

Capsular Polysaccharide (CPS) Release by Serotype 3 Pneumococcal Strains Reduces the Protective Effect of Anti-Type 3 CPS Antibodies

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The efficacy of the serotype 3 (ST3) pneumococcal conjugate vaccine (PCV) remains unclear. While the synthesis of capsular polysaccharide (CPS) of most serotypes is *wzy* dependent, the strains of two serotypes, 3 and 37, synthesize CPS by the synthase-dependent pathway, resulting in a polysaccharide that is not covalently linked to peptidoglycan and can be released during growth. We hypothesized that the release of CPS during growth reduces anti-type 3 CPS antibody-mediated protection and may explain the lower efficacy of the type 3 component of PCV than that of other PCVs. The *in vitro*-released CPS concentrations per 10⁷ CFU of ST3 and ST37 strains were significantly higher than those for the ST1, ST4, ST6B, and ST14 strains. Following intraperitoneal (i.p.) injection in mice, blood concentrations of CPS were significantly higher for the ST3 than for the ST4/5 strains. The opsonophagocytic killing assay (OPKA) titer of anti-type 3 CPS antibody was significantly reduced by type 3 CPS, culture supernatant, or serum from *Streptococcus pneumoniae* ST3 strain WU2-infected mice. Mice were injected with capsule-specific antibodies and challenged i.p. with or without the addition of sterile culture supernatant containing type-specific CPS. The addition of 0.2 μ l of culture supernatant from WU2 inhibited passive protection, whereas 100-fold-more culture supernatant from *S. pneumoniae* ST4 strain TIGR4 was required for the inhibition of protection. We conclude that released type 3 CPS interferes with antibody-mediated killing and protection by anti-CPS antibodies. The relative failure of ST3 PCV may be due to CPS release, suggesting that alternative immunization approaches for ST3 may be necessary.

Streptococcus pneumoniae remains a major cause of bacteremia, meningitis, pneumonia, and acute otitis media worldwide (1). For the 94 pneumococcal serotypes (ST) that have been identified, the synthetic mechanisms of capsular polysaccharide (CPS) can be classified into two pathways: a synthase-dependent and a *wzy*-dependent pathway (2–5). A major difference between the two pathways is that *wzy*-dependent synthesis results in CPS that is covalently linked to the peptidoglycan on the bacterial cell wall, whereas the synthase-dependent CPS is bound on phosphatidylglycerol or the synthase on the membrane (2). Therefore, synthase-dependent serotypes can release CPS either by dissociation from phosphatidylglycerol or by ejection from the synthase (6); this release can be detected *in vitro*, as shown in reference 7.

While the CPS synthesis of most serotypes is *wzy* dependent, strains of two serotypes, 3 and 37, synthesize CPS by the synthase-dependent pathway (3, 8). Whereas ST37 strains are rarely isolated from humans, ST3 isolates are an important cause of invasive pneumococcal disease, particularly pneumonia in both children and adults (9). With the expansion of the 7-valent to the currently used 13-valent pneumococcal conjugate vaccine (PCV13), serotype 3 conjugate was added to the formulation. The immunogenicity of the type 3 conjugate led to the expectation that both colonization and infection with strains of this serotype would decline significantly, as has been noted for the other serotypes included in pneumococcal conjugate vaccines. Surprisingly, however, there have been conflicting reports on the efficacy of the serotype 3 component of the PCV (9–11). Despite the predicted effectiveness at the accepted 0.35 μ g/ml enzyme-linked immunosorbent assay (ELISA) cutoff of 97% (11), to date, it does not appear that PCV13 has resulted in the same degree of reduction in the incidence of type 3 disease as that seen with other newly included serotypes, such as 19A.

We hypothesized that the unusual polysaccharide synthesis

pathway of ST3 strains may provide an explanation for these findings. To evaluate this, we first compared CPS release *in vitro* and *in vivo* by various serotypes and sought to determine whether the amount of released CPS from ST3 pneumococci is sufficient to inhibit antibody-dependent bacterial killing and protection against serotype 3 pneumococci.

