Protection against Pneumococcal Colonization and Fatal Pneumonia by a Trivalent Conjugate of a Fusion Protein with the Cell Wall Polysaccharide

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Received 22 December 2008/Returned for modification 23 January 2009/Accepted 19 February 2009

The current vaccines against Streptococcus pneumoniae (pneumococcus) are based upon the serotype-specific capsular polysaccharides, posing limitations with respect to serotype coverage and replacement (11). Certain pneumococcal antigens common to all serotypes of the species have been shown to have immunoprotective potential despite the encapsulation, e.g., the surface proteins PspA, PspC, PsaA, and the cytotoxin pneumolysin or pneumolysoid mutants (3, 6); the use of genomics and mutational libraries has identified several dozen additional species-common proteins (12, 38). Immunity has been induced by individual antigens in animal models (1, 2, 10, 39), but no vaccine based on a common antigen has been licensed to date. One disadvantage is that, given the genetic diversity of pneumococcus as well as its propensity for genetic transformation, any strategy relying on single proteins may prove to be evaded; further, antibody of a single common specificity may not bind to the capsulated cell in sufficient multiplicity to be effective. For this reason, vaccination with mixtures of species antigens has been proposed; in some studies, synergistic effects of mixtures of three proteins in systemic vaccination were demonstrated (25). We have shown that a mixture of three species-common proteins administered intranasally (i.n.) with cholera toxin (CT) adjuvant confers protection against colonization in an antibody-independent, CD4+–T-cell–dependent manner (3). We have also recently shown that protection against colonization by i.n. immunization of mice with a killed whole-cell antigen (WCA) is critically dependent on the cytokine interleukin-17A (IL-17A) and that measurement of IL-17A expression by peripheral cells of these mice in response to stimulation with WCA is an excellent predictor of resistance to carriage (17).

Here we have examined a covalent combination of three species antigens—a nontoxic derivative of pneumolysin, PdT (Asp385Asn, Cys428Gly, and Trp433Phe); the surface adhesin protein A (PsaA); and cell wall polysaccharide (CWPS)—selected with the following rationale. Pneumolysin has activity as a protective antigen per se, reducing the severity of pneumococcal infections in animal models (1), but also has potential adjuvanticity. We previously showed that the pneumolysoid PdT as well as native pneumolysin activates cells via Toll-like receptor 4 (TLR4) (18, 31). It has been suggested that immunization with an antigen and TLR agonists is more effective when the TLR agonist is part of the antigenic cargo rather than just added in solution with the antigen (5). The IL-17A pathway is also known to be dependent on TLR involvement (15). Thus, we chose PdT as one component of the construct. PsaA is antigenically common in all pneumococcal serotypes (22), and immunization with PsaA protected mice from both nasal colonization and lethal infection (26, 34). CWPS, with slight variation (30), is likewise common to all serotypes examined; antibodies directed against components of CWPS have been shown to be protective in some animal models (7, 32, 40) but not in others (33). Independently of this disagreement, however, we showed that i.n. vaccination with CWPS, albeit at a

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† Published ahead of print on 2 March 2009.
high dosage, induces an antibody-independent, IL-17A-mediated immunity (20). Here we wished to test whether covariant association of CWPS with a fusion protein consisting of PsaA and PdT would enhance protectiveness. Thus, a fusion protein of PsaA and PdT was coupled to CWPS, and this conjugate was evaluated with respect to immunogenicity and protection. Serum antibodies, priming for IL-17A production, and protection against nasopharyngeal (NP) colonization, in response to both i.n. and subcutaneous (s.c.) vaccination, were assayed, and the s.c. route was further examined in a fatal aspiration pneumonia model. The three separate antigens and the three bivalent constructs were included as controls in order to evaluate possible enhancement of immunogenicity and/or protection.

MATERIALS AND METHODS

Materials. “Pneumococcal cell-wall polysaccharide, purified” (CWPS) was from Statens Serum Institut, Copenhagen. Pneumococcal WCA consists of ethanol-killed cells of a capsule-deficient pneumococcus strain, Rx1AL, as previously reported (19). CT was from List Biological Laboratories (Campbell, CA). The monoclonal antibody to the phosphorylcholine (PC) determinant of CWPS (TEPC-15), 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, and triethylamine were from Sigma (St. Louis, MO). Other chemicals used were of analytical grade.

