Novel Protein-Based Pneumococcal Vaccines Administered with the Th1-Promoting Adjuvant IC31 Induce Protective Immunity against Pneumococcal Disease in Neonatal Mice

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Streptococcus pneumoniae is responsible for many vaccine-preventable deaths, annually causing around 1 million deaths in children younger than 5 years of age. A new generation of pneumococcal vaccines based on conserved proteins is being developed. We evaluated the immunogenicities and protective efficacies of four pneumococcal protein vaccine candidates, PcsB, StkP, PsaA, and PspA, in a neonatal mouse model. Mice were immunized three times and challenged intranasally with virulent pneumococci. All four proteins were immunogenic in neonatal mice, and antibody (Ab) responses were significantly enhanced by the novel adjuvant IC31, which consists of an antibacterial peptide (KLKL₅KLK) and a synthetic oligodeoxynucleotide, ODN1a, that signals through Toll-like receptor 9 (TLR9). Two single proteins, StkP and PspA, combined with IC31 significantly reduced pneumococcal bacteremia but had no effects on lung infection. Three proteins, PcsB, StkP, and PsaA, were evaluated with alum or IC31. IC31 enhanced Ab responses and avidity to all three proteins, whereas alum enhanced Ab responses and avidity to StkP and PsaA only. Mice receiving the trivalent protein formulation with IC31 had significantly reduced bacteremia and lung infection compared to unvaccinated mice, but the level of protection was dependent on the dose of IC31. When PspA was added to the trivalent protein formulation, the dose of IC31 needed to obtain protective immunity could be reduced. These results demonstrate that a novel pneumococcal protein-based vaccine is immunogenic at an early age of mice and emphasize the benefits of using a combination of conserved proteins and an effective adjuvant to elicit potent protective immunity against invasive pneumococcal disease.

Streptococcus pneumoniae, or the pneumococcus, can cause life-threatening invasive diseases (meningitis and bacteremia) and pneumonia and frequently causes otitis media in children. The pneumococcus remains a major cause of vaccine-preventable deaths. Current vaccines are based on capsular polysaccharides (PSs), which are a major virulence factor and the basis for the classification of pneumococci into more than 90 different serotypes. Therefore, the effect of a polysaccharide-based vaccine is restricted to the serotypes included in the vaccine, and the coverage varies due to different geographical serotype distributions (13). The pneumococcal PS (PPS) vaccine contains purified PSs of 23 serotypes (PPV23) that account for 85 to 90% of invasive disease in adults in the United States. However, PSs are not immunogenic in children <2 years of age (8, 16, 18) and do not induce immunological memory (26). These limitations were overcome by conjugating pneumococcal PS to proteins, converting the PSs from T-cell-independent (TI) to T-cell-dependent (TD) antigens (Ags) (37) that elicit immune responses in infants and induce immunological memory, affinity maturation, and isotype switching of antibodies (Abs). The seven-valent pneumococcal conjugate vaccine (PCV7), which contains 7 PPS conjugated to a non-toxic mutant of diphtheria toxin (CRM₁₉₇), is highly efficacious against invasive disease caused by the vaccine serotypes in children (5). Due to geographical differences in serotype distribution, PCV7 lacks many serotypes that cause serious pneumococcal disease in developing countries (12). Also, serotype replacement in the nasopharynx following vaccination with PCV7 has been reported (44), resulting in pneumococcal diseases caused by serotypes not included in the vaccine (14, 15, 38, 43). Ten-valent and 13-valent PCVs have recently been licensed, increasing the sero-

type coverage, but the conjugation technology is expensive, limiting access to PCVs in resource-poor countries.

A new generation of protein-based pneumococcal vaccines is being developed. Proteins are immunogenic at birth and induce immunological memory. Well-conserved protein vaccines could provide broad serotype and geographical coverage and be a less expensive alternative to PCVs. Conventional vaccine development is based on Ags with known roles in bacterial pathogenesis, but more recent searches for vaccine candidates are based on genomic approaches. Reverse vaccinology (35) utilizes the whole bacterial genome sequence to predict proteins that are suitable vaccine candidates and has proven effective for various pathogens, in particular *Neisseria meningitidis* serogroup B (33). The ANTIGENome technology is another approach developed to select novel pneumococcal vaccine candidates using genomic surface display libraries to identify proteins recognized by Abs in sera from patients recovering from invasive disease as well as healthy noncarrier adults exposed to the pathogen (24). The ANTIGENome technology identified the majority of known pro-

