Vaccine 32 (2014) 39–47

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A modified surface killing assay (MSKA) as a functional *in vitro* assay for identifying protective antibodies against pneumococcal surface protein A (PspA)

Kristopher R. Genschmer[a], Mary Ann Accavitti-Loper[b], David E. Briles[a,b,c,*]

[a] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, United States
[b] Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, United States
[c] Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294, United States

ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form 8 October 2013
Accepted 24 October 2013
Available online 6 November 2013

Keywords:
Streptococcus pneumoniae
Pneumococcal surface protein A
PspA
Surface killing assay
Polymorphonuclear leukocytes
Functional assay

ABSTRACT

Streptococcus pneumoniae causes otitis media, meningitis and pneumonia in patients worldwide; predominantly affecting young children, the elderly, and the immune compromised. Current vaccines against invasive pneumococcal disease are based on the polysaccharide capsules of the most clinically relevant serotypes. Due to serotype replacement, non-vaccine serotypes of *S. pneumoniae* have become more clinically relevant and as a result pneumococcal vaccines are becoming increasingly complex. These events emphasize the need to evaluate the potential for pneumococcal cross-reactive proteins to contribute to future vaccines. Antibody elicited by the immunization of humans with pneumococcal surface protein A (PspA) can passively protect mice from infection. However, robust *in vitro* functional assays for antibody to PspA are not available to predict the protective capacity of immune serum. For polysaccharide based vaccines, a standardized opsonophagocytosis killing assay (OPKA) is used. Antibody to PspA, however, does not work well in the standard OPKA. The present studies take advantage of past observations that phagocytosis is more efficient on tissue surfaces than in solution. In a modified surface killing assay (MSKA), monoclonal antibody to PspA, in the presence of complement, opsonized pneumococci for killing by phagocytes on an agar surface. Five monoclonal antibodies to PspA were tested; three demonstrated increased amounts of killing compared to the diluent control and protected mice by passive protection against type 3 pneumococci. The two antibodies that were not functional in the MSKA also failed to protect mice. Thus, an MSKA might be useful as a functional assay for immunity to PspA.

1. Introduction

Pneumonia and pneumonia related illnesses are the number one cause of mortality in children under the age of five worldwide resulting in close to 20 percent of all deaths in this age group [1]. *Streptococcus pneumoniae* is the number one cause of serious pneumonia and can also cause meningitis, sepsis, and otitis media. In some developing countries, including some in Africa, *S. pneumoniae* can account for over 50% of all pneumonia deaths [2]. There are over 90 different capsular serotypes of *S. pneumoniae* based on the structure of the polysaccharide capsules [3]. Pneumococci possess a number of virulence factors, including its polysaccharide capsule [4]. The capsule enables the pneumococci to evade entrapment by the mucus secretions that line the nasal cavity, which is usually the first surface of the host the bacterium encounters [5,6]. The capsule also helps protect the pneumococcus against opsonization and killing by phagocytes [7–9]. A 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in the US in 2000 and more recently, in 2010, a 13-valent pneumococcal conjugate vaccine (PCV13) was licensed. The use of PCV7 greatly reduced the number of bacteremic pneumococcal infections in immunized children with the 7 vaccine types [10–12]. However, the use of PCV7 was associated with an increase in incidence of pneumococcal disease caused by serotypes not included in PCV7 [13–15]. Because of serotype replacement and the high cost of the conjugate vaccines, interest has increased for the development of a vaccine containing cross-reactive proteins that that could protect against multiple serotypes [16] and could be inexpensive enough to produce to be used in the developing countries without the need for large international subsidies [17].

S. pneumoniae possesses various surface-associated proteins that contribute to its virulence and many of which are able to elicit measurable protection in mice. Pneumococcal surface protein A (PspA) is present on almost all strains of pneumococci and has been shown to be good a candidate antigen for a protein-based

* Corresponding author at: Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, United States. Tel.: +1 205 934 1880; fax: +1 205 934 0605.
E-mail address: dbriles@uab.edu (D.E. Briles).

0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.10.080

vaccine [18–22]. Antibodies to PspA elicited in humans and animals can protect mice from challenge when given passively [23,24]. One problem with the development of vaccines based on PspA and other protein antigens is that quantitative *in vitro* functional assays do not exist that can predict the protective effects of the elicited antibody.

For antibody to pneumococcal capsular polysaccharides, an opsonophagocytic killing assay (OPKA) has been developed that is valuable as an *in vitro* surrogate that can evaluate the potential protective effects of conjugate vaccines [25–27]. The OPKA can evaluate the ability of human immune sera from immunized patients to opsonize pneumococci for killing by phagocytes in the presence of complement. The standard UAB OPKA [25,28] is relatively specific for antibody to capsular polysaccharide but does not efficiently detect protective antibody to PspA without the addition of sub-protective levels of anti-capsule antibody (Daniels et al. submitted). Thus, it was important to devise an *in vitro* functional assay to see if antibody to PspA could, by itself, mediate complement-dependent, phagocyte-dependent killing of wild-type pneumococci.

Weinberger et al. described the ability of neutrophils to phagocytose and kill bacteria on the surface of agar plates in the absence of antibody and complement [29]. While the observation that surface phagocytosis is extremely efficient is not new [30], Weinberger et al. revealed that in the absence of antibody and complement, bacteria of capsular types with larger capsules were less likely to be phagocytized and killed in a surface killing assay, similar to the observations made by Kim et al. in regards to bacteria in suspension [31]. We have used the fact that pneumococci with the type three capsule able to significantly resist surface phagocytosis in the absence of antibody and complement, to develop a complement-dependent assay to detect the protective effects of antibody to PspA. In this "Modified Surface Killing Assay" (MSKA) we have used human PMNs, and complement obtained from unimmunized mice to evaluate the functional activity of monoclonal antibodies (MAbs) to PspA.

2. Materials and methods

2.1. Bacterial strains

The highly virulent type 3 pneumococcal strain A66.1, strain BR240.1 (a PspA$^-$ A66.1), the type 2 strain D39, and the type 4 strain TIGR4 [32–34] were all grown separately in Todd-Hewitt broth containing 0.5% added yeast extract (THY) at 37 °C until they reached an optical density at 600 nm of 0.40–0.45. The bacterial stocks were then washed and resuspended in THY supplemented with 8% glycerol, aliquoted in 1 mL volumes, and stored at −80 °C until used. The exact CFUs/mL of these stocks was determined after the aliquots had been frozen for two weeks by plating a single quick-thawed aliquot on 5% sheep's blood TSA agar plates (Becton, Dickinson and Company). The calculated bacterial concentration was used subsequently to make dilutions for experiments from aliquots thawed at later times. In each experiment the actual concentration of CFU injected was determined by plating on blood agar at the time of the assay.