Protein purification. A nonhemolytic variant of pneumolysin (PdT) with mutations W433F, D385N, and C428G, which render the molecule nontoxic (4), was purified from an Escherichia coli mutant strain carrying a pQE30 vector expressing PdT (31). PsaA was a kind gift from Edward Aes, CDC. Fusion protein PsaA-PdT was generated by linking truncated PsaA with PdT with a GSGGGGS sequence in the C terminus. This DNA piece was inserted between the His6 tag and the signal peptide and transmembrane domain and has a GSGGGGS sequence in the starting codon of PdT. The sequence of the final product was verified in the Children’s Hospital, Boston, molecular genetics core facility (the structure of the fusion protein is shown in Fig. 1).

\[ \text{His-tag} \rightarrow \text{PsaA (22-309)} \rightarrow \text{PdT} \]

\[ \text{Histagged proteins were eluted with 10 mM EDTA.} \]

Purified proteins were extensively dialyzed against phosphate-buffered saline (PBS). Proteins were then treated with EtOH to elute (Cellufine, MA) to remove any residual lipopolysaccharide contamination.

Conjugation of CWPS to proteins. The method of Lees et al. (16) was used to make all the protein-poly saccharide conjugates. In brief, 5 mg of CWPS was dissolved in saline at 10 mg/ml, 3.75 mg of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (100 mg/ml in acetonitrile) was added while vortexing, 30 s later 75 μl of 0.2 M triethylamine was added, and 5 mg protein was added after 2 min. Incubation was continued overnight at 4°C and terminated with 100 μl 1 M ethanolamine. The reaction mixture was applied to a Sepharose S300 column and eluted with PBS. Protein-conjugated CWPS was separated by collecting void volume fractions. The protein concentration was determined by the bi cinchoninic acid method calibrated with bovine albumin (Pierce, Rockford, IL), and the CWPS content was determined by the anthrone reaction method (27). The compositions of the protein-poly saccharide conjugates in the constructs were as follows: PdT-CWPS conjugate protein/CWPS ratio, 4:5; PsaA-CWPS conjugate (protein/CWPS ratio, 1:2; and PsaA/PdT-CWPS conjugate ratio, 1:1:1.

Assay of IL-17A production in whole blood samples. Fifty microliters of heparinized blood was added to 450 μl Dulbecco modified Eagle medium (BioWhittaker, Walkersville, MD) containing 10% low-endotoxin defined fetal bovine serum (HyClone, Logan, UT) and ciprofloxacin (10 μg/ml; Cellgro, Manassas, VA). Except for the unstimulated control, the cultures were incubated at 37°C for 6 days with 10^7 cells of pneumococcal WCA or with purified antigens as specified. Supernatants were collected following centrifugation and stored at −80°C until analyzed by enzyme-linked immunosorbent assay (ELISA) for IL-17A concentration (R&D Systems, Minneapolis, MN).

Immunization of mice. Mice were immunized s.c. and i.n. with CWPS (5 μg/ml), 3.75 mg of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, and triethylamine were from Sigma (St. Louis, MO). Other chemicals used were of analytical grade.

Neutrophil chemotaxis assay. Neutrophils were purified from the anterior tibia of unanesthetized mice, a procedure that puts no detectable strain on the animal. The neutrophil chemotactic assay was performed as described by Leifson et al. (17).

Neutralization of pneumolysin. To evaluate whether antibodies against pneumolysin may have the capacity to neutralize the hemolytic activity of the molecule, we developed a neutralization assay. Briefly, 100 μl of a solution containing pneumolysin at 100 mg/ml in PBS-0.1% bovine serum albumin with 10 μM dithiothreitol (Sigma) was incubated for 30 min at 37°C with serial dilutions of sera from mice immunized s.c. three times with the trivalent conjugate or alum alone. Following this incubation, 50 μl of sheep red blood cells was added and incubated for another 30 min in the same conditions. After spinning at 2,000 × g for 5 min, supernatants were harvested and their optical density at 420 nm measured to quantify hemolysis. These values were read against a standard consisting of twofold-diluted samples of fully hemolyzed red blood cells; we then compared the dilutions of serum at which 50% hemolysis was observed between groups.