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MATERIALS AND METHODS

Bacterial strains and reagents. Purified pneumococcal CPS of various serotypes was purchased from ATCC; this was used for all vaccine preparations and ELISA. The pneumococcal strains used in this work are listed in Table 1. All strains were grown in Todd-Hewitt broth containing 0.5% yeast extract (THY) at 37°C with 5% CO₂ until an optical density at 600 nm (OD₆₀₀) of 0.5 was reached. Bacterial stocks were stored in THY medium with 20% glycerol at –80°C until use. A CPS deletion (Δ CPS) ST3 strain was generated by deleting the operon that contains two genes, *cps3D* and *cps3S*, which are essential for serotype 3 CPS biosynthesis (12), using the Janus cassette strategy (13). A Δ CPS *S. pneumoniae* TIGR4 strain was

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TABLE 1 Pneumococcal strains used in the current work

Serotype	Strain	Source ^a
1	0101	CDC/ABC
	0102	CDC/ABC
	0850-94	CDC/ABC
	0853-99	CDC/ABC
	2550-97	CDC/ABC
3	0301	CDC/ABC
	0302	CDC/ABC
	0303	CDC/ABC
	0305 (WU-2)	RM
	0306	DEB
4	0401	CDC/ABC
	0403 (TIGR4)	CDC/ABC
	0543-99	CDC/ABC
	3748-99	CDC/ABC
6B	0601	CDC/ABC
	0602	CDC/ABC
	0604	CDC/ABC
	0618	CDC/ABC
	0619	CDC/ABC
14	88	KOB
	231	KOB
	314	KOB
	407	KOB
	530	KOB
37	BG6308	DEB
	FZ3701	RM
	FZ3702	RM

^a Strains were obtained from the ABCs collection of the CDC (CDC/ABC), David Briles (DEB), Kate O'Brien (KOB), or a collection of clinical isolates in the Richard Malley laboratory (RM).

also generated by deleting the whole *cps* gene cluster (14). Capsule deletion was confirmed by PCR, colony appearance on blood agar plates, the Quellung reaction (Statens Serum Institut, Denmark), and/or inhibition ELISA.

To generate a large volume of serum against ST3, ST4, or ST5 capsule, rabbits were immunized with affinity-coupled complexes of target CPS, using the Multiple Antigen Presentation System (MAPS) technology we described previously (15). Briefly, ATCC ST3, ST4, and ST5 CPS were activated by 1-cyano-4-dimethylaminopyridinium (CDAP) tetrafluoroborate and then labeled with EZ-Link amine-PEG3-biotin (Pierce). Free EZ-Link amine-PEG3-biotin was removed by dialysis. MAPS complexes were assembled by incubating biotinylated CPS with purified egg white avidin (Sigma) at room temperature overnight and then purifying by size-exclusion chromatography (SEC). Mouse serum against multiple pneumococcal polysaccharides was generated by immunizing mice subcutaneously three times at a 2-week interval with two-fifths the adult dose of PCV13 (Pfizer). Mouse serum against ST37 capsule was generated by subcutaneous immunization with MAPS complex made from biotinylated ST37 CPS and egg white avidin.

CPS-specific IgG antibodies were measured by ELISA using the endpoint titer. Ninety-six-well ELISA plates were coated with purified CPS of various serotypes (ATCC), according to the conditions described in the WHO instructions for pneumococcal serology (24) or the conditions optimized in our laboratory. Briefly, ELISA plates were incubated with purified CPS at 2 µg/ml for type 1, 1 µg/ml for type 3, 0.5 µg/ml for type 4, 10 µg/ml for type 6B, 1 µg/ml for type 14, and 0.5 µg/ml for type 37 at

37°C for 5 h and then at 4°C overnight before use. The endpoint titer was defined as the highest dilution of serum that did not give any signal. All sera were heat inactivated before use in killing assays or passive immunizations.