2.2. Monoclonal antibodies

Table 1 lists the monoclonal antibodies used. MAb 16.3 is a mouse IgG3 monoclonal antibody against capsule type 3 and was generated and shown to protect mice from type 3 infection as described previously [35,36]. MAb XiR278 is a mouse IgG1 monoclonal antibody raised against clade 2 PspA from a non-encapsulated mutant of *S. pneumoniae* strain D39 that expresses the same PspA as D39 mutant Rx1 [37]. Strains of the same clade show >78% amino acids sequence homology with each other in the clade determining region of the alpha helical region of PspA. Strains of the same family show >65% sequence homology in the "clade determining region of PspA [38]. MAbs 8b2.19, 1b2.21, 5c6.1, and 6e5.5 were all produced for this study from spleen cells of mice immunized with a mixture of the α-helical domains of PspA from strains Rx1 (family 1, clade 2), EF3296 (family 2, clade 3), and EF5668 (family 2, clade 4) by the University of Alabama at Birmingham Epitope Recognition and Immunoreagent Core Facility. Of the three immunogens, MAbs 8b2.19 and 1b2.21 reacted with the purified clade 2 (Rx1) PspA on western blot, but not purified clade 3 EF3296 PspA. MAbs 5c6.1 and 6e5.5 reacted with purified clade 3 EF3296 PspA on western blot [39], but not with clade 2 Rx1 PspA. None of the MAbs reacted with clade 4 PspA of strain EF5668. MAbs to PspA are specific for individual epitopes and bind these epitopes on diverse PspAs, but are generally neither PspA family nor clade specific [38]. All three MAbs that reacted with clade 2 Rx1 also reacted with clade 2 A66.1. The two MAbs that recognized clade 3 EF3296 failed to react with the PspA of A66.1.

Table 1
Monoclonal antibodies used.

Monoclonal antibody	Antibody reactivity	Reference
16.3	Type 3 polysaccharide capsule	[35,36]
1b2.21	PspA of strains Rx1 and A66.1[a]	This paper
8b2.19	PspA of strains Rx1 and A66.1	This paper
5c6.1	PspA of strain EF3296[a]	This paper
6e5.5	PspA of strain EF3296	This paper
XiR278	PspA of strains Rx1 and A66.1	[37]

[a] The PspA of strain Rx1 is in family 1 clade 2, the PspA of strain EF3296 is in family 2 clade 3 PspA. The MAbs each bind an epitope on one of these two PspA. However, since all epitopes on any PspA clade or family [38] are not found in all clades or families of the same designation, these MAbs are not necessarily clade or family specific.

The antibodies were all used as diluted ascites fluid to avoid any potential denaturation that might results from purification and concentration of the MAbs. Dilutions were in Ringer's Infection Solution (Abbot Labs, Chicago). To determine the concentration of each antibody in the respective ascites fluids, quantitative microzone electrophoresis was used to separate the protein components of the ascites on nitrocellulose [40]. The protein bands were stained using Ponceau S red [41] and subjected to densitometry of each band. The total amount of protein contained in each ascites fluid was determined using a Bio-Rad protein quantification assay. Amount of monoclonal antibody in the ascites was calculated by determining the fraction of the total densitometry of the scan that was contained in the monoclonal band in the gamma globulin region and multiplying this fraction times the total μg protein/mL in the ascites fluid. MAb concentrations were expressed in (μg/mL). This approach was based on the fact that in mice with MAb-containing ascites, the vast majority of immunoglobulin is comprised of the MAbs.

2.3. Human neutrophil purification

10 mL of whole blood was collected and 5 mL of blood was layered on top of 5 mL of Polymorphprep™ density gradient media (Axis-Shield) in a 15 mL conical tube and centrifuged for 30 min at 2500 rpm. The layer containing the polymorphonuclear cells (PMNs) was pipetted out of each of the two tubes and combined in a clean 15 mL conical tube. 14 mL 1× Phosphate Buffer Saline (PBS) was added to wash cells and tube was centrifuged for 5 min at 1500 rpm. PBS was decanted and 1 mL double distilled water was added to lyse any remaining red blood cells then 13 mL of PBS was added and tube spun again. Wash process repeated twice more or until no red blood cells remained. Next the PMNs were

resuspended in Hank's Buffered Salt Solution supplemented with 1% bovine serum albumin, counted in a hemocytometer, and diluted to a concentration of 2×10^6 PMNs/mL.

2.4. Modified surface killing assay

A66.1 or BR240.1 bacterial stocks were thawed and diluted to a concentration of 5×10^3 CFU/mL in Hanks Balanced Salt Solution supplemented with 1% Bovine Serum Albumin (BSA). MAbs to be tested were also diluted in HBSS with 1% BSA to the desired concentrations. 200 μL of the bacterial solution was added to each tube along with 80 μL of the desired MAb dilution in 1× PBS (or 80 μL of 1× PBS for the no-antibody control). Tubes were then incubated with shaking for 30 min at 37 °C. 20 μL of the complement source, naïve BALB/ByJ mouse serum (NMS), was added to each tube. Tubes were then incubated for an additional 30 min with shaking at 37 °C.

After incubation, 15 μL of the bacteria and MAb suspension was then placed on a trypticase soy agar with 5% sheep blood plate in six spots. In each experiment, with each target strain of pneumococci, there was also a blood plate with 6-total spots that contained complement and bacteria only; no antibody. This was the control plate. All spots were then allowed to "soak" into the plate *via* air drying at room temperature. Once spots were dry, a faint outline of them remained on the agar surface. Next, 20 μL of the 2×10^6 PMNs/mL (in 1%BSA in 1× HBSS) were placed over 3 of the six spots so that each original spot was entirely covered with the PMNs solution. These PMNs spots were also given time to "soak in" *via* air drying. Once all spots had soaked in, the plates were incubated for 10 h at 37 °C in 5% CO_2. Longer incubation was avoided to keep the colonies from growing together. Growth was stopped by moving the plates to the lab bench.

The following day all CFUs in each spot were counted under 2× magnification and percent killing was tabulated. To calculate the background killing for experiment, the average remaining CFUs counted on the plate with no antibody were averaged and then compared with each individual spot count with CFU remaining, using the following equations:

C = CFU remaining in C' + PMN control (also contains PMN)

E = CFU remaining in presence of C' + PMN + antibody

C^i and E^i are individual replicate points.

% Background Killing for individual plating replicates

$$= \frac{C_{ave} - C^i}{C_{ave}} \times 100$$

% Increase in killing in the presence of antibody over C' + PMN for individual plating replicates $= \frac{C_{ave} - E^i}{C_{ave}} \times 100$

Each spot was plotted on a graph and reported as percent increase in killing over C'-only control. Within each assay each data point was examined in sextuplicate. Each experiment was repeated in at least three times, utilizing different PMN donors and the data reported was an average of the results from the three or more experiments. Results with different donors were generally similar but never exactly the same. As a result the reported data takes into account the variation in human neutrophil phagocytic activity among donors.

In the MSKA assay, the amount of killing was probably dependent not only on the antibody and complement but also on whether a PMN was in the proximity of each pneumococcal target. Thus, the maximum killing probably was much less than 100% as the assay has been performed here. In all experiments there were 2 duplicate plates for each antibody concentration, resulting in six data points for each antibody concentration. To determine if the killing observed with a given concentration of MAbs was statistically significant the Mann–Whitney test was used to compare all 6 data points with antibody to those the C'-only control that lacked antibody.

In an attempt to shorten the total assay time, we tried adding the complement immediately prior to plating, instead of incubating with complement for 30 min with shaking. While there was still an antibody-dependent increase in killing, the average killing was around 25–30% less at the highest MAb dilutions (data not shown), compared to the standard method described above.

2.5. Use of cytochalasin-D to inhibit phagocytosis

To inhibit the phagocytic activity of the purified human neutrophils, the neutrophils were subjected to cytochalasin-D to inhibit actin polymerization. Following published protocols, 10 μg/mL cytochalasin-D was added to the purified neutrophil suspension for 30-min to inhibit phagocytosis [42].