Immunization and challenge of mice. C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in all the experiments. The age at time of first immunization was between 4 and 6 weeks. i.n. immunization was done by instilling 10 μl of saline, adjuvant. The following antigen or antigen was specified intranasally into unanesthetized mice, a procedure that puts no detectable strain on the lungs; secondary immunizations were given after 1 week. s.c. immunization was done by mixing the antigen with 200 μl of alum hydroxide; Accurate Chemical & Scientific Co, Westbury, NY) in a volume of 200 μl and injection into the dorsal surface of the mouse. The amounts of all antigens were normalized to 5 μg PsaA per mouse. To determine susceptibility to NP colonization, i.n. challenge with live encapsulated pneumococci was done as described previously (19): 4 weeks after the second immunization, mice were challenged with 10^7 CFU of serotype 6B strain 0603 or of a strain of serotype 19F in the TIGR4 background (35) in 10 μl of PBS applied as just described. To determine whether protection against colonization was CD4 T-cell dependent, a small subset of mice received intraperitoneal injections of 0.35 mg of rat anti-mouse CD4 monoclonal IgG2b (purified from hybridoma GK1.5; American Type Culture Collection [ATCC], Manassas, VA) 1 day prior to and on day 3 of challenge as we have done previously (3, 21). To determine NP colonization, a
nasal wash was done by instilling sterile saline retrograde through the transected trachea, collecting the first 6 drops (about 0.1 ml) from the nostrils, and plating neat or diluted samples on blood agar plates containing 2.5 g gentamicin/ml. The figures show the CFU per nasal wash sample of individual mice and geometric means as a horizontal bar; a sterile sample was assigned half of the lower limit of detection, or 0.8 CFU/nasal wash. As a model for pneumonia and sepsis, the heavily encapsulated serotype 3 strain WU2 (7) was used as described previously (20). The mice were given $10^6$ CFU in 10 µl of PBS i.n. to establish NP colonization; after 2 days, to mimic the aspiration of pneumococci, the mice were anesthetized with isoflurane and given an additional dose of $10^6$ CFU of the

FIG. 2. Responses to i.n. immunization with bivalent conjugates or uncoupled antigens. (A to C) Mice were vaccinated weekly twice with 1 µg of CT as adjuvant. Antigen dosages, coupled or not, were 8 µg of PdT and 10 µg of CWPS. Blood was taken 3 weeks after the second immunization. (A and B) Serum IgG antibody responses to PdT (A) and CWPS (B) were assayed. Mice immunized with the PdT-CWPS conjugate made significantly more anti-PdT and anti-CWPS antibodies than mice that received CT alone and more anti-CWPS antibodies than mice that received the mixture of PdT and CWPS. (C) The mice were challenged i.n. with the serotype 6B strain at 4 weeks postimmunization, and the density of colonization was determined 7 days later by plating dilutions of nasal washes. No protection against colonization was observed in any of the immunization groups. (D and E) Mice were immunized with CT with or without the addition of a conjugate of PsaA (5 µg per dose) and CWPS (10 µg per dose). Immunization with PsaA-CWPS resulted in significantly higher levels of anti-PsaA antibodies than in control mice (D), but no protection was observed following i.n. challenge (E). For all panels, horizontal lines represent geometric means, and statistical analysis was performed using the Kruskal-Wallis test with Dunn’s correction or the Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$. 

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serotype 3 strain in 100 μl by the i.n. route. In control animals, this challenge produced death within 4 to 5 days in most mice and bacteremia in all mice. A control group in which mice received 1 ml of human antipneumococcal capsular antibody (BPIG-8, a plasma concentrate from volunteers immunized with bacterial polysaccharides, including pneumococcal serotype 3 [28]) on the day before challenge was included.

Statistical analysis. NP colonization densities were compared by use of the Mann-Whitney U test or the Kruskal-Wallis test when indicated using PRISM.
WHEN THE KRUSKAL-WALLIS TEST WAS USED, EACH GROUP WAS COMPARED TO THE GROUP RECEIVING ADJUVANT ALONE, WITH DUNN’S CORRECTION FOR MULTIPLE COMPARISONS. DIFFERENCES IN SURVIVAL WERE ANALYZED WITH THE KAPLAN-MEIER TEST, USING PRISM AS WELL.