Measurement of released CPS concentration following *in vitro* growth. A frozen aliquot was thawed and incubated in THY medium at 37°C with 5% CO₂ until an OD₆₀₀ of 0.8 was reached. Bacterial CFU were determined by plating dilutions of bacterial culture on blood agar plates. Bacterium-free culture supernatant was collected by centrifugation for 10 min at 13,000 rpm and then was filtered through a 0.65-µm-pore-size filter (Millipore, Billerica, MA); sterility was confirmed by plating an aliquot on a blood agar plate. To measure the CPS on the bacterial surface, the bacterial pellet was washed with phosphate-buffered saline (PBS) (pH 7.4) and fixed with 4% paraformaldehyde at 4°C overnight. The amount of CPS on the bacterial surface or in the culture supernatant was measured by inhibition ELISA using sera from mice immunized with PCV13 for types 1, 3, 4, 6B, and 14 or sera from mice immunized with MAPS complex for type 37. A standard curve of inhibition was generated by adding different concentrations of purified CPS (range, 0.1 to 100 µg/ml). For each serotype, the dilution of antiserum was optimized according to the detection range of the standard curve. Inhibition of anti-CPS antibody binding to the plate by different dilutions of bacterial supernatant or fixed pneumococci was evaluated against the standard curve. The concentration of CPS in the supernatant or on the bacterial surface was then normalized by the CFU count of the culture.

OPKA. An opsonophagocytic killing assay (OPKA) was performed using the protocol described by Romero-Steiner et al. (16). Briefly, HL-60 cells (ATCC, Manassas, VA) were propagated in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum (FBS) and then differentiated into granulocytes by adding 100 mM dimethylformamide for 5 days. Heat-inactivated immune rabbit serum was diluted with Hanks balanced salt solution (HBS) (Corning Cellgro) containing 10% FBS. Frozen bacterial stock was thawed, washed, and resuspended with Hanks balanced salt solution containing 10% FBS. To determine the OPKA titer, 10 µl of bacteria (1,000 CFU) was incubated with 20 µl of serial dilutions of rabbit serum in a 96-well microtiter plate for 30 min at room temperature and shaken at 600 rpm. Forty microliters of differentiated HL-60 cells (4×10^5 cells) and 10 µl of baby rabbit complement were then added (final volume, 80 µl per well), and the mixture was incubated at 37°C for 45 min with shaking. After incubation, 5 µl of mixture was plated onto a blood agar plate in triplicate. Blood agar plates were incubated for 6 to 8 h at 37°C with 5% CO₂ and then placed at room temperature in ambient air overnight to facilitate colony counting of the serotype 3 strains, which are very mucoid. The highest serum dilution that resulted in 100% killing was selected for OPKA inhibition analysis.

To measure the inhibitory effect of released type 3 CPS on bacteria killing, an OPKA was performed with the addition of culture supernatant or mouse serum obtained at 24 h following i.p. injection with 10^3 CFU of the *S. pneumoniae* WU2 strain. In each well, 10 µl of diluted rabbit serum was mixed with 10 µl of strain WU2 (1,000 CFU) and 10 µl of culture supernatant or mouse serum for 30 min at room temperature before HL-60 cells and complement were added. An inhibitory standard curve was generated by adding different amounts of purified type 3 CPS using either the ΔCPS WU2 culture supernatant or naive mouse serum as the diluent. The percent inhibition of *in vitro* bacterial killing was compared to that with the OPKA with the addition of ΔCPS WU2 culture supernatant or naive mouse serum containing no purified CPS. Experiments performed to determine the OPKA titer and percent inhibition of killing were each repeated at least three times.

Measurement of CPS release in mouse blood following intraperitoneal infection. Female 6- to 8-week-old C57BL/6J mice were used (Jackson Laboratories, Bar Harbor, ME) for all animal experiments. All animal protocols were approved by the Boston Children's Hospital IACUC. Intraperitoneal (i.p.) infection was performed with 10^3 CFU of an ST3, ST4, or ST5 strain in a 200-µl volume. Each mouse was sacrificed 24 h after