2.6. Passive protection against lethal sepsis

Each passive immunization test was performed by injecting the mouse intraperitonealy with 100 μl ascites fluid that contains 20 μg (or other dilution as indicated) of the MAb to be tested diluted in Ringer's injection solution. Control mice received 1:6 dilution of pooled normal mouse serum (NMS); adjusted to approximate the amount of total albumin in our ascites fluids. Four hours later the mice were challenged i.v. with A66. *S. pneumoniae*. CBA/N mice were challenged with 400–450 CFU of A66.1 and C57BL/6 mice were challenged with 1×10^5 CFU of A66.1. Infected mice were monitored every six hours to determine the time-until-moribund. Each experiment ended after 14 days or when all mice became moribund. Mice were considered moribund when their body temperatures were 26 °C or lower or they were not responsive to touch. Mann–Whitney test was used to compare the hours to moribund for each MAb immunized group with the hours to moribund for the control group. Surviving mice were assigned 14.5 days as their time to moribund for calculation of *p*-values. All moribund mice and mice surviving 14 days were euthanized by CO_2 narcosis, and subsequently cervically dislocated. All animal protocols were conducted in accordance with AAALAC guidelines approved by the UAB IACUC.

3. Results

MAb to capsule type 3 polysaccharide increases killing of A66.1 in MSKA. Since MAb 16.3 is known to be able to protect mice against otherwise fatal sepsis caused by type 3 pneumococci [35], it was used as an initial test of the MSKA using target strain A66.1. As seen in Fig. 1A, MAb 16.3 was able to facilitate a significant increase in killing of the bacteria on the surface of the agar at concentrations of the MAb from 100 μg/mL down to 0.1 μg/mL. However at 0.01 μg/mL MAb 16.3 failed to show a statistically significant increase in killing over the C'-only control. The increase in killing over the C'-only control with MAb 16.3 was as high as around 44% for the 100, 30% at 10 μg/mL and around 18% for the 1 and 0.1 μg/mL concentrations.

MAb to clade 2 PspA increases killing of A66.1 in MSKA. A66.1 *pneumococcus* possesses family 1 clade 2 PspA on its surface. To determine if the MSKA would be able to detect killing mediated by Ab to PspA, A66.1 bacteria were incubated with MAbs to PspA in the MSKA. Two MAbs to clade 2 PspA were chosen for testing, 1b2.21 and 8b2.19. Both of these MAb ascites were used in the MSKA at 100 μg/mL, and at four sequential 1:10 serial dilutions. As

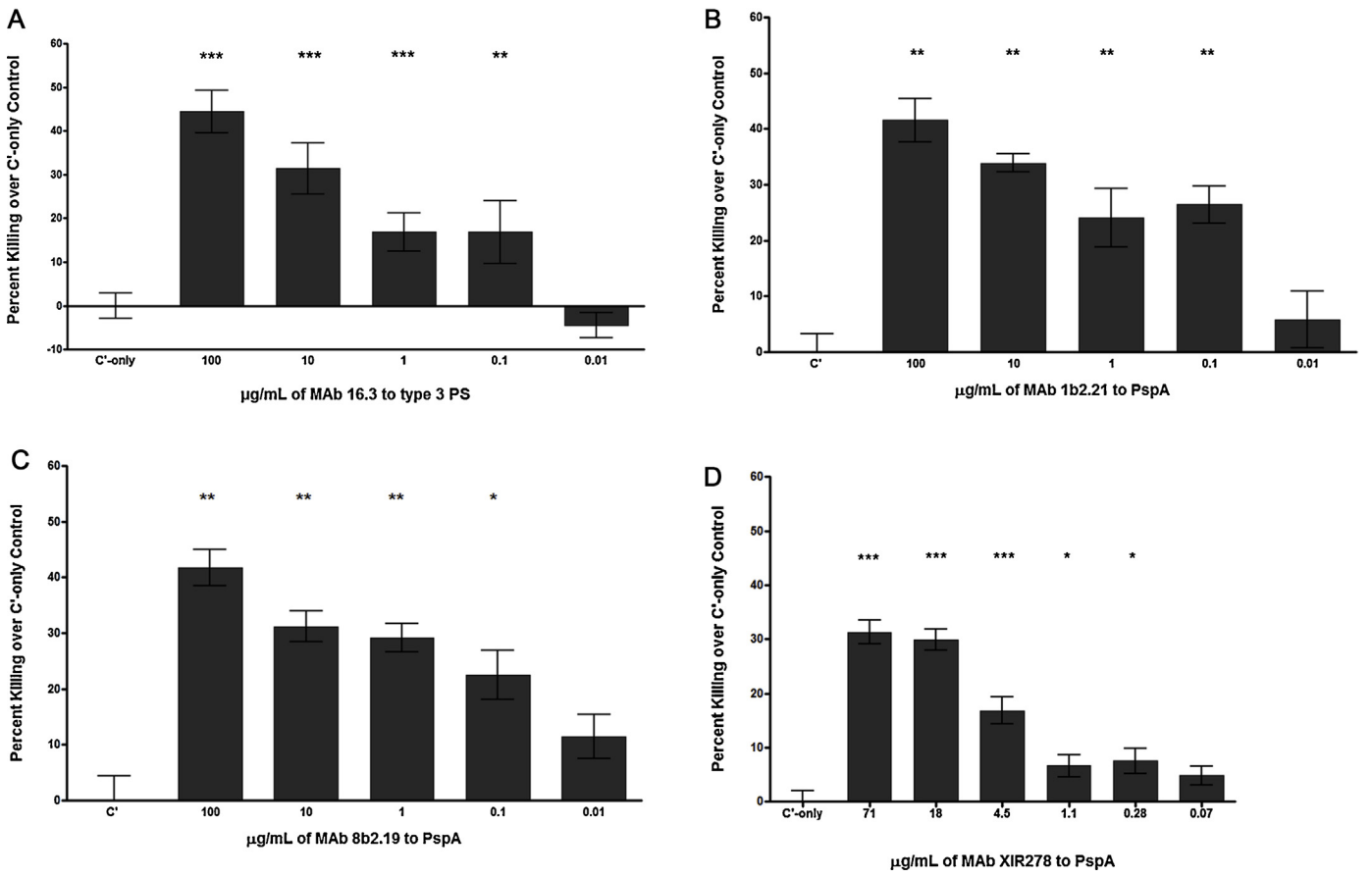

Fig. 1. (A) Killing of type 3 strain A66.1 in the MSKA is mediated by anti-type 3 PS MAb 16.3. Significant killing was not seen at concentrations of MAbs <0.1 μg/mL. Significance was determined by comparing each dilution to C'-only control. ***, $p < 0.001$; **, $p < 0.01$. All other comparisons had $p \geq 0.05$. Killing of type 3 strain A66.1 in the MSKA is mediated and anti-PspA MAbs (B) 1b2.21 and (C) 8b2.19, which each react with A66.1 PspA. Significance was determined by comparing results with each MAb concentration to results with C'-only control. *, $p < 0.05$; **, $p < 0.01$. All other comparisons had $p \geq 0.05$. (D) Killing of type 3 strain A66.1 is mediated by MAb XiR278, which is reactive with A66.1 PspA. Significance was determined by comparing results with each MAb concentration to results with C'-only control. ***, $p < 0.001$. All other comparisons had $p \geq 0.05$.

seen in Fig. 1B, the increase in killing of A66.1 over the C'-only control due to MAb 1b2.21 was about 42% ($p < 0.01$) down to around 27% at 0.1 μg/mL of MAb. Below this concentration there was no significant increase in killing observed (Fig. 1B).