RESULTS

I.N. IMMUNIZATION WITH BIVALENT CONJUGATES PDdT-CWPS AND PSAA-CWPS OR UNCOPLED CONTROLS. MICE WERE I.N. IMMUNIZED TWICE AT A 1-WEEK INTERVAL WITH CWPS, PDdT, MIXTURES OF CWPS AND PDdT, OR THE BIVALENT CONJUGATE PDdT-CWPS, ALL WITH AMOUNTS OF EACH ANTIGEN EQUALIZED; 1 µg OF CT WAS USED AS AN ADJUVANT AND WAS ALWAYS TESTED ALONE AS ONE CONTROL. THE SERUM IgG ANTIBODY RESPONSES TO PDdT AND CWPS MEASURED BY ELISA IN SERA TAKEN 3 WEEKS AFTER THE LAST IMMUNIZATION ARE SHOWN IN FIG. 2. ANTI-CWPS (FIG. 2B) ANTIBODY RESPONSES IN THE PDdT-CWPS CONJUGATE-IMMUNIZED MICE WERE GREATER THAN THOSE IN MICE IMMUNIZED WITH MIXTURES OF THE TWO ANTIGENS, BUT THE ANTI-PDdT RESPONSES IN THE PDdT-CWPS CONJUGATE-IMMUNIZED MICE WERE NO GREATER THAN THOSE IN MICE GIVEN THE RESPECTIVE MIXTURE (FIG. 2A). THE MICE WERE THEN CHALLENGED I.N. WITH THE SEROTYPE 6B PNEUMOCOCCUS STRAIN 4 WEEKS AFTER THE SECOND IMMUNIZATION. THE DOES OF AND NUMBER OF IMMUNIZATIONS (2) WITH THE UNCOUPLED IMMUNOGENS WERE CHOSEN BASED ON PRIOR EXPERIMENTS IN OUR LABORATORY WHICH SHOWED MINIMAL PROTECTION AGAINST CARRIAGE (DATA NOT SHOWN). THE CFU PER MOUSE IN NASAL WASHES COLLECTED 1 WEEK AFTER CHALLENGE ARE SHOWN IN FIG. 2C. NO STATISTICALLY SIGNIFICANT REDUCTION OF BACTERIAL COLONIZATION, COMPARED TO THE CONTROLS RECEIVING CT ALONE, WAS OBSERVED IN ANY OF THE IMMUNIZATION GROUPS.

NEXT WE EVALUATED A BIVALENT CONJUGATE CONSISTING OF PSAA AND CWPS. AS SHOWN IN FIG. 2D, MICE IMMUNIZED WITH THIS CONJUGATE HAD SIGNIFICANTLY HIGHER ANTIBODY RESPONSES THAN MICE IMMUNIZED WITH THE ADJUVANT ALONE, BUT THERE WAS NO OBSERVABLE PROTECTION AGAINST COLONIZATION (FIG. 2E).