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protective pneumococcal proteins, including PspA and PspC, and further discovered two novel antigens, PcsB and StkP, that both have almost 100% amino acid identity between different serotypes and strains and are thus promising vaccine candidates (10). PcsB is important for pneumococcal cell wall synthesis and survival (27, 28). It is not essential for the pneumococcus, but a PcsB deletion causes greatly reduced growth *in vitro* and a complete loss of virulence (10). StkP has amino acid sequence homology to serine/threonine kinases and was suggested previously to play a role in cell wall synthesis, cell-cell signaling, virulence, and resistance to stress conditions (9, 29, 32). StkP deletion mutants are less virulent in mice, and electron microscopic studies revealed an altered cell shape, indicating a defect in cell division (10).

Young infants are the prime targets for protein-based pneumococcal vaccines. We therefore evaluated the immunogenicities and protective efficacies of four pneumococcal proteins, PcsB, StkP, PsaA, and PspA, in a neonatal mouse model using intranasal (i.n.) challenge with virulent pneumococci to mimic the natural route of infection. Since infants respond weakly to vaccinations (42), we tested the effects of two potent adjuvants, alum and IC31, on the immune response to the pneumococcal proteins. Alum is still the most extensively used human adjuvant and the only adjuvant used with pneumococcal vaccines. In recent years, three novel adjuvants, MF-59, ASO3, and ASO4, have been licensed for human use (reviewed in reference 7). Although alum has been shown to improve the Ab response to several Ags, including diphtheria and tetanus toxoids, it is a poor inducer of Th1 immune responses and may thus be suboptimal for neonates that have Th2-dominant immune responses. Effective Th1 responses are required to protect against several infectious diseases, including tuberculosis (TB), HIV/AIDS, and hepatitis C. Furthermore, Th17 responses have been shown to play an important role in protecting mice against pneumococcal colonization (19, 20). The total amount of alum acceptable for human vaccines (1.0 to 1.5 mg/vaccination) restrains the number of Ags that can be used within the same formulation. IC31 is a novel two-component adjuvant consisting of an antibacterial peptide (KLKL₅KLK [KLK]) and the synthetic oligodeoxynucleotide ODN1a that signals through Toll-like receptor 9 (TLR9). Unlike alum, IC31 enhances both Th1- and Th2-associated humoral responses and a high level of production of the Th1-associated cytokine gamma interferon (IFN- γ) (1, 36, 39). Results from human trials of IC31 showed that when combined with the TB vaccine candidate Ag85B-ESAT6, it was well tolerated and highly immunogenic, with strong Th1 responses persisting more than 2.5 years following vaccination (48). We have previously shown that IC31 enhances the murine neonatal Ab response to a monovalent PCV and improves protection against pneumonia and lung infection (31).

In this study, we demonstrate protective effects of a protein-based pneumococcal vaccine in a neonatal murine model. However, the levels of protection depend on the combination of protein candidates and the dose of the Th1-promoting adjuvant IC31.

MATERIALS AND METHODS

Mice. Adult NMRI mice were purchased from M&B AS (Ry, Denmark) and allowed to adapt for 1 week before matching. They were kept in microisolator cages with free access to commercial food pellets and water and were housed under standardized conditions with regulated daylight humidity and temperature. Breeding cages were checked daily, and pups

were kept with their mothers until weaning at 4 weeks of age. The study was approved by the Animal Experimental Committee of Iceland.

Vaccine and adjuvants. PcsB, StkP, PsaA, and PspA were identified and produced by Intercell AG (Vienna, Austria) as described previously (10). The adjuvants used were IC31, provided by Intercell, and aluminum hydroxide gel adjuvant (Brenntag Biosector, Denmark), here called alum.

Immunization. Neonatal (7-day-old) mice (8 per group) were immunized subcutaneously (s.c.) in the scapular girdle region with 50 μ l of each vaccine formulation. Sixteen days later, mice received a second dose (100 μ l) of the vaccine formulation, and a third dose (200 μ l) was administered 2 weeks later. Tris buffer (10 mM Tris–70 mM NaCl [pH 6]) was added to the vaccine formulation to obtain the right volume. Each mouse received 20 μ g of each protein, i.e., PcsB, StkP, PspA, and PsaA, alone or combined. IC31 was given at three different doses: 15.75 nmol KLK and 0.63 nmol ODN1a (IC31 lower low dose [LLD]), 50 nmol KLK and 2 nmol ODN1a (IC31 low dose [LD]), or 90 nmol KLK and 3.6 nmol ODN1a (IC31 high dose [HD]), as indicated in the respective figure legends. Alum was used at a dose of 0.48% aluminum hydroxide gel adjuvant (Brenntag Biosector, Denmark) per 1 μ g of protein.