As seen in Fig. 1C, the MAb 8b2.19 also facilitated increased killing of A66.1 compared to the C'-only control of A66.1. The increased killing over control observed with this MAb was just over 40% at 100 μg/mL down to around 22% at 0.1 μg/mL. Below this concentration there was no statistically significant increase in killing observed.

MAb XiR278 was raised against a clade 2 PspA [37]. It is known to protect mice when given i.p. prior to an otherwise lethal i.v. challenge of type 3 pneumococci [43]. Fig. 1D shows the increase in killing of A66.1 over the C'-only control when incubated with XiR278. The increase in killing over control was as high as 30 percent at 71 μg/mL of MAb and around 17 percent at 4.5 μg/mL. 1.1 μg/mL and 0.28 μg/mL showed slight increase in killing around 15–17%. There was no statistically significant effect at a lower concentration.

The results of these MSKA experiments illustrate that all 3 MAbs that reacted with the PspA on the surface of A66.1 mediated increased killing of the bacteria compared to a C'-only control, and that for at least two of the MAb the killing was similar on a μg basis to that of MAb 16.3 to the type 3 capsule of the challenge strain.

Killing mediated by MAb to PspA is dependent on complement activity. To determine if the killing mediated by MAbs to PspA was dependent on complement activity we repeated the study above using 18, and1.1 μg/mL of MAb 1b2.21 in the presence of fresh frozen mouse complement and 71, 18, 4.5, and 1.1 μg/mL of the same MAb in the presence of heat inactivated complement. With fresh complement, we observed 20% or more killing by PMNs with both 18 and 1.1 μg/mL of MAb 1b2.21, but with the same complement that had been heat inactivated, we observed no killing greater than 3% and in no case were the results statistically significant compared to the heat-inactivated complement control (data not shown). We also performed the assay in the absence of PMNs and observed all killing was dependent on phagocytes and that nothing in the complement or ascites fluid was responsible on its own for killing observed (data not shown).

MAb to PspA did not mediate killing of a pspA mutant of A66.1 (BR240.1) that did not express PspA. If the ability of the MAbs studied in Figs. 1B and C to mediate killing of A66.1 was because they bound PspA, then the MAb-containing ascites fluid would be expected to not mediate killing of a mutant of A66.1 that lacked PspA. To address this question, BR240.1, a mutant of A66.1 that lacks surface PspA [39], was used as a target strain in the MSKA and incubated with 71 μg/mL MAb 1b2.21. As a positive control, A66.1 was used as the target strain in a parallel experiment. Fig. 2 illustrates that there was no antibody-dependent killing of the BR240.1, whereas the MAb 1b.2.21 facilitated killing of A66.1 as expected. These results indicate that the killing seen in MSKA with MAbs to PspA is dependent on the presence of PspA on the target strain.

Antibodies that were not reactive with the PspA expressed by A66.1 did not increase killing of A66.1 in the MSKA. To further test the possibility that the enhanced killing might have been due to something

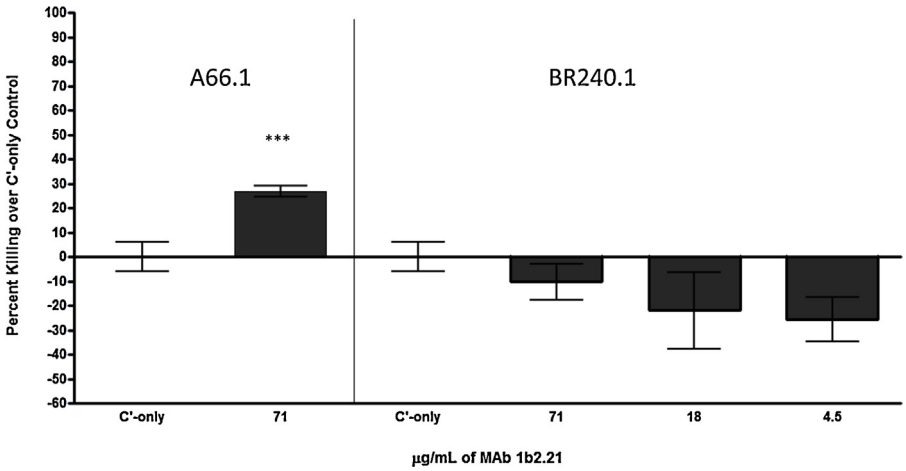

Fig. 2. MAb 1b2.21 was not able to mediate killing of strain BR240.1, a mutant of A66.1 that lacks PspA expression, even though MAb 1b2.21 did mediate killing of A66.1 in the same experiment. Significance was determined by comparing results with each MAb concentration to results with C'-only control against the same target strain. ***, $p < 0.001$. All other comparisons had $p \geq 0.05$.

in the ascites fluid other than the MAb specific for A66.1 PspA, we examined MAbs that lacked specificity for A66.1 PspA. MAb 6e5.5 and 5c6.1 are reactive with clade 3 PspA of EF3296 but not the clade 2 PspA of A66.1. These two MAbs do not bind to the surface of A66.1 by FACS analysis [44] (data not shown). A66.1 was separately incubated with 5c6.1 or 6e5.5 at concentrations of 143, 71, 18, and 4.5 μg/mL. At no concentration did either MAb show killing that differed from that of the C'-only control (data not shown). Thus, to mediate killing in the MSKA, the MAbs must bind PspA of the target strain, and other components of the ascites fluids appeared unable to mediate killing in the MSKA in the absence of the MAbs to PspA.

Cytochalasin-D stops killing of bacteria by neutrophils in the MSKA. To test the possibility that the neutrophil-mediated killing in the MSKA might involve a mechanism such as killing by neutrophil extracellular traps (NETs), we examined killing in the MSKA in the presence and absence of cytochalasin-D. Cytochalasin-D inhibits the cell's ability to polymerize actin and therefore its ability to phagocytose [45] but does not inhibit killing by NETs [46]. Neutrophils were incubated with cytochalasin-D prior to layering them on the blood agar plate and the results were compared with those of the same experiment using untreated neutrophils. To best illustrate the effects of cytochalasin-D on killing mediated in the presence of Ab + C', in the presence of C' only, and in the presence of heat inactivated C', we have plotted this data as total killing rather than percent killing over the C'-only control. As in the experiments above, any killing in the absence of PMNs was subtracted from the data shown.

This experiment used A66.1 and its *pspA* mutant, BR240.1 as target bacteria. In this assay MAb 1b2.21 mediated killing of A66.1 as compared to complement alone as expected (Fig. 3A). From this figure it is also clear that significant killing occurs in the presence of PMNs and C' alone. This greater killing with complement alone, as compared to heat-inactivated complement, was expected because prior data showed that even for pneumococci expressing PspA and capsule, some complement is deposited on pneumococci even in the absence of specific antibody [8,47,48]. Fig. 3A shows the PMN-dependent killing obtained without cytochalasin-D and Fig. 3B shows an experiment conducted in parallel where the PMNs were pre-treated with cytochalasin-D. Virtually all killing of A66.1, with our without antibody and with and without active complement, was blocked with cytochalasin D (Fig. 3B), indicating that in each case, the PMN-dependent killing was mediated by phagocytosis rather than NETS.

