Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Trivalent pneumococcal protein vaccine protects against experimental acute otitis media caused by *Streptococcus pneumoniae* in an infant murine model

Qingfu Xu *, Karin Pryharski, Michael E. Pichichero

Center for Infectious Diseases and Immunity, Rochester General Hospital Research Institute, Rochester, NY 14621, USA

ARTICLE INFO

Article history:
Received 14 September 2016
Received in revised form 31 October 2016
Accepted 10 November 2016
Available online xxxx

Keywords:
Streptococcus pneumoniae
Infant murine model
Pneumococcal protein vaccine
Acute otitis media

ABSTRACT

Background: Currently licensed serotype-based pneumococcal vaccines are effective in preventing invasive pneumococcal diseases, but less effective in preventing non-bacteremic pneumonia and acute otitis media (AOM). We previously reported that a trivalent pneumococcal protein recombinant vaccine (PPrV) protected against pneumonia in a murine model. Here we evaluated PPrV protection against AOM in an infant murine model.

Methods: C57BL/6J mice were intramuscularly vaccinated at 1–3 weeks of age with monovalent pneumococcal histidine triad protein D (PhtD), or pneumococcal choline binding protein A (PcpA), or detoxified pneumolysin (PlyD1), or trivalent vaccine, and transtympanically challenged at 7–8 weeks of age with 1×10^2 CFU of pneumococcal strain BG7322 (6A) or 1×10^4 CFU of pneumococcal nontypeable strain 0702064 MEF. Serum IgG titers were determined by ELISA. At 24 and 48 h post infection (hpi), animals were sacrificed and middle ear fluid (MEF) samples were collected to determine pneumococcal CFUs.

Results: We found that vaccination of infant mice with monovalent and trivalent pneumococcal proteins elicited significant serum IgG antibody responses to corresponding component proteins. Vaccination with PhtD reduced BG7322 bacterial burdens in MEF at both 24 ($p = 0.05$) and 48 hpi ($p = 0.16$). Vaccination with PcpA significantly reduced the bacterial burdens in MEF at both 24 ($p = 0.02$) and 48 hpi ($p = 0.004$), and PlyD1 significantly reduced bacterial burden in MEF at 48 hpi ($p = 0.02$). Vaccination with trivalent PPrV (PhtD, PcpA and PlyD1) significantly reduced *Spn* burdens in MEF at both 24 ($p = 0.001$) and 48 hpi ($p < 0.0001$). Similar reductions of bacterial burdens were found when the vaccinated animals were challenged with a non-typeable *Spn* strain. Vaccinated mice had significantly milder inflammatory cytokine levels (IL-1β, IL-6, TNF-α, MIP-2 and KC) in middle ears at 24 hpi (all p values < 0.05).

Conclusion: Trivalent PPrV confers protection against pneumococcal AOM in an infant murine model.

1. Introduction

Invasive pneumococcal disease (IPD) and non-IPD represent a significant global public health concern [1,2]. IPD includes meningitis, bacteremia/sepsis, bacteremic pneumonia and non-IPD includes non-bacteremic pneumonia, otitis media, sinusitis and conjunctivitis [2–4]. According to WHO, it is estimated that there are approximately 333,000–529,000 children < age of 5 that die of pneumococcal infections each year worldwide [5].

Acute otitis media (AOM) is the most common pediatric respiratory bacterial infection where antibiotics are prescribed [6,7] and accounts for about $6 billion in healthcare costs annually in the US [8]. There are about 709 million AOM cases and 21,000 deaths, due to complications of OM each year worldwide [7]. Most children experience at least one episode of AOM by three years of age [6] and 40% have more than 6 episodes by the age of 7 [7]. The most common bacterial pathogens of AOM are *Streptococcus pneumoniae* (*Spn*), *Haemophilus influenzae*, and *Moraxella catarrhalis* [9].

