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Nontoxic outer membrane vesicles efficiently increase the efficacy of an influenza vaccine in mice and ferrets

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ABSTRACT

In this study, we developed a further-modified outer membrane vesicle (fmOMV) from the Δ*msbB*/Δ*pagP* mutant of *Escherichia coli* transformed with the plasmid, pLpxF, in order to use it as an adjuvant for pandemic H1N1 (pH1N1) influenza vaccine. We evaluated the efficacy of the pH1N1 influenza vaccine containing the fmOMV in animal models as compared to the commercial adjuvants, alum or AddaVax™. The fmOMV–adjuvanted pH1N1 influenza vaccine induced a significant increase in the humoral immunity; however, this effect was less than that of the AddaVax™. The fmOMV–adjuvanted vaccine displayed pronounced an enhanced protective efficacy with increased T cell immune response and reduced the viral load in the lungs of the infected mice after challenging them with a lethal dose of the homologous virus. Moreover, it resulted in a significantly higher cross-protection against heterologous virus challenge than that of the pH1N1 vaccine with alum or with no adjuvants. In ferrets, the fmOMV–adjuvanted vaccine elicited a superior antibody response based on the HI titer and efficiently protected the animals from the lethal viral challenges. Taken together, the nontoxic fmOMV could be a promising adjuvant for inducing robust T cell priming into the pH1N1 vaccine and might be broadly applicable to the development of preventive measures against influenza virus infection.

1. Introduction

For the development of a successful influenza virus vaccine, an adjuvant is required for promoting the antibody-mediated hemagglutination inhibition (HI) activity against the hemagglutinin (HA) antigen to neutralize the virus infectivity, and for preventing heterologous virus infections by increasing the cross-protective immunity [1]. Most of the vaccine adjuvants reported to date are proven of their ability to induce robust antibody responses, but their ability to stimulate the cell-mediated immune (CMI) responses is limited [2].

CMI responses induced by influenza vaccination are considered to play an important role in the protection of human against the infection with heterologous virus subtypes [3,4]. It provides cross-reactive T cell responses that may reduce the virus replication and clearance at the infection site [5]. In addition, it is desirable to develop a vaccination that is better at inducing the production and release of type-1 cytokines, such as interferon-γ (IFN-γ) [6,7], because, improved induction of these T helper type-1 (Th1)–associated cytokines would encourage the production of the IgG2a antibody subclass, which contributes to the protection against influenza infection in mice [8].

Outer membrane vesicles (OMVs) are released from Gram-negative bacteria and consist of the bacterial outer membrane components, such as outer membrane proteins, lipopolysaccharide (LPS), and the periplasmic constituents [9]. LPS-mediated endotoxicity would be largely attenuated by under-acylation of lipid A moiety, which can be achieved *via* inactivation of the *msbB* gene, a well-known target for reducing the potential side effects of the bacterial LPS component [10–12]. Furthermore, strictly penta-acylated lipid A can be produced from *Escherichia coli* (*E. coli*) by additional *pagP* gene inactivation in the *msbB* mutant background [13,14]. Thereby, the modified OMV (mOMV) composed of the

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strictly penta-acylated LPS could be generated from the Δ*msbB*/Δ*pagP* mutant of *E. coli* W3110. In addition, the mOMV was evaluated as a much less endotoxic adjuvant than the wild type OMV containing intact LPS [15].

Moreover, we have recently developed a further-modified OMV (fmOMV) from the mOMV producer (*E. coli* W3110 Δ*msbB*/Δ*pagP*) by introducing LpxF (lipid A 4′-phosphatase) activity in order to generate a more effective but non-toxic version of the mOMV-based adjuvant retaining the T cell stimulating activity, which is required for the development of viral vaccines.

In this study, we synthesized fmOMV and evaluated its adjuvanticity in influenza vaccination. We examined the effects of the fmOMV on the protective efficacy of a pandemic H1N1 (pH1N1) influenza vaccine against a lethal challenge of homologous or heterologous virus in mouse and ferret models.

2. Materials and methods

2.1. Preparation of the fmOMV adjuvant

OMVs were isolated from the *E. coli* W3110 Δ*msbB*/Δ*pagP* mutant transformed with pWSK29-LpxF (pLpxF) plasmid [16] expressing the lipid A 4′-phosphatase, as described previously [10,15]. Briefly, the culture of the fmOMV producer harboring pLpxF was prepared by growing the strain in lysogeny broth containing 100 μg/mL ampicillin at 37 °C (A_{600} = 0.5), and then the culture supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside was grown overnight. Then, OMVs in the culture supernatant were harvested and purified as described in a previous report [10].

The change of lipid A composition according to pLpxF expression in *E. coli* W3110 strains was confirmed by thin-layer chromatography (TLC), as previously described [16]. To visualize the OMVs by using transmission electron microscopy (CM20 TEM, Philips, the Netherlands).

