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Intranasal vaccination with pneumococcal surface protein A plus poly(I:C) protects against secondary pneumococcal pneumonia in mice

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ABSTRACT

Effective pneumococcal vaccines are required for preventing secondary bacterial pneumonia, a life-threatening condition, during epidemics of influenza. We examined whether nasal administration of a low dose of pneumococcal surface protein A (PspA) plus polyinosinic–polycytidylic acid (poly(I:C)) could protect against a fatal secondary pneumococcal pneumonia after influenza A virus infection in mice. PspA-specific IgG but not IgA level was higher in the airways and blood of mice nasally administered a low dose of PspA plus poly(I:C) than in mice nasally administered PspA alone or poly(I:C) alone. Binding of PspA-specific IgG increased C3 deposition on the bacterial surface. The survival rate during secondary infection was higher in mice immunized with PspA plus poly(I:C) than in mice immunized with poly(I:C) alone. The significant reduction in bacterial density in the lung and blood was associated with increased survival of immunized mice with secondary pneumonia. Passive transfer of sera from mice immunized with PspA plus poly(I:C) increased the survival of mice infected with secondary pneumonia. Our data suggest that an intranasal PspA vaccine has promising protective effects against secondary pneumonia after influenza and that PspA-specific IgG plays a critical role in this protection.

1. Introduction

Although influenza is a seasonal viral infection associated with significant morbidity and mortality [1], most of the deaths during the 1918 influenza pandemic were caused by secondary bacterial pneumonia, primarily *Streptococcus pneumoniae* [2,3]. Although recent studies on the 2009 pandemic of H1N1 virus infection showed that the most frequent cause of death was viral pneumonia [4–6], bacterial coinfection by *S. pneumoniae* was found in fatal cases or cases with severe respiratory failure associated with confirmed pandemic H1N1 virus infection [7,8].

Although pneumococcal conjugate vaccine provides protective immunity against pneumonia and invasive disease in infants [9,10], polysaccharide-based vaccines are not ideal because they must include multiple polysaccharide serotypes and do not protect against strains with nonvaccine serotypes [11]. Previous investigators have examined several pneumococcal proteins as potential vaccine candidates with promising results [12–15]. One of these candidates is pneumococcal surface protein A (PspA), which is a choline-binding protein exposed on the cell surface [16–18]. PspA is present on all pneumococcal strains, and anti-PspA antibody increases bacterial clearance and induces cross-protection against infection from strains with different serotypes [19]. Anti-PspA antibodies neutralize the anticomplementary effect of PspA, increasing C3 deposition on PspA-bearing bacteria [20,21]. Human antibody to PspA could protect mice from fatal pneumococcal infection, suggesting that PspA can be an effective human vaccine [22].

We reported previously on increased bacterial clearance in the lung of mice given intranasal immunization of PspA with a Toll-like receptor (TLR) ligand [23]. Intranasal immunization of each TLR agonist in combination with PspA works as a potent mucosal adjuvant in the induction of PspA-specific antibodies in the airway and blood. In the present study, we developed a murine model of secondary pneumococcal pneumonia and studied the effect of intranasal immunization with PspA with polyinosinic–polycytidylic acid (poly(I:C)), a double-stranded RNA (dsRNA) ligand for both TLR3 and melanoma-associated 5 [24] in this model.

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2. Materials and methods

2.1. Viral and bacterial strains

Influenza virus (H1N1) A/New Caledonia strain was obtained from The Research Foundation for Microbial Diseases, Osaka University. *S. pneumoniae* WU2 strain (serotype 3) was grown in Todd-Hewitt Broth (BD, Franklin Lakes, NJ) supplemented with 0.1% yeast extract (THY) to mid-log phase and washed twice with Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Sigma–Aldrich, St. Louis, MO). Bacteria were suspended in THY, and the aliquots were snap frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Immunogen and adjuvant

The recombinant plasmid pUAB055 [25] containing the 0.9 kb *pspA* Rx1 gene encoding $PspA_{32–333}$ was transformed into *Escherichia coli* strain BL21 (DE3) (Invitrogen, Carlsbad, CA) for protein production. The recombinant PspA (rPspA) was purified with Ni-NTA agarose (Qiagen, Valencia, CA). The purified His-tag PspA was purified further with an ion exchange column Mono Q 5/50 GL (GE Healthcare Bio-Sciences, Piscataway, NJ), followed by gel filtration chromatography with Superdex 75 10/300 GL (GE Healthcare Bio-Sciences). We used poly(I:C) (InvivoGen, San Diego, CA), which is a synthetic analog of dsRNA, as the adjuvant for PspA [23].