Currently marketed vaccines, including *Spn* polysaccharide vaccine (PPV) and *Spn* polysaccharide conjugate vaccines (PCVs), are highly effective in preventing invasive pneumococcal diseases (IPD) [10,11], but less effective in prevention of non-bacteremic pneumonia and AOM [12,13]. Although there has been a reduction in *Spn* disease caused by strains expressing the vaccine serotypes following introduction of PCVs, there has been an increase in *Spn* infections caused by non-vaccine replacement serotypes [14,15].

* Corresponding author at: Rochester General Research Institute, Rochester General Hospital, 1425 Portland Ave, Rochester, NY 14621, USA.
E-mail address: Qingfu.Xu@rochesterregional.org (Q. Xu).

http://dx.doi.org/10.1016/j.vaccine.2016.11.046
0264-410X/

Spn protein-based vaccine candidates that target all *Spn* strains have been sought for decades to replace or complement current vaccines.[2–4,12].

Pneumococcal histidine triad protein D (PhtD), pneumococcal choline binding protein A (PcpA) and a genetically detoxified pneumolysin mutant (PlyD1) are highly conserved, immunogenic vaccine candidates that elicit protection against *Spn* disease in animal models, and they have been tested in clinical trials [16–20]. We have shown that children colonized by *Spn* produce serum [21,22] and mucosal [23] antibody responses to PhtD, PcpA and PlyD1. Moreover, we recently showed that naturally acquired mucosal antibody levels to these three vaccine candidates are associated with protection against AOM in young children [23].

In a previous study, using an infant murine pneumonia model, we have found that vaccination with PPrV containing PhtD, PcpA and PlyD1 elicited significant serum and lung antibody responses to the three antigens, increased phagocytic activity of lung granulocytes after challenge, and protected against bacteremic pneumonia [24,25]. In this study, infant mice were immunized with PPrV at 1–3 week of age, immunologically similar to 6 months to 2 years of age in children [26], and we investigated protection against AOM.

2. Materials and methods

2.1. Animals and vaccination

Animals, experimental design and vaccination were the same as described previously [25] with minor modifications. Briefly, infant C57BL/6J mice of a same litter were randomly allocated to vaccinated and unvaccinated groups. Vaccinated groups were given monovalent PhtD (5 μg), PcpA (5 μg) or PlyD1 (5 μg), or trivalent vaccine containing 0.9 μg of PhtD, 0.2 μg of PcpA and 5 μg of PlyD1 with aluminum hydroxide gel adjuvant (Alhydrogel, Invitrogen, San Diego, CA), in 50 μl, intramuscularly at age 6–8 days, and boosted twice at age 13–15, 19–21 days with the same dose of PhtD, PcpA, PlyD1 or trivalent PPrV. Control groups were given adjuvant in parallel. The mice were anesthetized with isoflurane (1%) in 100% oxygen with a delivery rate of 5 L per minute. Parenting mice, 8–10 weeks old, were purchased from Jackson Laboratory (JAX®Mice) to breed. Newborn mice were weaned at 4 weeks of age, and thereafter the female and male mice were housed separately, 4–5 mice per cage. Animals were housed in a SPF BSLII murine facility at the Rochester General Hospital Research Institute (RGHRI), and all experiments were approved by Institutional Animal Care and Use Committee at RGHRI.

2.2. Bacteria and challenge

Spn strain BG7322 (serotype 6A) was obtained from Sanofi Pasteur. Strain 0702064MEF was an infection-causing nontypeable *Spn* (NT-*Spn*) clinical isolate from MEF of a child with AOM described in a previous study [27]. The bacteria were freshly cultured to mid-logarithmic growth phase in Todd Hewitt Broth containing 1% yeast extract (THBY, Difco), and re-suspended in PBS after harvesting by centrifugation.