2.2. Viruses and cells

The influenza viruses, A/California/04/09 [H1N1], A/Philippines/2/82 [H3N2], and A/Brisbane/10/07 [H3N2], were grown in the allantoic cavities of 10-day-old embryonated chicken eggs at 37 °C for 48 h. Madin-Darby canine kidney (MDCK) cells maintained in minimal essential medium (Invitrogen, USA) containing 10% fetal bovine serum (Gibco, USA). The MDCK cells were inoculated with influenza virus and cultured in serum-free MEM containing 30 μg/mL bovine serum albumin (BSA; Sigma-Aldrich, USA) and 1.0 μg/mL TPCK-trypsin (Worthington Biochemical, USA).

2.3. Vaccination and virus challenge

All procedures for animal experiments and care were performed in accordance with Korea Research Institute of Bioscience and Biotechnology. Six-week-old female C57BL/6 mice were intramuscularly immunized into the caudal thigh with two doses of the vaccine containing 0.5 μg of HA (pH1N1 split vaccine antigen) with or without an adjuvant (50 μL per leg) separated by two weeks. The fmOMV, Imject® alum (Thermo Fisher Scientific; alum hereafter), or AddaVax™ (MF59-like squalene-based oil-in-water nanoemulsion adjuvant, InvivoGen, USA; Addavax hereafter) was used as an adjuvant. The fmOMV was used at a dose of 20 μg per mouse, whereas alum or Addavax was mixed with the vaccine at a ratio of 1:1 (v/v) for injection per mouse. Two weeks after the second vaccination, the mice were challenged intranasally with 10 50% lethal dose (LD_{50}) of pH1N1 virus or 5 LD_{50} of human H3N2 (hH3N2) virus (A/Philippine/2/82). Serum samples were collected before the second vaccination and virus challenge. Body weight change and survival rate was monitored for 14 days after the virus challenge. Mice losing more than 25% of their body weight were considered to have reached the experimental endpoint and were euthanized.

Three- to eight-month-old ferrets were purchased from Triple F Farms (USA). Ferrets were intramuscularly immunized into the caudal thigh (500 μL per leg) with two doses of the vaccine containing 25 μg of HA with or without an adjuvant (1 mg of fmOMV or 250 μL of Addavax). Two weeks after the second vaccination, the ferrets were challenged intranasally with 1 ml of 6.5 $\log_{10}$ 50% egg infectious dose per mL (EID_{50}/mL) of pH1N1 or A/Brisbane/10/07 viruses. Serum samples were collected before the second vaccination and virus challenge, and the animal health condition was monitored for 14 days.

2.4. ELISA

The ELISA plates were coated with the pandemic vaccine antigen (0.5 μg/mL), and incubated at 4 °C for 16 h. The unbound sites were blocked with 5% skimmed milk in phosphate-buffered saline (PBS), and the plates were incubated with serially diluted serum samples at 37 °C for 2 h. The plates were then treated with the horseradish peroxidase (HRP)-conjugated anti-mouse IgG, IgG2c and IgG1 detection antibodies (Southern Biotech, USA). An hour later, the plates were incubated with the chromogenic tetramethylbenzidine substrate (BD Biosciences, USA). The colorimetric reaction was terminated with the stop solution (0.5 N H_2SO_4 in water), and the optical density (OD) was measured at 450 nm by using an ELISA plate reader. The antibody titers defined as the reciprocal of the highest serum dilution producing an OD above cutoff value. The cutoff value is determined as the OD of the plate background [17].

2.5. Hemagglutination inhibition assay and virus neutralization assay

The HI assay and VN assay was performed as described previously [18]. Briefly, to determine the HI antibody titer, the receptor-destroying enzyme (RDE)-treated sera in PBS were incubated with 4 agglutinating units of virus at room temperature for 30 min. The HI titer determined by addition of chicken red blood cells (cRBC; 0.5% in PBS) to antigen-antibody mixture.

To determine the VN antibody titers, each heat-inactivated serum was serial twofold dilutions in a serum-free MEM medium (0.3% BSA and 1.0 μg/mL TPCK-trypsin). The diluted sera were mixed with 2000 50% tissue culture infectious dose ($TCID_{50}$) per mL of A/California/04/09 virus at 37 °C for 1 h. The serum-virus mixture was transferred to 96-well cell culture plates where MDCK cells were cultured, and the plates were incubated at 37 °C for 72 h. The supernatant was harvested, and the endpoint of hemagglutination was determined.

2.6. ELISPOT assay

ELISPOT assay was carried out according to the manufacturer protocol (ELISPOT kit; BD Bioscience). Isolated splenocytes (5×10^5 cells/100 μL per well) were dispended into each capture antibody-coated well, and stimulated with the UV-inactivated A/California/04/09 virus (500 $TCID_{50}$/100 μL per well) at 37 °C for 1 h. After washing with PBST, the plates incubated with the provided biotin-conjugated detection antibody (2 μg/mL) at room temperature for 2 h. After wash, streptavidin-conjugated HRP was added to each well. An hour later, after washing, incubated with AEC (3-amino-9-ethly carbazole) substrate solution. The colorimetric reaction was terminated by washing the well with water. The plates were then dried, and the spots were counted using an ELISPOT plate reader (Cellular Technology Ltd., USA).

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