value indicates higher bactericidal activity. The assay was not performed with strain WU2.

Mouse protection is expressed as percent survival. A higher value indicates higher protective efficacy. This experiment was not done with strain D39.