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Vaccination with three tandem repeats of M2 extracellular domain fused to Leismania major HSP70 protects mice against influenza A virus challenge

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ABSTRACT

Influenza viruses are globally important respiratory pathogens with high degree of morbidity and mortality during annual epidemics. Influenza A vaccination has been challenged due to genetic evolution. M2 protein is the surface antigen of the virus and facilitates virus entry into the host cells. N-terminal 24 amino acid residues that constitute the extracellular domain of M2 protein (M2e) show remarkable conservation among all subtypes of influenza A viruses. The aim of the present study was to investigate the effects of using HSP70 as an adjuvant fused to three tandem repeats of M2e (3M2e) to enhance the immune responses against influenza A challenge.

The mice were immunized three times by intradermal inoculations of 3M2e alone or in combination with Alum adjuvant or in fused form to HSP70. The specific immune responses were evaluated by measuring the serum antibody titers, lymphocyte proliferation, as well as Th1 and Th2 cytokines.

The results showed that, although 3M2e with no adjuvant could induce secretion of specific antibodies, significantly higher humoral immune responses were stimulated in combination with Alum adjuvant ($p < 0.05$). Moreover, analysis of specific immune responses revealed that the 3M2e-HSP70 chimer protein mainly stimulated IgG2a and IFN-γ responses indicating aTh1 bias which shows the ability of HSP as a powerful adjuvant for activation of cellular immune responses. This was supported by a higher IgG2a/IgG1 ratio, significantly increased IL-4 production and lymphocyte proliferation ($P < 0.001$) compared with mice vaccinated with 3M2e alone or supplemented with Alum, suggesting a mixture of Th1 and Th2 type cellular immune response with a Th1 bias.

The findings of this study indicated that 3M2e-HSP70 enhances humoral and cellular immune responses and improves immune protection against influenza challenge in mice. Thus, it has the potential to be used as a promising vaccine candidate.

1. Introduction

Despite the availability of seasonal vaccines, influenza is considered as an important source of morbidity and mortality worldwide by which 5–10% of adults and 20–30% of children are infected resulting in 3–5 million severe cases and 250,000–500,000 deaths worldwide (Rappazzo et al., 2016). Outbreaks of seasonal influenza A viruses are created by subtypes which have already spread among humans whereas pandemics of this disease are developed through reassortment of subtypes/strains of influenza A viruses in avian or porcine hosts (Alvarez et al., 2013). Currently available vaccines are based on the hemagglutinin (HA) protein that can provide strain-specific immunity (Kim et al., 2015). HA is a hypervariable glycoprotein and it is necessary to produce strain specific vaccines annually due to its genetic alterations (Treanor, 2015). To effectively control a pandemic outbreak, global vaccine strategies have been applied by protection against various strains of the virus (Subbarao and Matsuoka, 2013).

The extracellular domain of M2 protein (M2e) is a highly conserved region in all human isolates since 1933. It differs in just a few amino acids in avian strains and also in human strain emerged during 2009 flu pandemic (Mardanova et al., 2016). However, M2e is intrinsically low immunogenic peptide due to small size, low level incorporation into virion surface and covering effects by two larger surface proteins, hemagglutinin and neuraminidase (Black et al., 1993; Hutchinson et al., 2014). Therefore, several strategies have been tried to increase M2e immunogenicity including fusion to an adjuvant or carrier virus-like particle (Mardanova et al., 2016). Moreover, other strategies such as using tandem repeats of the peptide and different adjuvants have been evaluated (Denis et al., 2008; Huleatt et al., 2008; Zhang et al., 2011).

In addition, heat shock proteins (HSPs) as conserved proteins

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present in all prokaryotes and eukaryotes with high immunogenicity which can act as adjuvant. Therefore, they can play a critical role in integrating innate and adaptive immunity (Ebrahimi and Tebianian, 2011a).

Aluminum salts have been used for years as safe adjuvants with different vaccines. Alum adjuvant has potency to form antigen depots in the administration site and also helps to persistent and prolonged release of antigens (Awate et al., 2013). Alum adjuvant preferentially stimulates T Helper 2 (Th2) cytokines which skews the immune response toward Th2 phenotype and B cells which produce Th2-related antibodies, Immunoglobulin G1 (IgG1) and Immunoglobulin E (IgE) (Brewer et al., 1999; Bendelac and Medzhitov, 2002; Jordan et al., 2004; Gavin et al., 2006). Moreover, proinflammatory mediators including IL-1β, CC-chemokine ligand 2 (CCL2; MCP1), CCL11 (eotaxin), histamine and IL-5 are raised by Alum adjuvant along with neutrophils, eosinophils, inflammatory monocytes, myeloid dendritic cells (DCs), and plasmacytoid DCs (Pape et al., 1997; Kool et al., 2008).

In the present study, the effects of HSP70 and Alum on elevating specific anti-M2e immune responses were evaluated. A three tandem repeats of M2e peptide (3M2e) and HSP70 were produced in prokaryotic cells individually and in fusion form using pET expression system. The immunogenic potency and protection efficacy of 3M2e, HSP70 and 3M2e-HSP70 chimer protein was then evaluated in Balb/c mice.

2. Materials and methods

2.1. Materials

Mouse-adapted human influenza virus A/PR/8/34(H1N1), Madin-Darby canine kidney (MDCK) cell line, *E. coli* strains BL21 (DE3), and female mature Balb/c mice aged 6–8 weeks were obtained from Pasteur Institute of Iran. DMEM cell culture medium and Fetal Bovine Serum (FBS) were purchased from Gibco (USA). Penicillin/Streptomycin (Pen/Strep), Isopropyl β-D-1-thiogalactopyranoside (IPTG), Tetramethylbenzidine (TMB) and MTT salt (3-(4,5-dimethyl tetrazolyl-2) 2,5 diphenyl) tetrazolyumbromide were purchased from sigma-aldrich Company (USA). Acrylamide and Sodium Dodecyl Sulphate (SDS) were obtained from Merck Company (Germany). DNA extraction kit and Protino™ Ni-TED-IDA 1000 kit were provided by Bioneer Company (South Korea) and Macherey Nagel™ (Germany), respectively. All materials were of analytical grade. Distilled water was used throughout the study.