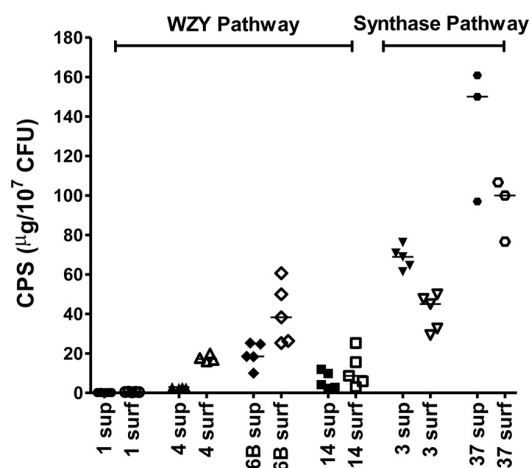


FIG 1 Comparison of surface CPS and CPS released in the culture supernatant by serotype and capsule synthesis pathway. Each symbol represents the mean measurement (3 to 4 repeat measurements per strain). The horizontal lines represent the medians of various strains in the same serotype. Sup, culture supernatant; Surf, surface.

infection. Blood samples were plated to determine the CFU count. Serum was then collected and filtered through 0.65- μ m-pore-size filter to remove live pneumococci. The concentration of CPS in serum was measured by inhibition ELISA, as described above, and normalized by the CFU count in the blood.

Passive protection and intraperitoneal challenge in mouse model.

To evaluate the extent to which released CPS could interfere with passive protection conferred by anti-capsular antibodies, the amount of serotype-specific anti-CPS antibody necessary to confer 80 to 100% protection against i.p. challenge by 10^3 CFU of the WU2 or TIGR4 strain was determined. The antibody was then administered to mice 24 h before i.p. challenge. For the challenge, pneumococci were resuspended in the culture supernatant of a Δ CPS strain with or without the addition of different volumes of culture supernatant derived from the growth of encapsulated strain WU2 or TIGR4. Mouse survival was monitored daily for 7 days after i.p. challenge. In this passive protection challenge model, the inhibitory potential of the culture supernatant was calculated by comparing the survival rates between mice that received the challenge strain diluted in supernatant from the Δ CPS mutant alone and those that received the supernatant from the Δ CPS strain to which supernatant purified from the growth of encapsulated strains was added.

Statistical analysis. The differences in CPS concentrations in culture supernatant and mouse serum by serotype were analyzed using the Mann-Whitney *U* test. Differences in the inhibitory effects of supernatant on mouse survival were assessed by the log rank test. For all comparisons, a *P* value of <0.05 was considered to represent statistical significance. All statistical analyses were performed using Prism (version 5.0d).

RESULTS

CPS release and bacterial content following *in vitro* growth by different serotypes. It has been known for some time that the type 3 polysaccharide in *S. pneumoniae* cultures can be associated with the bacterial cells and soluble in the culture medium (17), but the extent to which this occurs compared to other serotypes has not been fully explored. We were also interested in seeing whether this release of polysaccharide (PS) in the medium would occur with type 37 strains, for which capsular polysaccharide synthesis is also synthase dependent. Here, different strains representative of the two synthetic pathways (synthase dependent, ST3 and 37; and *wzy*

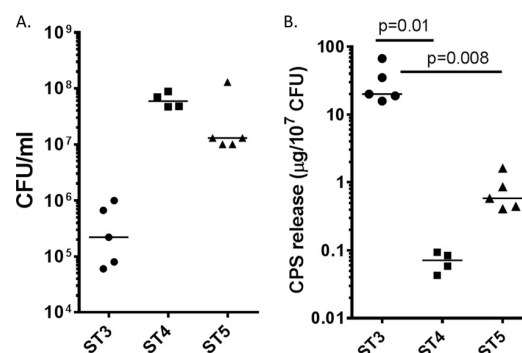


FIG 2 CPS release in blood at 24 h following intraperitoneal infection by various serotypes. (A) CFU counts in serum after infection. Each symbol represents one mouse ($n = 5$ mice per group). The horizontal lines represent the medians. (B) CPS concentration in serum after infection (normalized to μ g/ 10^7 CFU). Each symbol represents one mouse. The bars represent the medians. Statistical analysis was performed using the Mann-Whitney test.

dependent, ST1, 4, 6B, and 14; see Table 1) were grown in THY, and the supernatants were harvested. Serotype-specific CPS concentrations in the bacterium-free culture supernatant and on the bacterial pellet were measured by inhibition ELISA. The mean released CPS per 10^7 CFU of ST3 (60 μ g) and ST37 (130 μ g) strains were significantly higher than those for ST1, 4, 6B, and 14 (0.4 to 10 μ g) (Fig. 1). The ratio of CPS released into the culture supernatant to that present on the bacterial surface was >1 for synthase-dependent serotypes, whereas it was <1 for *wzy*-dependent serotypes (Fig. 1).