Blood samples. Three weeks after the priming and then weekly, mice were bled from the tail vein, and serum was isolated and stored at -20°C until use.

Pneumococci and mouse challenge. Two weeks after the second immunization or 4 weeks after priming, mice were challenged intranasally (i.n.) with *S. pneumoniae* serotype 1 (ATCC 6301; American Type Culture Collection, Rockville, MD). A stock solution was maintained in tryptose broth plus 20% glycerol at -70°C . One day before challenge, the bacteria were plated onto blood agar made of tryptone soya agar (Oxoid, Cambridge, United Kingdom) supplemented with gentamicin and horse serum (Keldur, Reykjavik, Iceland) and incubated at 37°C in 5% CO_2 overnight. Isolated colonies were transferred into Todd-Hewitt broth (Oxoid), cultured at 37°C to log phase for 3.5 h, and resuspended in sterile saline. Serial 10-fold dilutions were plated onto blood agar to determine the challenge dose as CFU in 50 μ l. The challenge dose for each experiment is given in the representative figure legends.

Twenty-four hours after i.n. challenge, the mice were sacrificed, blood samples were taken from the tail vein, and 10-fold serial dilutions were plated onto blood agar that included Staph/Strep selective supplement containing nalidixic acid and colistin sulfate (Oxoid) and incubated at 37°C in 5% CO_2 overnight. Bacteremia was determined as the number of CFU per ml of blood. Lungs were removed, homogenized, and diluted into 3 ml saline, and serial dilutions were plated onto blood agar plates, which were incubated for 48 h at 37°C under anaerobic conditions. Pneumococcal lung infection was expressed as CFU per ml of lung homogenate. Depending on the first dilution used, the detection limits were 2.2 log CFU/ml lung homogenate and 1.3 log CFU/ml blood.

Antibodies to PcsB, StkP, PsaA, and PspA. PcsB-, StkP-, PspA-, and PsaA-specific Abs (IgG) were measured by an enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates (Immulon 2HB; Thermo) were coated with 100 μ l/well of 1 μ g/ml of PcsB, StkP, PspA, or PsaA in phosphate-buffered saline (PBS) overnight at 4°C . Serum samples were diluted 1:50 in PBS containing 0.05% Tween 20 (Sigma, St. Louis, MO) and 2% bovine serum albumin (BSA) (Sigma). The plates were washed 3 times with PBS-Tween, and the serum samples were added, serially diluted, incubated in duplicates for 2 h at room temperature (RT), washed as described above, and incubated for 2 h at RT with horseradish peroxidase-conjugated goat anti-mouse IgG Abs (Southern Biotechnology Associates Inc., Birmingham, AL) diluted 1:20,000 in PBS-Tween–2% BSA. The plates were washed as described above, and the enzyme reaction was developed by the addition of 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) to the mixture. After 10 to 30 min, the reaction was stopped by the addition of 100 μ l of 0.18 M H_2SO_4 to each well. The absorbance was measured at 450 nm with an ELISA spectrophotometer (Original Multiscan Ex; Thermo Electron Corporation, Vantaa, Finland). Serial dilutions of a reference

serum, obtained by immunizing adult mice with each of the protein antigens and IC31, were included on each plate. The titer of the reference serum corresponded to the inverse of the serum dilution giving an optical density of 1.0. The titers of the test serum samples were calculated from the reference serum and based on a minimum of four data points and parallelism between the serum samples and the reference curve. The detection limit was 1.0 ELISA units (EU)/ml. The results were expressed as the median log ELISA units per ml with interquartile ranges.

Avidity. The avidity of antigen-specific IgG Abs in sera was measured by using the ELISA protocols described above but with the addition of a potassium thiocyanate (KSCN) elution step (11). Microtiter plates were coated with specific Ags as described above. Serum samples were diluted 1:50 in PBS-Tween. The plates were then washed three times with PBS containing 0.05% (vol/vol) Tween 20 (Sigma)–2% BSA, and the sera were serially diluted and incubated in duplicates in the Ag-coated plates for 2 h at RT. After washing 3 times with PBS-Tween, 2-fold dilutions of KSCN, ranging from 0.117 M to 7.5 M, or PBS-Tween (indicating 100% binding) were added, and incubation continued for 15 min at RT. After washing 3 times with PBS-Tween, the plates were incubated for 2 h with alkaline-phosphatase-labeled anti-mouse IgG (Southern Biotech, Birmingham, AL) that was diluted to a working concentration of 1:3,000 in PBS-Tween. The reaction mixture was developed by using 5-mg tablets of phosphatase substrate (Sigma) in substrate buffer (pH 9.8), and the absorbance was read at 405 nm by use of a Multiscan Ex spectrophotometer. The results were expressed as the avidity index (AI), which is defined as the M KSCN needed to displace 50% of bound IgG antibodies.