Mice at 6–8 weeks of age were anesthetized using a subcutaneous injection of ketamine (67 μg/gbw^{-1}) and xylazine (13 μg/gbw^{-1}). 1×10^2 CFU of BG7322 or 1×10^4 CFU of 0702064MEF in 5 μl of PBS per ear were injected transtympanically into middle ears under direct visualization of microscopy using a 31 gauge needle as described previously [28]. Challenge dosages were calculated based on OD600 values and an optimized conversion ratio (an OD600 of $1.0 = 5 \times 10^8$ cells/ml), and the actual challenge CFU was determined by plating on 5% sheep blood agar.

2.3. Sample collection and bacterial burden

Blood samples were obtained 3 weeks after last vaccination and 1 week before challenge for measurement of antibody levels. Blood and MEF samples were collected 24 and 48 h after challenge for measurement of bacterial burden. Blood was collected under anesthesia with ketamine/xylazine. MEF samples were collected after animal sacrifice. 10 μl (for MEF) or 100 μl (for blood) samples were serially diluted 10-fold in PBS, plated onto 5% sheep blood agar plates overnight at 37 °C, and the number of CFUs of *Spn* in blood and MEF was measured.

2.4. Detection of surface expression of PhtD and PcpA on bacteria from middle ear

Indirect immunofluorescence and flow cytometry detection was carried out to determine expression of PhtD and PcpA on *Spn* strains, and the accessibility of antibodies to bind to PhtD and PcpA to intact *Spn* in middle ear during infection. Three C57BL/6J mice at 6–7 weeks of age were transtympanically injected with 1×10^3 CFU per ear of *Spn* BG7322 or 1×10^5 CFU per ear of 0702064MEF in 5 μl PBS. 24 h after infection, animals were sacrificed and MEF samples were collected. The MEF samples of three animals were pooled and animal tissue debris and other non-bacterial particles were removed by centrifugation at 200*G* for 5 min and re-suspended in staining buffer after 2 washes with PBS. As a control, both strains of *Spn* were cultured *in vitro* to log phase in Todd Hewitt broth + 1% yeast extract, and harvested in staining buffer. All bacteria from MEF samples of three mice and approximately 2×10^7 bacteria from culture were used for this indirect immunofluorescence experiment using flow cytometry as described previously with modification [29,30]. Briefly, bacteria were incubated with a 1:5 diluted mouse serum containing polyclonal antibodies for specific antigens PhtD and PcpA. After incubation at RT for 60 min, bacteria were washed with PBS and incubated with a F(ab′)2 fragment of goat anti-mouse IgG (H + L) secondary antibody conjugated to FITC label (Biolegend) at a dilution of 1:100 and incubated for 30 min at RT. Bacteria were then washed in PBS and subjected to flow cytometry using a LSR II flow cytometer (BD Biosciences). The data were collected and analyzed using FlowJo software (FlowJo, LLC).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Antibody titers in serum were determined by ELISA as described previously [24,25].

2.6. Cytokines and chemokines measurement

Cytokines and chemokines in serum and MEF samples were quantified using Milliplex Map Mouse Cytokine/Chemokine Magnetic Bead Panel including G-CSF, IFNγ, IL-1β IL-4, IL-6, IL-10, IL-12 (p70), IL-17, KC, LIX, MIP-1α, MIP-2, and TNF-α on a Bio-Plex 200 instrument (Bio-Rad) according to the manufacturer's instructions.

2.7. Statistics

The statistical tests were performed using Prism software (Graph Pad, La Jolla CA). Differences between vaccinated and non-vaccinated groups were analyzed by unpaired non-parametric two-tailed Mann-Whitney *U* test for antibody levels, bacterial loads, and intracellular bacterial CFU, and unpaired two-tailed *T* test for cytokine/chemokine levels. For the purpose of statistical analysis, undetectable samples were arbitrarily assigned a value equivalent to half the lower limit of detection.

Please cite this article in press as: Xu Q et al. Trivalent pneumococcal protein vaccine protects against experimental acute otitis media caused by *Streptococcus pneumoniae* in an infant murine model. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.11.046