CPS release in blood following i.p. challenge. Next, we wished to examine whether PS release from the bacterial cell could also be detected in experimental bacteremia in mice. Mice were infected i.p. with strains of serotype 3, 4, or 5 at a dose of 10^3 CFU ($n = 5$ mice per group). Bacteremia was documented in all mice at 24 h following i.p. challenge. The CFU counts in the blood ranged from 6×10^4 CFU/ml to 1.3×10^8 CFU/ml, depending on the serotype of the infecting strain (Fig. 2A). At 24 h following i.p. injection, the median serotype 3 CPS release in the blood per 10^7 bacteria was 31.2 μ g, an amount that is significantly higher than that seen following infection with a strain of serotype 4 (<0.1 μ g, $P = 0.016$) or serotype 5 (0.8 μ g, $P = 0.008$) (Fig. 2B).

Inhibition of bacterial killing *in vitro* by addition of mouse serum or filtered culture supernatant. To evaluate the impact of released type 3 CPS *in vitro* or *in vivo* on the antibody-mediated killing of pneumococci, we performed OPKA against the WU2 strain in the presence of culture supernatant or mouse serum post-WU2 infection. We first determined the minimum amount of anti-type3 CPS rabbit serum that would be sufficient to achieve between 90 and 100% bacterial killing in a standard OPKA (Fig. 3A). This amount of antibody was then used in further assays to measure the inhibition of bacterial killing. A standard inhibition curve was developed by the addition of purified type 3 CPS (diluted with the culture supernatant of Δ CPS WU2 strain), ranging from 0.15 to 150 ng. About 50% inhibition of bacterial killing was observed when 12 ng of purified type 3 CPS was added to the OPKA (Fig. 3B, solid line). A similar inhibition capacity was observed with the culture supernatant of the wild-type (WT) WU2 strain (containing 150 μ g/ml released type 3 CPS). As shown in Fig. 3B (dashed line), the bactericidal activity of the anti-serotype