Strain BR240.1, a mutant of A66.1 expressing no PspA, was much more efficiently killed by neutrophils than A66.1 in both the presence and absence complement (compare Fig. 3A and C), indicating that PspA expression was also able to protect against killing by PMNs even in the absence of complement activity. This means that PspA has an anti-phagocytic role in addition to its ability to interfere with complement deposition [49]. However, as with A66.1, the killing of BR240.1 by PMNs in the presence of either complement or heat-inactivated complement was completely inhibited by cytochalasin-D indicating that the enhanced killing of type 3 pneumococci lacking PspA in both the presence and absence of complement was dependent on phagocytosis (Fig. 3C).

Passive immunization studies showed that MAbs to PspA that facilitated increased killing in MSKA also protected mice from lethal challenge. As an initial test of whether the MSKA might serve as an *in vitro* functional assay to predict protection, we compared the ability of the different MAbs 1b2.21, 8b2.19, XiR278, 5c6.1, and 6e5.5 to passively protect mice. We used CBA/N mice as our model since these mice have no serum Ab to phosphocholine and are thus, highly susceptible to pneumococcal infection [36]. Moreover, any protection observed would probably be due to the MAbs to PspA acting by itself since these mice lack known antibody to other pneumococcal antigens [36]. Their high susceptibility to PspA allows a low challenge dose to be used making it easy to distinguish protected from unprotected mice [50]. This model has been shown to be sensitive model for protection by Ab to PspA [23,36]. Control mice received 1/6 NMS diluted in Ringer's lactate. We used 7–10 mice in each treatment group.

In this experiment, all of the CBA/N mice in the negative control group, and the CBA/N mice in the two groups passively immunized with the MAbs that did not increase killing in MSKA (5c6.1 and 6e5.5) become moribund around 24–48 h after challenge (Fig. 4A). Conversely, all of the mice passively given MAbs that show increased killing in MSKA (8b2.19, 1b2.21, and XiR278) all survive to the endpoint of the experiment, 14 days ($p < 0.001$) (Fig. 4A).

We conducted a similar experiment conducted in C57BL/6J mice to make sure that our results with CBA/N mice were not dependent on that particular model. Passive protection by 20 μg of 1b2.21 against a lethal dose of A66.1 in C57BL/6 mice resulted in a longer time to moribund as compared to mice given a 1/6 dilution of NMS ($p < 0.02$). Mice given 20 μg of 5C6.1 had no significant difference in time to moribund compared to NMS (Fig. 4B). This data indicates that MAbs that show an ability to increase killing of bacteria in the MSKA can also passively protect mice against lethal challenge.

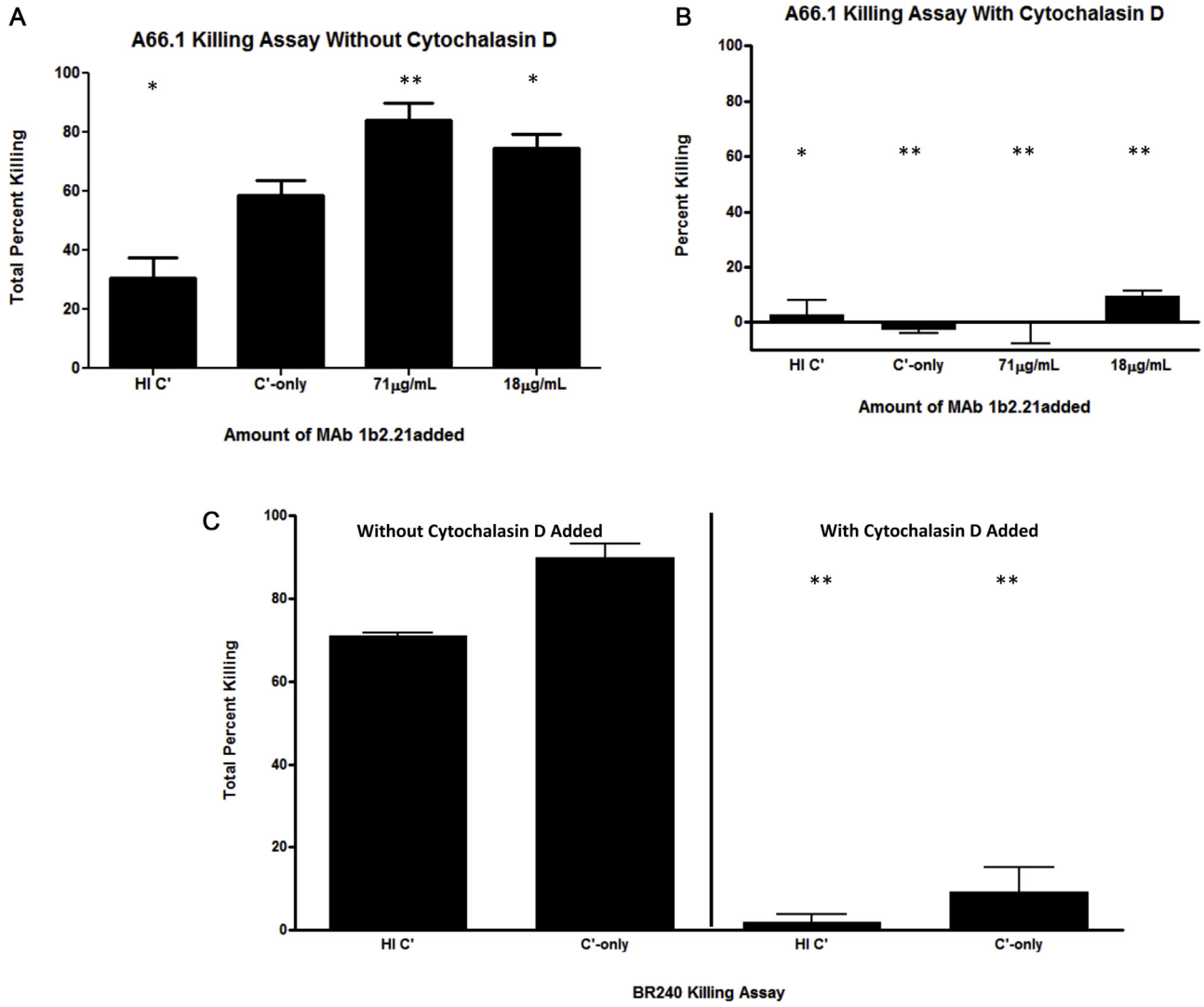

Fig. 3. Inhibition of killing by treatment of neutrophils with cytochalasin-D. Unlike prior figures, in this figure the data is plotted as total percent killing rather than percent killing over C'-only control. (A) The total percent killing of A66.1 by PMNs with and without MAbs to PspA. The statistical differences of each group from the C'-only group are shown: **, $p < 0.01$; *, $p < 0.05$. (B) All of the killing seen in the previous figure was blocked by the addition of cytochalasin-D to the neutrophils. Killing in groups in panel B were different from the corresponding groups in panel A by **, $p < 0.01$; *, $p < 0.05$. (C) The effect of cytochalasin-D of the killing by PMNs of BR240.1, a PspA non-expressing mutant of A66.1, in the presence and absence of complement. The statistical difference between the corresponding groups with and without cytochalasin is shown: **, $p < 0.01$. All of the killing shown was dependent on neutrophils since any bacterial death in the absence of neutrophils was subtracted away. The experiments in panels A, B, and C were done simultaneously to permit direct comparisons of the results.