Statistical analysis. The overall *P* value was calculated by using the Kruskal-Wallis nonparametric analysis of variance (ANOVA). The nonparametric Mann-Whitney rank sum test and the Fisher exact test were used for statistical comparisons between groups. The Fisher exact test was performed to compare the frequencies of protected mice between the groups using CFU/ml lower than the mean CFU plus 2× the standard deviation in the control group as a measure of protection. The program Sigma Stat was used for statistical analysis. A *P* value of <0.05 was considered statistically significant.

RESULTS

Neonatal immunization with the quadrivalent protein formulation and the adjuvant IC31 reduces bacteremia and lung infection. Neonatal (7-day-old) mice were immunized with a single pneumococcal protein, PcsB, StkP, PsaA, or PspA (20 µg of each protein), with IC31 (50 nmol KLK and 2 nmol ODN1a, which corresponds to half of the dose previously used for adult mice [39]) or all four proteins combined (quadrivalent protein formulation), with or without IC31. Mice receiving Tris buffer were used as a negative control. The immunizations were repeated twice at 2-week intervals. Two weeks after the third immunization, the mice were challenged with *S. pneumoniae* serotype 1 to evaluate protection against bacteremia and lung infection.

All four single proteins were immunogenic in neonatal mice when administered with IC31. Due to poor immune responses early in life, the single proteins were not tested without adjuvant, but the immune responses were compared with those elicited by the quadrivalent formulation given with and without IC31. When immunized with the quadrivalent protein formulation, IC31 significantly enhanced the Ab responses to PcsB (*P* = 0.003), StkP (*P* < 0.001), and PspA (*P* < 0.001) compared to the Ab responses to the quadrivalent formulation administered without an adjuvant (Fig. 1). Furthermore, the Ab response was more rapid for the adjuvanted formulation, and higher Ab levels were observed against all proteins except PsaA at all time points measured, 2 weeks after the first, second, and third doses.

A significant reduction in the level of bacteremia compared to that

of the negative-control group was observed after immunization with two single proteins given with IC31, StkP (*P* < 0.05) and PspA (*P* < 0.05) (Fig. 2A), but none of the single proteins admixed with IC31 reduced lung infection (Fig. 2B). A significant (*P* < 0.05) reduction of both bacteremia and lung infection was observed only for mice receiving the quadrivalent protein formulation and IC31, whereas the quadrivalent formulation without adjuvant reduced neither bacteremia nor lung infection (Fig. 2).

IC31 induces higher-level and broader Ab responses than alum in combination with a trivalent protein formulation. The immunogenicity and protective efficacy of a vaccine formulation consisting of the three proteins PcsB, StkP, and PsaA (trivalent protein formulation) in combination with alum or IC31 at different doses (lower dose, 50 nmol KLK and 2 nmol ODN1a; higher dose, 90 nmol KLK and 3.6 nmol ODN1a) were evaluated by using the same experimental setup as that described above. Mice receiving only alum or IC31 were used as negative controls.

Mice receiving the trivalent protein formulation without adjuvant showed significant Ab responses to StkP (*P* = 0.0003) and PsaA (*P* = 0.0002) compared with control mice that received only adjuvant (alum/IC31), but no PcsB-specific Ab response was observed (Fig. 3). Alum and either dose of IC31 significantly enhanced neonatal IgG Ab responses to StkP (*P* < 0.001) and PsaA (*P* = 0.0002), and IC31 but not alum enhanced PcsB-specific Ab responses (lower dose, *P* = 0.0185; higher dose, *P* = 0.0004) compared with the trivalent protein formulation without adjuvant. The higher dose of IC31 given with the trivalent protein formulation enhanced the Ab responses to PcsB (*P* < 0.001) and PsaA (*P* = 0.008) compared with alum. The higher dose of IC31 enhanced the StkP-specific Ab response more than the low dose of IC31 (*P* = 0.0052).