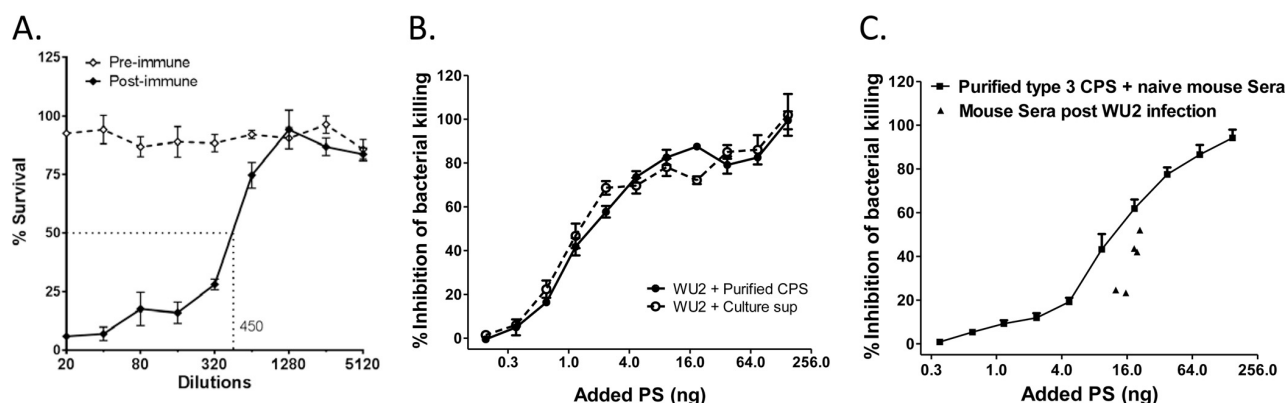


FIG 3 Inhibition of opsonophagocytic killing in the presence of released type 3 CPS. (A) OPKA titer of rabbit serum pre- and postimmunization with type 3 MAPS complex. Dotted lines indicate the dilution of serum that is associated with 50% killing of the inoculum. (B) Inhibition of opsonophagocytic killing against WU2 in the presence of different amounts of purified type 3 CPS (diluted in the culture supernatant of Δ CPS WU2 strain) or wild-type WU2 culture supernatant (containing 150 μ g/ml of released type 3 CPS). The killing rate in the culture supernatant of Δ CPS WU2 strain without added CPS is defined as 100%. (C) Inhibition of opsonophagocytic killing against WU2 in the presence of mouse sera post-WU2 i.p. infection. The concentration of released type 3 CPS in mouse serum was measured by inhibition ELISA. Thirty microliters of mouse serum was added to the OPKA. The killing rate in the presence of naive mouse serum was defined as 100%. A standard inhibition curve was generated by adding different amounts of purified type 3 CPS diluted in naive mouse serum. For all three graphs, error bars represent the standard errors of the means.

3 antibody was significantly reduced in the presence of WU2 culture supernatant in a CPS concentration-dependent manner.

Next, we compared the killing activity of anti-type 3 CPS antibody in the presence of naive mouse serum (defined as 100% killing) or mouse serum (from 5 mice) following i.p. challenge with the WU2 strain. As shown in Fig. 3C, the addition of 30 μ l of mouse serum post-i.p. infection with WU2 (containing 0.4 to 0.7 μ g/ml released type 3 CPS) showed 26% to 52% inhibition of bacterial killing, comparable to what was observed when an equivalent amount of purified CPS was added.

In vivo inhibition of protection in a passive transfer-i.p. challenge model. Next, we wished to compare the relative capacity of bacterial culture supernatants from type 3 or 4 strains to inhibit protection by passive transfer of anti-capsule antibodies in an intraperitoneal sepsis model. First, we determined the minimum amount of capsule-specific rabbit serum that provided $\geq 80\%$ protection against i.p. challenge: 40 and 20 μ l were required to achieve this degree of protection against serotypes 3 and 4, respectively. These volumes of serum were then administered intraperitoneally 24 h prior to i.p. challenge with 1,000 CFU of the serotype 3 (WU2) or serotype 4 (TIGR4) strain, respectively. Filtered culture supernatants of the Δ CPS WU2 or TIGR4 strain were used as negative controls. In mice that were passively immunized with serum containing the relevant anti-capsule antibodies, the survival rate was $\leq 20\%$ in the presence of as little as 0.2 μ l of WU2 culture supernatant (containing about 0.03 μ g of type 3 CPS), whereas about 25 μ l of TIGR4 culture supernatant (containing about 0.12 μ g of type 4 CPS) was required to abolish protection against the type 4 challenge (Fig. 4).

DISCUSSION

Currently, a 0.35 μ g/ml threshold of anti-capsular antibody for each serotype is used as a correlate of protection for the licensure of pneumococcal conjugate vaccines. This correlate of protection has been used for the licensure of vaccines containing additional serotypes without the need for formal efficacy studies prior to regulatory approval. A recent study from the United Kingdom

evaluated serotype-specific effectiveness and determined the correlates of protection for each serotype included in PCV13. Serotype-specific correlates of protection varied widely, with a correlate of protection of >0.35 for several serotypes (11). In particular, for serotype 3, the calculated correlate of protection was 2.83 μ g/ml, the highest for all the serotypes represented in PCV13, of course raising the concern that thresholds for this and other serotypes may need to be revised.

Our study points to a possible mechanistic explanation for the finding that higher anti-capsular antibody concentrations may be required for protection against type 3. Indeed, our results demonstrated that compared to other serotypes included in PCV13, the ST3 strain releases the most CPS during *in vitro* growth and infection in mice, which then interferes with antibody-mediated killing in an opsonophagocytic assay and reduces the protective efficacy of preexisting anti-CPS antibody (Ab) in a murine model of sepsis. The extent to which the phenomenon of CPS release observed *in vivo* occurs in human infections remains to be determined, but this is a plausible explanation for the observation that higher concentrations of antibodies may be required in infants and children to ensure protection against type 3 pneumococcal disease. This hypothesis also is consistent with the finding that OPA titers that apparently suffice in conferring protection against other pneumococcal serotypes are insufficient to ensure protection against serotype 3 (11).

