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Research paper

Nasal immunization with major epitope-containing ApxIIA toxin fragment induces protective immunity against challenge infection with *Actinobacillus pleuropneumoniae* in a murine model

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ABSTRACT

Actinobacillus pleuropneumoniae is an infective agent that leads to porcine pleuropneumonia, a disease that causes severe economic losses in the swine industry. Based on the fact that the respiratory tract is the primary site for bacterial infection, it has been suggested that bacterial exclusion in the respiratory tract through mucosal immune induction is the most effective disease prevention strategy. ApxIIA is a vaccine candidate against *A. pleuropneumoniae* infection, and fragment #5 (aa. 439–801) of ApxIIA contains the major epitopes for effective vaccination. In this study, we used mice to verify the efficacy of intranasal immunization with fragment #5 in the induction of protective immunity against nasal challenge with *A. pleuropneumoniae* and compared its efficacy with that of subcutaneous immunization. Intranasal immunization of the fragment induced significantly higher systemic and mucosal immune responses measured at the levels of antigen-specific antibodies, cytokine-secreting cells after antigen exposure, and antigen-specific lymphocyte proliferation. Intranasal immunization not only efficiently inhibited the bacterial colonization in respiratory organs, but also prevented alveolar tissue damage in infectious condition similar to that of a contaminated pig. Moreover, intranasal immunization with fragment #5 provided acquired protective immunity against intranasal challenge with *A. pleuropneumoniae* serotype 2. In addition, it conferred cross-protection against serotype 5, a heterologous pathogen that causes severe disease by ApxI and ApxII secretion. Collectively, intranasal immunization with fragment #5 of ApxIIA can be considered an efficient protective immunization procedure against *A. pleuropneumoniae* infection.

Abbreviations: APP2, *Actinobacillus pleuropneumoniae* serotype 2; APP5, *Actinobacillus pleuropneumoniae* serotype 5; BALF, bronchoalveolar lavage fluid; BHI, brain heart infusion; CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot; FBS, fetal bovine serum; HE, hematoxylin and eosin; HRP, horseradish peroxidase; IN, intranasal; MLD, minimal lethal dose; NALT, nasopharynx-associated lymphoid tissue; OD, optical density; SC, subcutaneous.

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1. Introduction

Actinobacillus pleuropneumoniae is known to be an etiological agent of contagious porcine pleuropneumonia (Frey, 1995). *A. pleuropneumoniae* strains are classified as two biotypes on the basis of nicotinamide adenine dinucleotide dependency (Bossé et al., 2002; Nicolet, 1992; Nielsen et al., 1997; Schaller et al., 2001) and are differentiated into 15 serotypes according to surface polysaccharide characteristics (Blackall et al., 2002; Dubreuil et al., 2000). The symptom phase of porcine pleuropneumonia can broadly range from pre-acute to chronic clinical signs including fever, dyspnea, anorexia, vomiting, coughing, frothy hemorrhage, diarrhea, and cyanosis (Ajito et al., 1996; Bossé et al., 2002; Taylor, 1999). During disease outbreak, the infected bacteria are generally found in organs of the respiratory tract and are capable of inducing severe tissue damage in the low respiratory tract (Bossé et al., 2002; Sidibe et al., 1993). Dissemination of *A. pleuropneumoniae* is accomplished by inhalation of pathogenic bacteria through aerosols or close contact with both contaminated and clinically healthy carrier animals (Chiers et al., 2002; Fenwick and Henry, 1994; Jobert et al., 2000; Rycroft and Garside, 2000; Sidibe et al., 1993; Torremorell et al., 1997). Characteristics of development and dissemination of porcine pleuropneumonia reasonably suggest that prevention or eradication of the infectious pathogen in the pulmonary mucosal surface is essentially required for disease control. Consequently, mucosal vaccination, which is capable of establishing protective measures through inducing both IgA-mediated mucosal immunity at relevant mucosal sites and IgG-mediated systemic immunity throughout the body, is important (Belyakov and Ahlers, 2009; Chen and Cerutti, 2010). However, there is no effective and safe subunit vaccine to induce protective immunity in the respiratory tract, although live or modified whole cells have been used for mucosal vaccine development (Maas et al., 2006; Ramjeet et al., 2008).