The adjuvant effect on the IgG subclass pattern induced by the trivalent protein formulation was assessed. The higher dose of IC31 enhanced PcsB-specific IgG1 titers (*P* < 0.001) compared with the IgG1 titers obtained with no adjuvant (Fig. 4). Alum and the low and high doses of IC31 significantly enhanced IgG1 responses specific for StkP (*P* < 0.001) and PsaA (*P* = 0.010, *P* = 0.007, and *P* = 0.011, respectively) compared with the responses with the trivalent formulation without an adjuvant. The high dose of IC31 enhanced PcsB- and StkP-specific IgG1 responses compared with those induced by alum (*P* = 0.001 and *P* < 0.001, respectively) or the lower dose of IC31 (*P* < 0.001 and *P* = 0.002, respectively). Alum enhanced IgG2a-specific Abs for PsaA (*P* = 0.038) but not for PcsB or StkP compared with the trivalent protein formulation without an adjuvant. The high dose of IC31 significantly enhanced IgG2a specific for PcsB (*P* = 0.042) but not for StkP or PsaA compared with the proteins without an adjuvant. IgG2a Ab levels specific for PsaA were highest in mice receiving the lower dose of IC31 and significantly higher than when alum (*P* < 0.001) or the high dose of IC31 (*P* = 0.008) was given with the trivalent protein formulation.

Alum and both the lower and higher doses of IC31 significantly enhanced the avidity of IgG Abs for StkP (*P* = 0.002, *P* = 0.017, and *P* = 0.005, respectively) and PsaA (*P* = 0.020, *P* = 0.006, and *P* = 0.006, respectively) (Fig. 5). However, the avidity of PcsB-specific Abs was increased by IC31 at the low (*P* = 0.0298) and high (*P* = 0.0006) doses but not by alum.

The level of protection elicited by the trivalent formulation is increased by IC31 in a dose-dependent manner, and PspA further improves protective efficacy. The effects of increasing doses

FIG 1 IgG antibody responses to PcsB, StkP, PsaA, and PspA are enhanced by IC31. Shown are data for PcsB (A)-, StkP (B)-, PsaA (C)-, and PspA (D)-specific Ab responses elicited by immunizations with each protein alone or in a quadrivalent protein formulation with or without IC31 (LD, 50 nmol KLK and 2 nmol ODN1a). IgG levels were measured by ELISA 2 weeks after one (week 2), two (week 4), and three (week 6) immunizations. The results for one experiment are shown ($n = 8/\text{group}$). Bars are shown as medians of each group with interquartile ranges. An asterisk indicates statistically higher Ab levels than those in the group receiving Tris buffer ($P < 0.05$). P values are shown for comparison between the groups receiving the 4 proteins with and without IC31, when statistically significant. The overall P values, calculated by using the Kruskal-Wallis test, were a P value of 0.0006 (week 2) and a P value of <0.0001 (weeks 4 and 6) for PcsB-specific Abs; a P value of <0.001 (weeks 2, 4, and 6) for StkP-specific Abs; a P value of 0.0381 (week 2), a P value of 0.0031 (week 4), and a P value of 0.0004 (week 6) for PsaA-specific Abs; and a P value of 0.0003 (week 2), a P value of <0.0001 (week 4), and a P value of 0.0002 (week 6) for PspA-specific Abs.

of IC31 on neonatal Ab responses to four proteins and protective capacity were evaluated. A clear dose-dependent effect on protection was observed.

The trivalent protein formulation (PcsB, StkP, and PsaA) given with the lower dose of IC31 significantly increased the frequency of mice protected against bacteremia ($P = 0.026$) but not lung infection (Fig. 6). The level of bacteremia (CFU/ml) did not differ significantly between the groups due to high variability in CFU/ml within the immunized groups. However, when PspA was added to the trivalent protein formulation and given with the lower IC31 dose, a reduction in levels of both blood and lung infection was observed (Fig. 2). Preliminary data suggest that PspA also adds to the protection against lung infection provided by the trivalent protein vaccine when administered with the higher dose of IC31 (see Fig. S1 in the supplemental material). A higher IC31 dose (up to 90 nmol KLK and 3.6 nmol ODN1a) was needed for the trivalent protein formulation to induce a significant increase in the number of mice protected against both bacteremia ($P = 0.029$) and lung infection ($P = 0.029$) compared to the number of mice protected with adjuvant only (Fig. 6). Mice that received the trivalent protein formulation alone or with alum were not protected against pneumococcal infections (Fig. 6). Neither single proteins

nor the quadrivalent protein formulation with a further reduced dose of IC31 (LLD, 15.75 nmol KLK and 0.63 nmol ODN1a) elicited protection against bacteremia or pneumonia (see Fig. S2 in the supplemental material).