Since in each of our experiments all CBA/N mice given 20 μg of a protective MAb to PspA survived, we suspected that this amount of antibody exceed the minimal protective dose. To further examine the ability of the MAbs to PspA to protect, we made 1:5 serial dilutions of MAb 8b2.19, beginning with 5 μg and ending with 0.008 μg. Groups of 5 CBA/N mice were passively immunized with 5, 1, 0.2, 0.04, or 0.008 μg of 8b2.19, or 1/6 dilution of NMS. After 4 h the mice were challenged with 400CFU A66.1 and monitored. As seen in Fig. 4C, the mice immunized with 5 μg or 1 μg of MAb 8b2.19 all survived to end of experiment (14days). In the 0.2 μg group, one mouse became moribund within 48 h, while rest lived to end of experiment. In the 0.04 μg and 0.008 μg, as well as the NMS groups, the mice all died within 48 h, with the exception of one mouse in the NMS group, which died around 110 h. These findings indicate that the amount of this MAb required for passive protection is very similar to what was observed previously [23] for polyclonal human Ab to PspA.

4. Discussion

It is well established that antibody to PspA can elicit protective immunity in mice and that protection can be transferred to mice with passive antibody [37,51,52]. It has also been shown that PspA is well exposed on the bacterial surface and that the presence of capsule does not greatly affect the binding of antibody to PspA on the pneumococcal surface [44,53]. Finally, is it is well established that antibodies to PspA can mediate complement deposition on pneumococci [54–56], and complement dependent phagocytosis *in vitro* [57]. These findings supported a hypothesis that antibody to PspA may be protective because it mediates complement-dependent phagocytosis and killing *in vivo*. This hypothesis was also consistent with earlier studies showing that protection with antibody to PspA is not seen *in vivo* if C3 levels are depressed by treatment of the mice with cobra-venom factor [58].

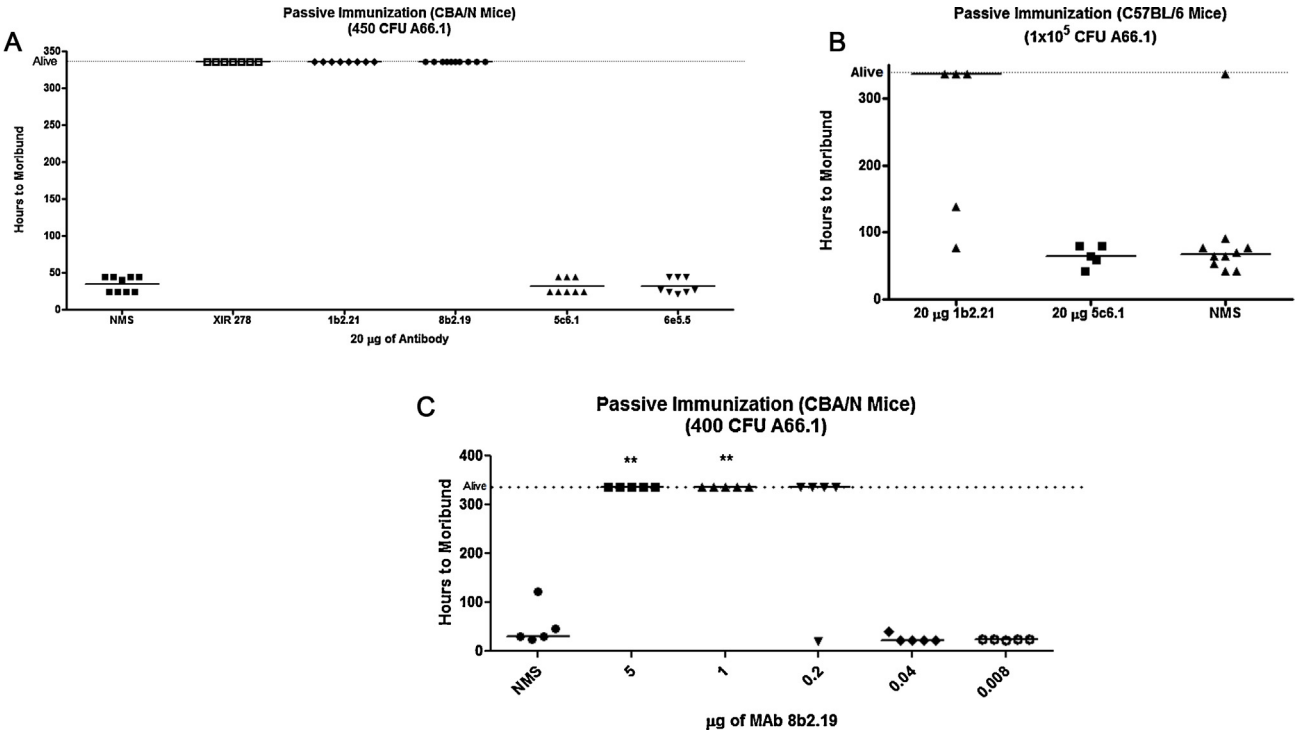

Fig. 4. Passive immunization of mice against lethal i.v. challenge with A66.1. (A) CBA/N mice passively immunized i.p. with 20 μg of anti-clade 2 MAbs 8b2.19, 1b2.21, XiR278, or anti-clade 3 MAbs 5c6.1 or 6e5.5. Control mice were given 1/6 NMS. Four hours later the mice were challenged i.v. with 450 CFU A66.1. Hours to moribund monitored. Mice given each of the three MAbs to clade 2 PspA showed a significantly greater time to moribund as compared to NMS control ($p < 0.001$). Mice given the two MAbs to clade 3 PspA had a time to moribund that was not statistically different from the NMS controls. (B) C57BL/6 mice were injected i.p. with 20 μg of either MAb to clade 2 PspA (1b2.21), MAb to clade 3 PspA (5c6.1), or a 1/6 dilution of NMS and challenged four hours later i.v. with 1×10^5 CFU of A66.1. Mice immunized with 1b2.21 showed a significant increase in time to moribund compared to the 1/6 NMS control group ($p < 0.02$). The mice given MAbs to clade 3 PspA showed no protection compared to the NMS control. The graphs shows hours to moribund for the individual mice in each group. (C) CBA/N mice passively immunized i.p. with serial dilutions of anti-clade 2 MAb 8b2.19. Control mice were given 1/6 NMS. Four hours later the mice were challenged i.v. with 400 CFU A66.1. Mice immunized with 5 μg and 1 μg of MAb had 100% survival. The 0.2 μg group had 80% survival while all other groups, including control, had 0% survival.

However, one finding did not fit with the hypothesis that protection by antibody to PspA was due to complement-dependent opsonophagocytosis and killing by phagocytes. Prior studies that compared antibody to capsular polysaccharide to antibody to PspA revealed that antibody to PspA did not readily mediate opsonophagocytic killing *in vitro* [59] (Daniels et al. In press Clin Vaccine Immunol 20: issue 10 2013) We reasoned that phagocytosis and killing probably occurs more efficiently *in vivo* where the phagocytes are bathed in a natural milieu and where phagocytes can capture bacteria on a surface, which appears to me much more efficient than *in vitro* phagocytosis in solution [29,60].

