Veterinary Microbiology 177 (2015) 142–153

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Nasal immunization with M cell-targeting ligand-conjugated ApxIIA toxin fragment induces protective immunity against *Actinobacillus pleuropneumoniae* infection in a murine model

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ARTICLE INFO

Article history:
Received 29 October 2014
Received in revised form 4 March 2015
Accepted 6 March 2015

Keywords:
Actinobacillus pleuropneumoniae
M cell
Nasal immunization
Vaccine

ABSTRACT

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia and severe economic loss in the swine industry has been caused by the infection. Therefore, the development of an effective vaccine against the bacteria is necessary. ApxII toxin, among several virulence factors expressed by the bacteria, is considered to be a promising vaccine candidate because ApxII toxin not only accompanies cytotoxic and hemolytic activities, but is also expressed in all 15 serotypes of bacteria except serotypes 10 and 14. In this study, we identified the peptide ligand capable of targeting the ligand-conjugated ApxIIA #5 fragment antigen to nasopharynx-associated lymphoid tissue. It was found that nasal immunization with ligand-conjugated ApxIIA #5 induced efficient mucosal and systemic immune responses measured at the levels of antigen-specific antibodies, cytokine-secreting cells after antigen exposure, and antigen-specific lymphocyte proliferation. More importantly, the nasal immunization induced protective immunity against nasal challenge infection of the bacteria, which was confirmed by histopathological studies and bacterial clearance after challenge infection. Collectively, we confirmed that the ligand capable of targeting the ligand-conjugated antigen to nasopharynx-associated lymphoid tissue can be used as an effective nasal vaccine adjuvant to induce protective immunity against *A. pleuropneumoniae* infection.

Abbreviations: Ag, antigen; ANK, ankyrin; AR protein, protein with ANK repeat; APC, antigen-presenting cell; BALF, bronchoalveolar lavage fluid; CFU, colony-forming unit; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; Ig, immunoglobulin; NALT, nasopharynx-associated lymphoid tissue; SC, secreting cell.

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http://dx.doi.org/10.1016/j.vetmic.2015.03.005
0378-1135/

1. Introduction

Nasal immunization stimulates immune responses in the nasopharynx-associated lymphoid tissue (NALT) and is effective in inducing systemic immunity, together with mucosal immunity in the gastric mucosa, and in the upper respiratory and genital tracts (Lycke, 2012). Nasal administration of antigen (Ag) is considered to be an attractive immunization route because it makes needle-free vaccine delivery possible and prevents disease transmission that can result from needle reuse (Thompson and Staats, 2011). In addition, taking into account the cost of vaccine injections in the animal industry, the needle-free vaccine delivery could present a great economic advantage.

NALT is a principle immune inductive tissue in nasal immunization and contains all the necessary lymphoid cells, including T cells, B cells, and Ag-presenting cells (APCs), for the induction and regulation of the introduced Ag-specific immune response (Kiyono and Fukuyama, 2004). NALT is also equipped with the molecular and cellular environments for the class switch recombination of a μ to α immunoglobulin (Ig) heavy chain gene for the generation of IgA-committed B cells and memory B cell induction (Shikina et al., 2004; Shimoda et al., 2001). M cells in NALT and the nasal passage play an important role in nasal immunization because the cells are highly involved in the introduced Ag uptake (Kiyono and Fukuyama, 2004). Also, B subunits of cholera toxin and heat-labile enterotoxigenic *E. coli*, which are capable of targeting Ags to M cells in NALT, have been considered to be a nasal vaccine adjuvant. It is important to note that possible deposition of Ags and toxins in the central nervous system through the olfactory bulbs and olfactory nerves limits the practical use of nasal immunization and requires explicit safety precautions (Thompson and Staats, 2011; Kiyono and Fukuyama, 2004). Consequently, there are only a limited number of licensed nasal vaccines including FluMist® and NASOVAC that use live attenuated influenza viruses (Azegami et al., 2014). Nevertheless, due to the great advantages of nasal immunization, development of safe and effective vaccine adjuvant is a promising avenue of research that will take advantage of the nasal immunization process and efficient mucosal and systemic immune induction.

Actinobacillus pleuropneumoniae is known to be an etiological agent of contagious porcine pleuropneumonia (Frey, 1995). Among many virulence factors of *A. pleuropneumoniae*, Apx toxins, which act to create pores in the host cell membrane, are known to be substantially involved in pathogenesis of pleuropneumonia (Chiers et al., 2010). The importance of Apx toxins as vaccine candidates has been demonstrated in many studies (Frey, 1995; Ramjeet et al., 2008). In particular, ApxII toxin, among the variety of Apx toxins, is a promising vaccine candidate because all serotypes of *A. pleuropneumoniae* (except serotypes 10 and 14) express ApxII Ag (Frey, 2011). 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* through subcutaneous immunization and an intraperitoneal challenge regimen in experimental mice (Seo et al., 2011). Given the pathology of porcine pleuropneumonia, in which pathogen infection occurs through the respiratory tract and disease severity is closely related to the protective immunity against pathogenic Ags in mucosal and systemic immune compartments, establishment of protective measures in both compartments is crucial for practical protective immunity against *A. pleuropneumoniae* infection (Ramjeet et al., 2008). In the previous study, we reported that nasal immunization of the Apx fragment induced more potent protective immunity than subcutaneous immunization against nasal challenge infection of the bacteria in a murine model (Seo et al., 2013). In this study, in order to develop the efficient adjuvant capable of targeting Ag into NALT, we selected a ligand and characterized its function in immune induction after nasal Ag administration.

2. Materials and methods

2.1. Chemicals and laboratory wares

Unless otherwise specified, chemicals and laboratory wares used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and SPL Life Sciences (Pocheon, Korea), respectively. Transwell® polycarbonate filter inserts (12 wells, pore diameter of 3 μm) were purchased from Corning Costar (New York, NY, USA), and oligonucleotide primers were purchased from Cosmo Genetech Inc. (Seoul, Korea).

2.2. Experimental animals

The BALB/C mice used in this study were purchased from Charles River Technology through Orient Bio, Inc. (Sungnam, Korea) and were maintained under general specific pathogen-free conditions with *ad libitum* access to food and water. Experimental procedures involving laboratory animals were 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 except for the challenge infection, where 10 mice were used, and a representative result from at least three independent experiments is shown in this study.

2.3. In vitro M-like cell co-culture model and biopanning of phage display library

M cell-targeting ligands were selected through phage display library panning against an *in vitro* co-cultured M-like cell model established by using Caco-2, a human colon carcinoma cell line, and Raji, a human Burkitt's lymphoma cell line, which were purchased from American Type Culture Collection (Manassas, VA, USA). The Caco-2 cells were cultured in DMEM with 10% (v/v) FBS (Hyclone Laboratories Inc., Logan, UT, USA), and the Raji cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS. The *in vitro* co-culture was assembled as described by Kernéis et al. (1997) and modified by Gullberg et al. (2000). The co-cultured cells were maintained for 2 days.