I.N. IMMUNIZATION WITH THE PSAA-PDdT FUSION PROTEIN CWPS CONJUGATE. HAVING FAILED TO SEE ANY PROTECTION AGAINST COLONIZATION FOLLOWING TWO DOES OF EITHER OF THE TWO BIVALENT CONJUGATES, WE NEXT IMMUNIZED MICE WITH THE TRIVALENT CONJUGATE CONSISTING OF THE FUSION OF PSAA AND PDdT CONJUGATED TO CWPS. A MIXTURE OF THE THREE ANTIGENS OR THE PSAA-PDdT FUSION PROTEIN ALONE (ALL GIVEN WITH THE ADJUVANT CT) WAS USED AS A CONTROL; ALSO INCLUDED AS CONTROLS WERE MICE VACCINATED WITH THE PNEUMOCOCCAL WCA AND CT. TITERS OF IgG ANTIBODIES TO PDdT, CWPS, AND PSAA AT 3 WEEKS AFTER IMMUNIZATION ARE SHOWN IN FIG. 3. THE CONJUGATE INDUCED HIGHER TITERS OF ANTICWPS THAN THE MIXTURE (FIG. 3B); THE CONJUGATE AND THE FUSION PROTEIN BOTH INDUCED HIGHER TITERS OF ANTI-PSA THAN THE MIXTURE (FIG. 3C). INTERESTINGLY, HOWEVER, THE CONJUGATE INDUCED LESS ANTI-PDdT IgG THAN THE MIXTURE OR FUSION PROTEIN (FIG. 3A). PRIMING FOR EXPRESSION OF IL-17A IN VITRO IS SHOWN IN FIG. 3D; MICE IMMUNIZED WITH MIXTURES OF ANTIGENS OR THE FUSION CONJUGATE PRODUCED SIGNIFICANTLY HIGHER IL-17A LEVELS THAN THOSE IMMUNIZED WITH THE ADJUVANT ALONE (P < 0.05 BY THE KRUSKAL-WALLIS TEST). IN PARTICULAR, MICE IMMUNIZED WITH THE TRIVALENT CONJUGATE HAD IL-17A EXPRESSION THAT WAS SIMILAR TO THAT OF THE MICE IMMUNIZED WITH WCA. THE RESULTS OF CHALLENGE WITH SEROTYPE 6B PNEUMOCOCCI ARE SHOWN IN FIG. 3E. THERE WAS NO SIGNIFICANT PROTECTION BY THE MIXTURE OR THE FUSION PROTEIN (ALTHOUGH THERE WAS A SUGGESTION OF ACTIVITY BY THE LATTER) COMPARED TO THE CT ADJUVANT ALONE (P > 0.05 BY THE KRUSKAL-WALLIS TEST WITH DUNN’S CORRECTION FOR MULTIPLE COMPARISONS).
Wallis test with Dunn's correction for comparisons of mixture or fusion proteins versus CT alone). In contrast, there was about 40-fold reduction in the geometric mean colonization of the conjugate-immunized mice (\(P < 0.01\) by the Kruskal-Wallis test with Dunn's correction). This protection was comparable to that produced by the pneumococcal WCA, which is known to be highly protective in this model (19).

s.c. immunization with \textit{PsaA-PdT–CWPS}. To examine the trivalent conjugate as a systemic immunogen, mice were injected s.c. (2-week intervals, alum adjuvant, either twice or three times) with the conjugate or the control mixture of PdT, CWPS, and PsaA. Antibodies were measured 2 weeks after the second immunization. Following two doses, the conjugate generated antibody titers similar to those of the mixture to PdT and PsaA (\(P > 0.5\) by the Mann-Whitney U test) but significantly higher CWPS titers (140-fold higher; \(P < 0.008\) by the Mann-Whitney U test) (Fig. 4A, B, and C). Antibody responses against WCA also were measured and were likewise greater (40-fold; \(P < 0.008\) by the Mann-Whitney U test) after immunization with the conjugate than with the mixture (Fig. 4D). Inhibition studies confirmed that the bulk of the antibody response was directed at the PC component: there was over a 23-fold reduction in antibody detection when serum was preincubated with PC (\(P < 0.008\) versus preincubation in PBS-Tween alone [Mann-Whitney U test]; data not shown). Thus, the fusion conjugate strongly elicited antibodies to its component antigens in configurations expressed in the pneumococcal cell. Priming for IL-17A responses and protection against colonization were determined after three s.c. injections. Cells from the s.c. vaccinated mice produced on average about 100-fold higher IL-17A than the CT control (Fig. 5A) (\(P < 0.001\) by the Mann-Whitney U test). In addition, there was an eightfold increase in the pneumolysin-neutralizing ability of sera from mice immunized three times with the trivalent conjugate compared to sera from alum-immunized mice (\(P < 0.0001\) [Mann-Whitney U test]; data not shown). The mice were nasally challenged (separately) with a strain of serotype 19F or serotype 6B. There was protection against both strains by about one log fold compared to the alum controls (\(P < 0.05\) by the Mann-Whitney U test) (Fig. 5B). At the time of challenge with the serotype 6B strain, antibodies to murine CD4 were administered to four mice previously immunized with the trivalent conjugate; although the number of mice included in this group was small, protection was completely abrogated in this group, suggesting that CD4 T cells (and likely IL-17A) are responsible for this protection (data not shown).