DISCUSSION

Protein-based pneumococcal vaccines should include conserved pneumococcal proteins that are able to provide serotype-independent, cost-effective protection at an early age. The selection of well-conserved proteins that play important roles in bacterial survival and growth reduces the risk of negative selection caused by vaccination. Two of the newly described proteins evaluated in this study, PcsB and StkP, fulfill these criteria. They are exceptionally well conserved ($>99.5\%$), and knockout strains showed reduced growth and virulence (10). PsaA is also a highly conserved lipoprotein produced by all pneumococci (34), whereas PspA can be grouped into three families, but significant cross-protection between families has been observed (22, 45).

We have demonstrated that all four proteins tested, PcsB, StkP, PsaA, and PspA, were immunogenic in neonatal mice, provided protection against bacteremia, and significantly reduced lung infection when combined and given with the adjuvant IC31. Fur-

FIG 2 A quadrivalent protein formulation admixed with a lower dose of IC31 (LD) protects neonatally immunized mice from both bacteremia and lung infections, whereas StkP and PspA as single proteins admixed with the IC31 LD reduce only bacteremia. Pneumococcal CFU/ml in blood (A) and lungs (B) 24 h after challenge with *S. pneumoniae* serotype 1 is shown for each mouse, and the median for each group ($n = 8$ /group) is indicated by a line. The IC31 LD consisted of 50 nmol KLK and 2 nmol ODN1a. The pneumococcal challenge dose was 1.18×10^7 CFU/mouse. P values are shown for comparison between the immunized groups and the control group receiving Tris buffer when statistically significant. The results are shown for one experiment ($n = 8$ /group). The overall P value for CFU in blood was 0.0009, and that for CFU in lungs was 0.0098. Further statistics are reported in Table S1 in the supplemental material.

thermore, two of the proteins, StkP and PspA, reduced bacteremia when given as single proteins with IC31. PspA is one of the most studied protein vaccine candidates and has been shown to reduce carriage (2, 49) and protect against lethal infection (23, 49, 50) in adult mice. More recently, a *Salmonella*-vectored vaccine expressing PspA was shown to be immunogenic in neonatal mice, but the level of protection was lower than that in adult mice (41). StkP and PcsB have been shown to reduce the bacterial load in lungs of adult mice following i.n. challenge with serotypes 3 and 19F, with StkP providing protection against serotype 19F comparable to that of PCV7 (10). However, PcsB but not StkP protected adult mice against death following i.n. challenge with serotype 1 (ATCC 6301, the same strain used in our study). Thus, despite using the same challenge route and pneumococcal strain, the different results may be explained by age-dependent immune responses and different endpoints, i.e., pneumococcal load in lung and blood in our neonatal experiment versus the survival of adult mice in the study by Giefing et al. (10). In our study, the pneumococcal load in lungs was significantly reduced when a quadrivalent protein formulation was given with IC31, demonstrating the benefits of eliciting Abs against several virulence factors of the pneumococcus. Other research groups have shown that a combination of different proteins increases the protective efficacy of a pneumococcal

FIG 3 IC31 induces higher and more rapid Ab responses to all three proteins in the trivalent formulation, whereas Alum enhances only StkP- and PsaA-specific Abs. Shown are the median IgG Ab levels specific for PcsB (A), StkP (B), and PsaA (C) 2, 4, and 6 weeks after the first neonatal immunization at 7 days of age. The IC31 LD consisted of 50 nmol KLK and 2 nmol ODN1a, and the IC31 HD consisted of 90 nmol KLK and 3.6 nmol ODN1a. The results for one of two comparable experiments are shown ($n = 8$ mice/group). The overall P values were a P value of 0.0676 (week 2) and a P value of <0.0001 (weeks 4 and 6) for PcsB-specific Abs, a P value of <0.0001 (weeks 2, 4, and 6) for StkP-specific Abs, and a P value of <0.0001 (weeks 2, 4, and 6) for PsaA-specific Abs.

protein-based vaccine. Thus, a double or a triple combination of PspA, PcsB, and PspC was shown previously to provide enhanced protection compared to that provided by each single protein alone (6, 30). Furthermore, when using proteins that exhibit strain-specific sequence variation, such as PspA, a combination of several different proteins will more likely protect against a wider variety of strains than any single protein.