One test of this hypothesis was to develop an *in vitro* functional assay for antibody to PspA where killing by phagocytes is complement-dependent, and then look for a correlation between the ability of antibody to PspA to protect mice and the ability of the same antibodies to mediate killing in the functional assay. In the MSKA we showed that the opsonization of pneumococci with MAbs to PspA in the presence of complement could enhance killing of the bacteria on an agar plate, and that the presence of both complement and phagocytes was required for the antibody mediated killing. Moreover, we demonstrate that antibody to PspA that could protect against infection also mediated killing in the *in vitro* functional assay. We showed that the ability of antibody to mediate killing in the MSKA appeared to be closely related with their ability to passively protect mice against lethal challenge. Moreover, the amount of MAb to PspA required to protect a mouse was quite similar to the amount of human antibody to PspA previously reported to protect mice from fatal infection with type 3 strain A66.1 [23].

An important result that was not anticipated was that in the absence of MAbs to PspA and complement that the mutant lacking PspA was more readily killed by PMNs than the wild type strain. This last observation needs further study but appears to show that PspA has an anti-phagocytic effect in addition to its ability to inhibit complement deposition [49].

The initial experiments utilizing a MAb to anti-type 3 capsule in the MSKA showed a significant amount of killing over the C'-only control when using the capsule 3 pneumococcal strain A66.1. This increased killing percentage was consistently a little higher than the amount of killing indicated by MAbs against clade 2 PspA. A likely reason for this difference is that MAb 16.3 can bind more densely on the bacterial surface because the repeating epitopes of the capsule are expected to be more densely expressed than individual PspA epitopes. By binding more densely on the pneumococcal surface, antibody to capsule can also probably cross-link C1q and activate complement more efficiently. In spite of this difference, however, the three MAbs to clade 2 PspA tested, (XiR278, 1b2.21, and 8b2.19) all displayed a significant increase in killing compared to the C'-only control.

All of the antibodies tested were used as whole ascites fluid. This permitted us to avoid the chance of denaturing the antibody during purification, but required controls to ensure that the antibody was the component of the ascites fluid that was protective. Our controls confirmed that MAbs which do not bind the PspA on the target pneumococci, mediated no killing and mutant pneumococci not expressing PspA showed no killing dependent on MAbs to PspA. However it should be noted that some concentrations of MAbs, there were actually more CFUs of the PspA negative bacteria recovered for with the C'-only control (Fig. 2), indicating that in this assay there is something present in the ascites that either has a slight inhibitory effect on the killing of the bacteria by

neutrophils or has a growth promoting effect on the pneumococci. In either case, the actual percent killing over the C'-only control of the MAbs to clade 2 PspA may be slightly higher than calculated.

The highest increases in killing observed with MAb to type 3 capsule was around 44% and that with MAb to PspA was as high as 42%. The lack of higher %s of observed killing is likely due to the methodology of the assay itself. When lower concentrations of MAb were used we saw a dose-response decrease in % killing. When the bacteria and PMNs are added to the surface of the agar plate and the fluid soaks in, the bacteria and PMN are probably no longer able to move freely. However, in order for the neutrophils to phagocytize the opsonized bacteria during the time course of the assay the bacteria probably need to be in very close proximity of a PMN. Thus, some bacteria may be able to escape phagocytosis due simply to a lack of proximity to a phagocyte.

5. Conclusions

In a previous phase I trial of a PspA-containing vaccine antigen, human pre- and post- immune serum were tested in mouse passive protection study and provided strong evidence that human antibody elicited to PspA could protect mice from fatal infection [61]. A passive protection assay is a powerful surrogate of protection by antibody to PspA, but because each pre- and post- immune human serum needed to be tested at several dilutions in groups of 10 or more mice per group, the assay was too expensive and cumbersome for the routine examination of large numbers of pre- and post-immune serum pairs that would be expected to be obtained in phase 2 and phase 3 vaccine trials.

In the present studies we observed that the three MAbs that recognized the PspA of the target strain A66.1 all mediated killing of A66.1 in the MSKA and were able to passively protect against otherwise fatal challenge of mice with A66.1. In contrast, the two MAbs to a clade 3 PspA, which both failed to bind the PspA of A66.1, did not mediate killing of A66.1 in the MSKA and did not passively protect against A66.1 infection. These results bode well for the possibility that a version of the present MSKA might be able to be used as an *in vitro* functional assay to predict protective immunity to PspA. Additional studies where pre- and post- immune human serum from volunteers immunized with PspA will need to be examined in both passive protection and in the MSKA to further test the potential that a MSKA may be a surrogate assay for protective immunity to PspA.

A partially automated version of the MSKA, where CFU are counted and results calculated automatically, as is done in the present opsonophagocytosis assays [28] may offer the potential for a high through-put functional assay that would not depend entirely on mouse infection studies. Likewise, the adaption of the assay to use with a phagocytic cell line such as HL60, as appears possible in preliminary results (Kim, Genschmer, and Briles, unpublished), also increases the likelihood that this assay could be adaptable to mass screening of human immune sera.

The MSKA could also be an invaluable tool in protein vaccine antigen discovery and it is possible that the assay could be used to rank the relative protective capacity of immune sera to different antigens for their potential to protect a mouse from lethal challenge. Finally, the assay may be useful as a highly sensitive screen for immunity to various portions of immunogenic antigens, or combinations of antigens, and to predict which ones might work the best as vaccine antigens.

Conflict of interest

The University of Alabama at Birmingham Research Foundation holds several patents on the use of PspA in vaccines. Dr. Briles is among the inventors on those patents.

Acknowledgements

We would like to thank Janice King and Yvette Hale for their work in blood acquisition and mouse work and the UAB Epitope Recognition and Immunoreagent Core Facility for its assistance in the production of the MAbs used in these experiments. We would like to acknowledge support from the NIH grants R01AI 021458 (DEB) and P30AR48311 (MAAL) and from R01 DC006452 (DEB) from the National Institute of Deafness and other Communicable Disorders.

References

[1] Bryce J, Boschi-Pinto C, Shibuya K, Black RE, WHO Child Health Epidemiology Reference Group. WHO estimates of the causes of death in children. Lancet 2005;365:1147–52.
[2] Wardlaw T, Salama P, Johansson EW, Mason E. Pneumonia: the leading killer of children. Lancet 2006;368:1048–50.
[3] Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J Clin Microbiol 2007;45:1225–33.
[4] Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 2008;6:288–301.
[5] Pletz MW, Maus U, Krug N, Welte T, Lode H. Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. Int J Antimicrob Agents 2008;32:199–206.
[6] Marriott HM, Dockrell DH. *Streptococcus pneumoniae*: the role of apoptosis in host defense and pathogenesis. Int J Biochem Cell Biol 2006;38:1848–54.
[7] Abeyta M, Hardy GG, Yother J. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. Infect Immun 2003;71:218–25.
[8] Brown EJ, Joiner KA, Gaither TA, Hammer CH, Frank MM. The interaction of C3b bound to *pneumococci* with factor H (beta 1H globulin), factor I (C3b/C4b inactivator), and properdin factor B of the human complement system. J Immunol 1983;131:409–15.
[9] Winkelstein JA, Abramovitz AS, Tomasz A. Activation of C3 via the alternative complement pathway results in fixation of C3b to the pneumococcal cell wall. J Immunol 1980;124:2502–6.
[10] Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, et al. Decline in invasive pneumococcal disease after the introduction of protein–polysaccharide conjugate vaccine. N Engl J Med 2003;348:1737–46.
[11] Shapiro ED, Berg AT, Austrian R, Schroeder D, Parcells V, Margolis A, et al. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. N Engl J Med 1991;325:1453–60.
[12] Shinefield HR, Black S. Efficacy of pneumococcal conjugate vaccines in large scale field trials. Pediatr Infect Dis J 2000;19:394–7.
[13] Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, Craig AS, et al. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998–2004. J Infect Dis 2007;196:1346–54.
[14] Hsu HE, Shutt KA, Moore MR, Beall BW, Bennett NM, Craig AS, et al. Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. N Engl J Med 2009;360:244–56.
[15] Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 2007;297:1784–92.
[16] Barocchi MA, Censini S, Rappuoli R. Vaccines in the era of genomics: the pneumococcal challenge. Vaccine 2007;25:2963–73.
[17] Saxenian H, Cornejo S, Thorien K, Hecht R, Schwalbe N. An analysis of how the GAVI alliance and low- and middle-income countries can share costs of new vaccines. Health Aff (Millwood) 2011;30:1122–33.
[18] Crain MJ, Waltman 2nd WD, Turner JS, Yother J, Talkington DF, McDaniel LS, et al. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. Infect Immun 1990;58:3293–9.
[19] Hollingshead SK, Baril L, Ferro S, King J, Coan P, Briles DE. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. J Med Microbiol 2006;55:215–21.
[20] Ferreira DM, Darrieux M, Silva DA, Leite LC, Ferreira Jr JM, Ho PL, et al. Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. Clin Vaccine Immunol 2009;16:636–45.