A. pleuropneumoniae is known to have many virulence factors such as membrane proteins, lipopolysaccharides, repetitive glycine-rich sequences in repeats-in-toxins (RTX toxins), fimbriae, transferrin binding proteins, and capsular polysaccharides (Cruijsen et al., 1995; Haesebrouck et al., 1997; Jacques, 1996; Jolie et al., 1995; Ramjeet et al., 2005). Among them, Apx toxins, the RTX toxins, act to create pores in the host cell membrane and are substantially involved in pathogenesis. Consequently, the presence of Apx toxins is recognized as a prerequisite to achieve full virulence by provoking severe tissue damage (Anderson et al., 1991; Frey, 1995; Ramjeet et al., 2008). The importance of Apx toxins as vaccine candidates has been demonstrated in many studies: antibodies neutralizing Apx toxins can rescue macrophages or neutrophils from necrosis and confer upon them an ability to digest the pathogens, and more importantly, animals vaccinated with Apx toxins were protected against bacterial infection (Beaudet et al., 1994; Byrd and Kadis, 1992; Cruijsen et al., 1992). Especially, ApxII toxin among the Apx toxins is a promising vaccine candidate because all serotypes of *A. pleuropneumoniae* except serotype 10 express the ApxII antigen (Bossé et al., 2002; Frey, 1995).

We previously verified the major epitopes of ApxIIA and reported that partial fragment #5 (aa. 439–801) of the toxin is sufficient to induce protective immunity against serotype 2 of *A. pleuropneumoniae* (APP2), applying subcutaneous immunization and intraperitoneal challenge regimen in experimental mice (Seo et al., 2011). Since the mucosal vaccination and mucosal challenge regimen is a practical strategy to test the efficacy of the vaccine against porcine pleuropneumonia, we investigated the immune responses induced by intranasal (IN) and conventional subcutaneous (SC) immunization with fragment #5 of ApxIIA antigen using mouse model. In addition, the immunization and protective efficacy against the nasal challenge with APP2 was also compared between the two different immunization procedures. Finally, we tested whether the intranasal immunization procedure could confer cross-protection against another serotype, *A. pleuropneumoniae* serotype 5 (APP5) which is one of the two most abundant serotypes of *A. pleuropneumoniae* in Korea together with APP2.

2. Materials and methods

2.1. Chemicals and laboratory wares

Unless otherwise specified, chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO) and SPL Lifesciences (Pocheon, Korea), respectively. Oligonucleotide primers were purchased from Cosmo Genetech Inc. (Seoul, Korea) and Integrated DNA Technologies Inc. (San Diego, CA).

2.2. Experimental animals

The female BALB/c mice used in this study were purchased from Charles River Technology through Orient Bio. (Seongnam, Korea) and were maintained under general specific pathogen-free conditions with food and water provided *ad libitum*. The experimental procedure using the laboratory animals was approved by the Institutional Animal Care and Use Committee of the Chonbuk National University (approval number: CBU 2009-0014) and followed the guidelines suggested by the committee. A group of 5 mice was used in each experiment and a representative result from at least three independent experiments is shown in this study.

2.3. Production of recombinant antigen protein

Recombinant antigen protein was produced and purified using the pCold II *Escherichia coli* expression system (Takara Bio., Shiga, Japan). Partial fragment #5 of the ApxIIA gene was amplified from the full-length ApxIIA gene (accession number: AF363362) through polymerase chain reaction as described previously (Seo et al., 2011). Recombinant antigen was produced in the BL21 bacterial system and refined by Ni-NTA agarose (Qiagen, Hilden, Germany). Authenticity of the antigen protein was confirmed through SDS-PAGE and Western blot analysis using both an anti-6× His tag and polyclonal anti-ApxII antibodies.