Since IC31 had proven potent in our neonatal model when given with a PCV (31), we compared its effect in comparison to that of alum on the neonatal Ab response to pneumococcal proteins. The trivalent vaccine formulation including StkP, PcsB, and PsaA combined with alum was thus compared to the formulation with IC31. Both alum and IC31 enhanced the Ab responses to StkP and PsaA, but only IC31 enhanced the response to PcsB. Measurements of the IgG1 and IgG2a titers and the avidity of the Abs further revealed that IC31 induced broader IgG1 and IgG2 responses to all three proteins with increased avidity, whereas the effects of alum were observed only for the Ab response to StkP and PsaA. IgG2a has been strongly correlated with opsonophagocytosis

FIG 4 IC31 enhances the levels of both IgG1 and IgG2a Abs to the three proteins in the trivalent vaccine more than alum. Shown are levels of IgG1 and IgG2a specific for PcsB (A), StkP (B), and PsaA (C) 2 weeks after the third immunization of neonatal mice with the trivalent protein formulation with high- or low-dose IC31, alum, or no adjuvant. The IC31 LD consisted of 50 nmol KLK and 2 nmol ODN1a, and the IC31 HD consisted of 90 nmol KLK and 3.6 nmol ODN1a. Bars represent the medians of each group with interquartile ranges. *, $P < 0.05$; **, $P < 0.001$ (compared to the group receiving alum/IC31). The results for one of two comparable experiments are shown. The overall P values were a P value of <0.0001 (IgG1) and a P value of 0.0134 (IgG2a) for PcsB-specific Abs, a P value of <0.0001 (IgG1) and a P value of 0.0024 (IgG2a) for StkP-specific Abs, and a P value of <0.0001 (IgG1 and IgG2a) for PsaA-specific Abs.

sis of pneumococci in the mouse (17), which is considered one of the main host defenses against pneumococcal infections. Both alum and IC31 induced IgG2a Abs but to different proteins. Overall, IC31 induced a higher level of protection against both bacteremia and lung infection than alum. Therefore, we concluded that the superior protective efficacy of the trivalent protein vaccine with IC31 compared to that with alum can be explained by a combination of higher levels of both the complement-fixing IgG2a and the Fc-binding IgG1 subclasses with an increased avidity of all protein-specific Abs. Although there was not a significant correlation between protein-specific Ab levels and CFU in lungs or blood, there was a tendency for mice with the highest pneumo-

FIG 5 Affinity maturation is enhanced by IC31 more than by alum. Shown are avidity indexes of IgG Abs specific for PcsB, StkP, and PsaA following three immunizations of neonatal mice with the trivalent protein formulation admixed with alum, a low or a high dose of IC31, or no adjuvant. The IC31 LD consists of 50 nmol KLK and 2 nmol ODN1a, and the IC31 HD consists of 90 nmol KLK and 3.6 nmol ODN1a. Results for one of two comparable experiments are shown. *, $P < 0.05$; **, $P < 0.001$ (compared to the group receiving the three proteins without an adjuvant). The overall P values were a P value of 0.0002 for PcsB-specific Abs, a P value of <0.0001 M for StkP-specific Abs, and a P value of <0.0001 for PsaA-specific Abs.

coccal loads to have Ab levels in the lower range, whereas the mice that were totally protected had Ab levels in the higher range of their respective groups. The lack of a correlation between infection and Ab titers for individual antigens indicates that the protective capacity of the protein vaccine is truly mediated by the combined effects of the protein-specific Abs, Ab subclasses, and avidity.

The protective immunity provided by IC31 combined with the trivalent protein formulation was clearly dose dependent. Only partial protection (only against bacteremia) was observed when the lower dose of IC31 was given, which corresponded to half of the adult mouse dose previously used. However, protection against both bacteremia and lung infection was observed when the higher dose (corresponding to a full adult dose) was given. No protection was observed when IC31 was reduced to 15.75 nmol KLK and 0.63 nmol ODN1a (approximately one-sixth of the adult dose). This is in contrast to our results for neonatal immunization with PCV, where the lowest dose (15.75 nmol KLK and 0.63 nmol ODN1a) of IC31 resulted in protection levels similar to those found when the highest dose (90 nmol KLK and 3.6 nmol ODN1a) was used in combination with proteins, with the latter inducing even higher vaccine-specific Ab levels (31). This finding suggests that higher levels of protein-specific Abs than PPS-specific Abs may be required to provide full protection against pneumococcal disease, possibly due to less exposure of the proteins on the bacterial surface than the PPS. Therefore, a higher dose of an effective adjuvant might be needed with the protein vaccine than with PCV. Two (31) and three (data not shown) doses of PCV with IC31 resulted in complete protection in our neonatal infection model, indicating the superior protective efficacy of the already licensed vaccine compared to the experimental protein vaccine, at least in this model. However, a direct comparison of the most promising protein vaccine combination with IC31 and PCV remains to be done, and the evaluation of these two vaccines upon colonization is an interesting future task.