PCVs prevent pneumococcal carriage and disease caused by the vaccine-containing serotypes. For example, immunization with the 7-valent PCV (PCV7) resulted in a marked decrease in the incidence of invasive diseases caused by the PCV7 serotypes (18). However, it is not yet clear to what extent type 3 conjugates are protective against disease or carriage caused by serotype 3. In studies from the United States and the United Kingdom, the protective efficacy for the serotype 3 component of PCV13 was not significant (9, 11, 19). In fact, prior to the licensure of PCV13, studies of an experimental PCV11 that contained a conjugate of serotype 3 surprisingly showed no protection against acute otitis

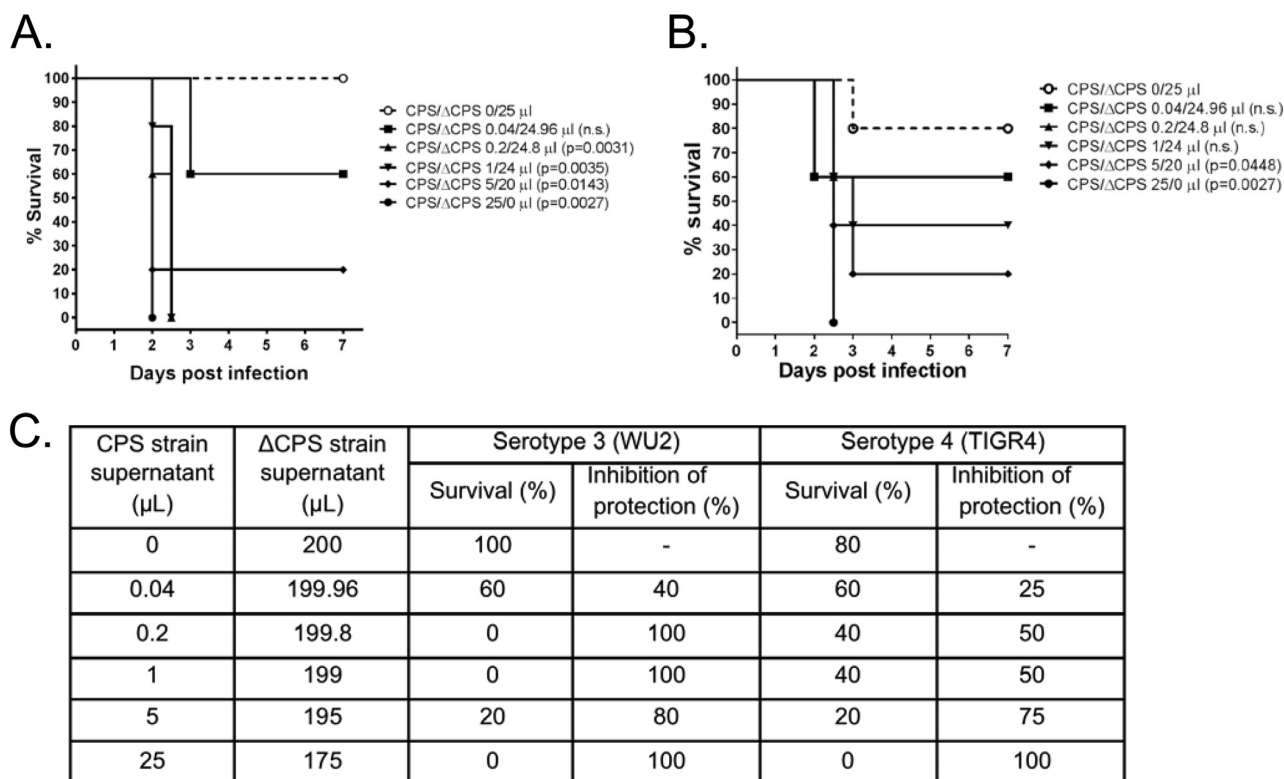


FIG 4 Inhibition of passive protection by addition of culture supernatant of ST3 or ST4 pneumococcal strain. Mice ($n = 5$ per group) received passive transfer with 40 μ L or 20 μ L of anti-ST3 or anti-ST4 rabbit serum, respectively, 1 day before infection. Mice were then infected by intraperitoneal injection with 1,000 CFU of WU2 or TIGR4 resuspended in the culture supernatant of the corresponding Δ CPS strain with the addition of different amounts of WU2 or TIGR4 culture supernatant. The WU2 culture supernatant contained 150 μ g/ml released type 3 CPS; the TIGR4 culture supernatant contained 4.7 μ g/ml released type 4 CPS. There was no detectable CPS in the culture supernatants of Δ CPS strains. The mice were monitored for 7 days after infection. (A and B) Survival curves after WU2 (A) or TIGR4 (B) i.p. infection. (C) Summary of the inhibitory impact of WU2 or TIGR4 culture supernatant on passive protection. Statistical analysis was done using the log rank (Mantel-Cox) test, comparing each group to the animals that received supernatant only from the related Δ CPS strain. n.s., not significant.

media (AOM) due to serotype 3 (19), which resulted in the removal of that particular serotype from the vaccine.

With respect to carriage, a randomized trial evaluating the comparative efficacy between PCV13 and PCV7 failed to demonstrate a reduction in the acquisition of nasopharyngeal colonization by serotype 3 among PCV13 recipients, whereas there was a clear and significant reduction in the cumulative acquisition of the serotypes that are included in PCV13 but not in PCV7 in recipients of PCV13 (with the exception of serotype 5, which was not prevalent enough to be evaluated) (20). A more recent study from Massachusetts showed no change in serotype 3 carriage rate after PCV13 introduction, although the numbers of serotype 3 cases overall admittedly were quite small (21).