Possible protection against invasive disease was tested using a fatal aspiration pneumonia model with the serotype 3 pneumococcal strain (20). Figure 6 shows that only 35% of the alum control mice survived. Survival of mice immunized with the antigen mixture (55%) was not significantly different. In contrast, 100% of mice vaccinated with the conjugate survived (\(P < 0.002\) versus alum controls by the Kaplan-Meier test).
DISCUSSION

Protection by the extant 23-valent capsular polysaccharide vaccine depends upon immunological maturity. In general, the vaccine is not effective for subjects <2 years of age, particularly infants, in whom pneumococcal disease is most prevalent. The 7-valent capsular conjugate vaccine (PCV7) has had a dramatic effect on the epidemiology of pneumococcal infections (13, 37); however, it does not include some serotypes prevalent in developing-world populations. Moreover, recent studies have shown that the use of PCV7 has been followed by increased colonization and infection by nonvaccine serotypes (13, 24, 29). One potential solution to the limited serotype coverage and serotype replacement problem is the addition of serotypes to the mixture, which increases the cost of an already expensive vaccine. Alternative approaches, particularly to address the large burden of pneumococcal disease in some areas of the developing world, are needed. A number of investigators have sought to develop vaccines based upon species-common antigens, but so much vaccine has yet been brought into clinical use.

Here, three such antigens, each known from prior work to have protective potential in different animal models, have been combined in a trivalent conjugate. This construct was significantly more protective than a mixture of its components or than any of the three bivalent combinations. The trivalent conjugate when given i.n. reduced NP colonization by a strain of serotype 6B, a major disease type in infancy. Although the conjugate elicited antibodies to all three of its component antigens, the protection against colonization appears more to depend upon priming for enhanced elicitation of IL-17A upon encounter with pneumococci. We recently showed that this cytokine promotes agar surface killing of pneumococci by polymorphonuclear leukocytes in the absence of antibody and complement, and we hypothesized that a similar mechanism might operate at the NP mucosa (17).

i.n. immunization using enterotoxin or related adjuvants such as CT, although immunologically advantageous, raise safety concerns due to possible entry into the central nervous system via the olfactory nerve and findings following the use of a killed nasal influenza vaccine in Switzerland (23). Thus, we also tested s.c. presentation with alum, the standard adjuvant in human vaccination. By this mode of immunization, the trivalent conjugate was active in antibody induction and priming for IL-17A, and it reduced NP colonization by the two tested serotypes, 19F and 6B. The antibody responses to the conjugate would be highly advantageous in an immunization strategy: if pneumococci evaded what we believe is a predominantly CD4+ T-cell-mediated protection against colonization (20, 21), the antibodies would represent an additional line of defense.

Protection by s.c. vaccination was also tested in a model of fatal disease due to aspiration of a highly capsulated serotype 3 strain. Here the conjugate, but not the mixture of its three component antigens, was highly protective, again demonstrating the superiority of the fusion conjugate over the mixture. Studies are under way to understand the increased immunogenicity and protective capacity of the fusion conjugate over the mixture of the three antigens. There was enhancement from coupling to CWPS, seen with the IL-17A priming, which may be merely a physicochemical effect or may be due specifically to the potential of zwitterionic polysaccharides to activate T<sub>17</sub> cells (8, 9, 14, 20, 36). The possible role of TLR4 in this enhanced protection is being pursued as well.

Our results may be generalizable to other proteins (beyond PsaA and involving pathogens other than pneumococcus). Thus, a construct consisting of CWPS conjugated to pneumolysin fused with another protein target should result in greatly enhanced immunity to the target protein and offer the possibility of providing protection against pneumococcus as well as other pathogens.

ACKNOWLEDGMENTS

We thank E. Ades for providing PsaA antigen and K. Trzcinski and D. Weinberger for providing the pneumococcal 19F strain. We gratefully acknowledge support from the Pamela and Jack Egan Fund. This work was supported by a grant from the National Institutes of Health (AI067737 to R.M.). We declare that we have no competing interests.

REFERENCES