Interestingly, reductions in both bacteremia and lung infection were observed when PspA was added to the trivalent protein formulation given with the lower dose of IC31. Thus, in the neonatal

FIG 6 Neonatal mice immunized with the trivalent protein formulation admixed with high-dose IC31 are protected against both bacteremia and lung infection. Pneumococcal CFU/ml in blood (A) and lungs (B) 24 h after challenge with *S. pneumoniae* serotype 1 is shown for each mouse. The median for each group ($n = 8$ /group) is indicated by a line. The IC31 LD consisted of 50 nmol KLK and 2 nmol ODN1a, and the IC31 HD consisted of 90 nmol KLK and 3.6 nmol ODN1a. The pneumococcal challenge dose was 2.80×10^8 CFU/mouse. The results for one of two comparable experiments are shown. The level of bacteremia (CFU/ml) did not differ significantly between the groups, as analyzed by the Mann-Whitney test. The Fisher exact test was performed to compare the frequencies of protected mice between the groups by using CFU/ml lower than the mean CFU minus $2 \times$ the standard deviation in the control group (3.48 CFU/ml in blood and 5.13 CFU/ml in lung) as a measure of protection. P values that were statistically significant by the Fisher exact test are shown. The overall P values were 0.8467 (blood infection) and 0.1698 (lung infection). Further statistical analysis is presented in Table S2 in the supplemental material.

mouse model, the highest dose of IC31 seems to be needed for the trivalent protein formulation to elicit protective immunity, but the IC31 dose can be reduced if PspA is added as the fourth protein. It should be noted that the infection dose was higher in the experiment evaluating the trivalent formula (Fig. 6) than when the quadrivalent vaccine was administered with the lower dose of IC31 (Fig. 2), which could overestimate the additional protection provided by PspA. However, our preliminary results demonstrated that PspA further added to the protection against lung infection provided by the high dose of IC31 when these two vaccinated groups were compared in the same experiments and thus challenged with same pneumococcal doses.

Pneumococcal proteins may induce T-cell responses, and CD4⁺ Th17 cells have been shown to reduce pneumococcal colonization in mice in an Ab-independent way (3, 19–21, 46, 47). Furthermore, interleukin-17A (IL-17A) was the cytokine most frequently secreted by human peripheral blood mononuclear cells (PBMCs) upon *in vitro* stimulation with any of the three proteins PcsB, StkP, and PsaA (40). We have shown that the immunization of neonatal mice with an influenza virus vaccine (Agrippal) and IC31 elicits high-level IFN- γ and IL-17 responses, indicating that IC31 is a potent Th1- and Th17-evoking adjuvant (T. A. Olafsdot-

tir et al., unpublished data). It is therefore conceivable that the pneumococcal protein vaccine combined with IC31 could also contribute to protection against pneumococcal colonization. However, the ability of protein Ags to induce IL-17 upon colonization has been shown to be the best indication of whether the proteins can protect against colonization in mice. Many proteins eliciting a Th17 response to colonization do not necessarily induce protective Abs against pneumococcal diseases (25). The protective capacity of the three-valent (PcsB, StkP, and PsaA) protein vaccine against colonization awaits further evaluation.

The ANTIGENome technology that identified and selected the two pneumococcal protein candidates PcsB and StkP is based on their capacity to induce natural immune responses in humans during exposure to or infection by the pneumococcus (10). PcsB and StkP are furthermore exceptionally well conserved. The results from our studies with neonatal mice support their potential for use in a protein-based vaccine for the youngest age group. However, the coadministration of an effective adjuvant that induces high levels of both complement-fixing and Fc-binding Abs of high avidity to all protein candidates in a vaccine is essential for protective efficacy, at least in the murine model. The translation of the murine data to humans is complicated by the fact that the human complement-fixing IgG1 and IgG3 subclasses (4) are not as directly associated with a Th1 response in humans as the IgG2a subclass is in the mouse. Still, murine models are the first choice to evaluate the protective efficacy of novel vaccine candidates. Therefore, a careful selection of an adjuvant enhancing mixed Ab subclasses as well as the avidity to all Ags is necessary to fully evaluate their protective potential. Furthermore, novel vaccine candidates should be evaluated in neonatal and infant models that exhibit limitations in the immune system comparable to those of the main human target group.

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