Some of the studies suggested that low immunogenicity to serotype 3 conjugate may be the underlying reason for breakthrough AOM or carriage (19, 20). While this is certainly possible, the data presented in our study suggest that the failure of the serotype 3 conjugate might also be due to CPS release by the organism or, put differently, that the phenomenon of CPS type 3 release results in the higher protective antibody threshold that is required. Inhibition of anti-CPS antibody by the released serotype 3 CPS can be explained in two different ways. Released CPS can inhibit anti-PCS antibody opsonization by absorbing free unbound antibody. It is also possible that the CPS on which antibody

was already bound can be released from the bacteria. From our data, the serotype 3 CPS concentration ratio of culture supernatant to bacterial surface was >1 , suggesting a higher antibody-absorbing capacity by the CPS in culture supernatant.

It is important, of course, to acknowledge that the data presented here rely only on *in vitro* and mouse *in vivo* studies. To what extent can the data shown in our studies be extrapolated to the pathophysiological context in humans? Our results showed that in mice, with a marginal protective anti-type 3 CPS antibody titer, the protection can be completely abolished in the presence of as little as 0.03 μ g of free type 3 CPS that could be released by approximately 10^4 CFU of WU2 strain *in vivo* (the average CPS release post-i.p. infection is about 30 μ g/ 10^7 CFU, as shown in Fig. 2). In contrast, for an ST4 strain, the abolishment of protection requires about 0.12 μ g of free type 4 CPS, which could not be reached even with 10^7 CFU of the ST4 strain *in vivo* (Fig. 2). Although the density of bacteremia associated with a significant disease in humans is not known, studies with *Haemophilus influenzae* type b revealed that 1,000 bacteria/ml of blood (counted by direct plating on solidified medium) was associated with meningitis (22).

In summary, our results suggest that the failure of the serotype 3 conjugate in PCV13 may be a direct consequence of the CPS release by the organism, a process that differentiates type 3 from other serotypes included in current conjugate vaccines. While

higher antibody concentrations can overcome this phenomenon, as shown here, alternative strategies (such as protein-based or whole-cell vaccines; see review in reference 23) may be required for optimal protection against serotype 3 disease.

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