2.2. Recombinant protein production

Recombinant pET-28a/3M2e, pET-28a/3M2e-HSP70 (chimer) and pET-28a/HSP70 were constructed in our lab previously (unpublished data). The M2e amino acid sequence (SLLTEVETPIRNEWGCRCNDSSD) used in the current study was originated from human influenza A viruses (H1 and H3) that circulated before the pandemic of 2009. The confirmed recombinant constructs were transformed into *E. coli* BL21 (DE3) strain using heat shock method. Briefly, competent bacteria were inoculated with recombinant vectors and cultured in LB agar containing Kanamycin (50 mg/ml) and tetracycline antibiotic (10 mg/ml). The plates were incubated at 37 °C for 18 h. Isolated colonies of BL21 (DE3) bacteria were cultured in 10 ml LB broth medium containing Kanamycin and incubated at 37 °C in shaker incubator (180 rpm) for 3 h. When OD_{600} reached to 0.6, the bacteria were induced using IPTG solution (0.2–1 mM) and incubated (37 °C, 200 rpm). In various time intervals, the induced bacteria were harvested and protein expression was evaluated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and confirmed by Western blotting using mouse monoclonal antibody to the M2 protein (Abcam, 5416 C).

In order to scale up the recombinant protein, LB broth medium were inoculated with a single colony harboring recombinant plasmid in 1 L Erlenmeyer containing 250 ml fresh medium and shaked (37 °C, 180 rpm). The protein expression was induced in desired time and the media were incubated for more 3 h. The bacteria suspension was then centrifuged (10,000 g, 4 °C and 10 min) and the pellet was stored at −20 °C. To extract the recombinant protein, lysis-equilibration-wash (LEW) buffer, urea (8 M) and sonication were used. LEW buffer contained 50 mM NaH2PO4 and 300 mM NaCl. Briefly, LEW buffer and urea were added to the pellet (in ratio of 5:1 W/W) and pipetted to obtain a homogeneous solution. Next, the suspension was incubated (4 °C, 30 min), sonicated (85 Hz, 10 times, 20 s) and centrifuged (10,000 g, 4 °C, 4 min). A sample of the supernatant and the pellet were separately taken for evaluation by SDS-PAGE technique. The process was repeated until the whole pellet was dissolved.

The protein purification was performed using Protino™ Ni-TED affinity chromatography technique according to the manufacturer. To remove urea and salt, gel filtration chromatography (Sephadex G-25 column, BioRad) and dialysis membrane bag (10,000 Da, Sigma) were used. Protein concentration was calculated by Bradford technique.

2.3. Immunization procedure

Seventy and five female Balb/c mice were randomly divided into 5 groups (15 mice/ each group). Two groups received recombinant 3M2e protein and 3M2e-HSP. One group was injected with 3M2e protein supplemented with Alum adjuvant and two groups received PBS or HSP in parallel as control. The amounts of recombinant proteins in all groups were equal to 15 μg for each dose. The compounds were administered intradermally in the volume of 100 μl in three doses with two weeks intervals. All experiments were carried out in accordance with the Animal care and use protocol of Pasteur Institute of Iran.

2.4. Measuring specific anti-M2e antibodies

Two weeks following the last injection, blood samples were taken from the mice and total IgG antibody and IgG subclasses specific for M2e protein were evaluated in blood serum using enzyme-linked immunosorbent assay (ELISA) technique. Briefly, 96-well ELISA plate (Greiner Sigma-Aldrich) was coated overnight at 4 °C with 100 μl of 10^{-4} mg/ml of M2e synthetic peptide (RP20206, GeneScripte). Mice sera were used as 1:1000 dilution. To evaluate total anti-M2e IgG, HRP conjugated anti-mouse IgG (Sigma) was used as secondary antibody and the optical density was measured at wavelength of 450 nm. The specific anti-M2e subclasses were investigated using goat anti-IgG1 or IgG2a subtype antibodies and HRP-conjugated rabbit anti-goat IgG (Sigma) as secondary and tertiary antibodies, respectively.

2.5. Lymphocyte proliferation assay (LPA)

To evaluate the cellular immune response, five mice from each group were sacrificed one week after the last immunization and the spleens were removed and splenocytes were isolated using a 40-μm cell strainer or syringe. Red blood cells were lysed with NH4Cl lysis solution and splenocytes were washed with RPMI 1640 medium (2x). Isolated cells were suspended in RPMI-1640 (supplemented with 10% fetal calf serum, 1% HEPES, 0.1% penicillin/streptomycin, 1% L glutamine and 25 mg/ml of amphotericin B) to reach the final concentration of 2×10^5 cells/ml, cultured in 96-well plate and incubated (37 °C, 5% CO2) with 1 μg/ml influenza M2e synthetic peptide as specific antigen (Griner Bio-One, Denmark) or left without stimuli (medium only). Colorimetric MTT assay was used for lymphoproliferation determination in which mitochondrial activity of living cells turns MTT to purple formazan. Forty eight hr. after adding the specific antigen, 30 μl of the 5 mg/ml MTT sterile solution was added to each well and the plate was incubated again. After 5 h. (allowing for MTT reduction), 100 μl dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. An automatic microplate reader at 540 nm wavelength was used to read the optical density (Anthos 2020, version 2.0.5). The results were