2.3. Immunization of mice

6–8-Week-old C57BL/6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were anesthetized by subcutaneous administration of ketamine (2.46 mg/mouse) and xylazine (0.216 mg/mouse). The anesthetized mice were immunized intranasally with 0.5 μg of PspA together with 10 μg of poly(I:C) or 10 μg of poly(I:C) alone in 12 μl of PBS once a week for 3 weeks. Mice were sacrificed 1 week after the last immunization, and serum and bronchoalveolar lavage (BAL) fluid were sampled for PspA-specific antibody assays. All animal experiments were performed in accordance with institutional guidelines for the Osaka University animal facility.

2.4. PspA-specific antibody assays

Microtiter plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight at 4 °C with 100 μl of 1 μg/ml of PspA. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T). Serially diluted serum and BAL fluid were added to the plates, and the plates were incubated for 30 min at 37 °C. The plates were washed three times with PBS-T and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA) for 30 min at 37 °C. After the incubation, the plates were washed three times with PBS-T, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich) diluted with substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$) was added, and the plates were incubated for 30 min at room temperature in the dark. The optical density was read at 405 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA).

2.5. Secondary pneumonia model

Anesthetized mice were infected intranasally with 1×10^3 plaque-forming units (PFU) of influenza virus (H1N1) A/New Caledonia strain in 30 μl of PBS and infected intranasally with 6×10^3 colony-forming units (CFU) of *S. pneumoniae* WU2 strain in 30 μl of PBS 5 days after the viral infection. Two weeks after the last immunization, immunized mice were similarly infected intranasally with influenza virus, followed 5 days later by nasal infection with *S. pneumoniae*. Mice were sacrificed and lung and blood samples were obtained 2, 6, 16, 24, 48, and 72 h after pneumococcal infection. The lungs were homogenized in 2 ml of PBS, and quantitative culture of the lung homogenates and blood samples were performed on sheep blood agar.

2.6. Lung histopathology

Lungs were excised from mice and fixed in 10% formalin. The fixed lungs were embedded in paraffin, and 5 μm-sliced sections were stained with hematoxylin–eosin. Four types of histopathological changes (peribronchiolitis, perivasculitis, interstitial pneumonitis, and alveolitis) were scored independently by a pathologist who was unaware of the animal status and were given histopathological scores using a 0–4 scale as described previously [26,27]. BAL fluid was obtained from the nasally immunized mice after pneumococcal infection at the indicated times. Cells were stained with Diff-Quik (Sysmex, Kobe, Japan), and the cell morphology was determined in cell monolayers prepared using a Cytospin 2 (Shandon Southern Products, UK).

2.7. Immunoblotting

S. pneumoniae WU2 strain and rPspA protein were lysed in sample buffer (60 mM Tris–HCl pH 6.8 containing 5% glycerol, 1.6% SDS, 0.1 M DTT, and 0.002% bromophenol blue), and the lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After the electrophoresis, the gel was transferred to a polyvinyl difluoride membrane (Bio-Rad Laboratories), the membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with sera from PspA-immunized mice at a dilution of 1:5000 for 1 h at room temperature. After the incubation, the membrane was washed three times and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) at a dilution of 1:10,000 for 1 h at room temperature. The separated bands were detected with an ECL kit (GE Healthcare Bio-Sciences).

2.8. Antibody binding and C3 deposition assay

S. pneumoniae WU2 strain was grown in THY to mid-log phase. The bacteria were harvested by centrifugation and washed once with PBS. The pellet was suspended with an appropriate amount of PBS to prepare a bacterial suspension at a concentration of 1×10^8 CFU/ml, and 100 μl of Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, Eugene, OR) or 90 μl of the bacterial suspension was incubated with 10 μl of heat-inactivated mouse serum for 30 min at 37 °C. After the incubation, the suspension was washed once with PBS, suspended in 90 μl of gelatin-veronal buffer with Mg^{2+} and Ca^{2+} with 10 μl of naive mouse serum, and then incubated for 30 min at 37 °C. Following the incubation, the bacterial suspension was washed once with PBS, suspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse C3 antibody (MP Biomedicals, Solon, OH), and incubated for 30 min on ice. After the incubation, the bacterial suspension was washed twice with PBS and suspended in 500 μl of 1% formaldehyde. The samples were kept on ice in the dark until analyzed by flow cytometry using a BD FACSCalibur™ with CELLQuest software.

2.9. Passive immunization

Mice were infected intranasally with 1×10^3 PFU of influenza virus and infected intranasally with 6×10^3 CFU of *S. pneumo-*