[21] Oma K, Zhao J, Ezoe H, Akeda Y, Koyama S, Ishii KJ, et al. Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice. Vaccine 2009;27:3181–8.
[22] Miyaji EN, Oliveira ML, Carvalho E, Ho PL. Serotype-independent pneumococcal vaccines. Cell Mol Life Sci 2013;70:3303–26.
[23] Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000;182:1694–701.
[24] Briles DE, Hollingshead SK, Swiatlo E, Brooks-Walter A, Szalai A, Virolainen A, et al. PspA and PspC: their potential for use as pneumococcal vaccines. Microb Drug Resist 1997;3:401–8.
[25] Burton RL, Nahm MH. Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. Clin Vaccine Immunol 2006;13:1004–9.
[26] Romero-Steiner S, Libutti D, Pais LB, Dykes J, Anderson P, Whitin JC, et al. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. Clin Diagn Lab Immunol 1997;4:415–22.
[27] Jodar L, Butler J, Carlone G, Dagan R, Goldblatt D, Kayhty H, et al. Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. Vaccine 2003;21:3265–72.
[28] Burton RL, Nahm MH. 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 2012;19:835–41.
[29] Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, Galagan J, et al. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 2009;5:e1000476.
[30] Berry AM, Paton JC. Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. Infect Immun 2000;68:133–40.
[31] Kim JO, Romero-Steiner S, Sorensen UB, Blom J, Carvalho M, Barnard S, et al. Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun 1999;67:2327–33.
[32] Avery OT, Macleod CM, McCarty M. 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 1944;79:137–58.
[33] Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, et al. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science 2001;293:498–506.
[34] Briles DE, Crain MJ, Gray BM, Forman C, Yother J. Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. Infect Immun 1992;60:111–6.
[35] Yother J, Forman C, Gray BM, Briles DE. Protection of mice from infection with *Streptococcus pneumoniae* by anti-phosphocholine antibody. Infect Immun 1982;36:184–8.
[36] Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. J Exp Med 1981;153:694–705.
[37] McDaniel LS, Ralph BA, McDaniel DO, Briles DE. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. Microb Pathog 1994;17:323–37.
[38] Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000;68:5889–900.
[39] Ren B, Li J, Genschmer K, Hollingshead SK, Briles DE. The absence of PspA or the presence of antibody to PspA each facilitate the complement-dependent phagocytosis of pneumococci *in vitro*. Clin Vaccine Immunol 2012;19:1574–82.
[40] Krause RM. The search for antibodies with molecular uniformity. Adv Immunol 1970;12:1–56.
[41] Bannur SV, Kulgod SV, Metkar SS, Mahajan SK, Sainis JK. Protein determination by Ponceau S using digital color image analysis of protein spots on nitrocellulose membranes. Anal Biochem 1999;267:382–9.
[42] Zhang B, Hirahashi J, Cullere X, Mayadas TN. Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation. J Biol Chem 2003;278:28443–54.
[43] McDaniel LS, Scott G, Kearney JF, Briles DE. Monoclonal antibodies against protease-sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. J Exp Med 1984;160:386–97.
[44] Daniels CC, Briles TC, Mirza S, Hakansson AP, Briles DE. Capsule does not block antibody binding to PspA, a surface virulence protein of *Streptococcus pneumoniae*. Microb Pathog 2006;40:228–33.
[45] Elliott JA, Winn Jr WC. Treatment of alveolar macrophages with cytochalasin D inhibits uptake and subsequent growth of *Legionella pneumophila*. Infect Immun 1986;51:31–6.
[46] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532–5.
[47] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and Antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2003;72:114–22.
[48] Li J, Glover DT, Szalai AJ, Hollingshead SK, Briles DE. PspA and PspC minimize immune adherence and transfer of pneumococci from erythrocytes to macrophages through their effects on complement activation. Infect Immun 2007;75:5877–85.
[49] Mukerji R, Mirza S, Roche AM, Widener RW, Croney CM, Rhee DK, et al. Pneumococcal surface protein A inhibits complement deposition on the pneumococcal surface by competing with the binding of C-reactive protein to cell-surface phosphocholine. J Immunol 2012;189:5327–35.
[50] Briles DE, Hollingshead SK, Jonsdottir I. Animal models of invasive pneumococcal disease. In: Siber GR, Klugman KP, Mäkelä PH, editors. Pneumococcal vaccines: the impact of conjugate vaccine. Washington, DC: ASM Press; 2008. p. 47–58.
[51] McDaniel LS, Scott G, Kearney JF, Briles DE. Monoclonal antibodies against protease sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. J Exp Med 1984;160:386–97.
[52] Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with rPspA elicits antibodies, which passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000;182:1694–701.
[53] Hakansson A, Roche H, Mirza S, McDaniel LS, Brooks-Walter A, Briles DE. Characterization of the binding of human lactoferrin to pneumococcal surface protein A (PspA). Infect Immun 2001;69:3372–81.
[54] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004;72:114–22.
[55] Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes AP, Ren B, et al. 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 2007;75:5930–8.
[56] Ochs MM, Bartlett W, Briles DE, Hicks B, Jurkuvenas A, Lau P, et al. Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. Microb Pathog 2008;44:204–14.
[57] Ren B, Li J, Genschmer K, Hollingshead SK, Briles DE. The absence of PspA or presence of antibody to PspA facilitates the complement-dependent phagocytosis of pneumococci in vitro. Clin Vaccine Immunol 2012;19:1574–82.
[58] Briles DE, Tart RC, Wu HY, Ralph BA, Russell MW, McDaniel LS. Systemic and mucosal protective immunity to pneumococcal surface protein A. Ann NY Acad Sci 1996;797:118–26.
[59] Briles DE, Forman C, Horowitz JC, Volanakis JE, Benjamin Jr WH, McDaniel LS, et al. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. Infect Immun 1989;57:1457–64.
[60] Smith MR, Wood Jr WB. Surface phagocytosis: further evidence of its destructive action upon fully encapsulated pneumococci in the absence of type-specific antibody. J Exp Med 1958;107:1–12.
[61] Briles DE, Hollingshead SK, Nabors GS, Paton JC, Brooks-Walter A. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. Vaccine 2000;19(Suppl. 1):S87–95.