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Original Article

Heamagglutinin Conserved Domain (HA2) Prepared in Prokaryotic System is Immunogenic in Mice but not Protective against Lethal Influenza Challenge

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Abstract

Background and Aims: Influenza vaccine production process is time-consuming with little-to-no cross-protection which requires annual adjustment. The construction of a universal vaccine to deal with the pandemics and epidemics which occasionally threat human population is the aim of many researches worldwide. Today, influenza vaccines are mostly against two major antigenic proteins, hemagglutinin and neuraminidase. As compared to high variable globular head, the hemagglutinin stalk domain is more conserved among different subtypes of influenza A viruses which could be a good candidate to develop a cross-protective vaccine.

Materials and Methods: In this study, recombinant HA2 protein comprising fusion peptide was expressed in E.coli, purified using Ni-TED columns, refolded and desalted by dialysis. BALB/c mice in different groups were immunized with HA2 alone or supplemented with Alum or Alum/CPG. Vaccinated mice sera were examined for anti-HA2 specific IgG responses. Finally, mice were challenged with one LD90 of mouse-adapted A/PR8 virus.

Results: The results showed that HA2 recombinant protein could provoke immunogenicity in BALA/c mice and this immune response could be elevated with Alum and Alum/CpG. Despite promising immune responses, there was insignificant protection of HA2-immunized mice when challenged with the mouse-adapted strain A/PR8.

Conclusions: Therefore, HA2 protein alongside with other influenza virus conserved proteins should be studied to achieve a suitable vaccine formulation for broad spectrum cross-reactive immune responses.

Keywords: Influenza virus, Hemagglutinin, CPG, Alum.

Introduction

Influenza viruses cause yearly repetition of contagious respiratory infections in animals and humans which results in severe illness and even a fetal end (1). This enveloped virus belongs to Orthomixoviridae family with negative-sense segmented ribonucleic acid genome (2). All three types of influenza A, B and C viruses, can cause epidemics, whereas only type A may lead to pandemics and has greater proportion of human infectivity (3).

Influenza A virus is affecting one billion people with almost 400,000 mortality rate throughout the world annually (4). Influenza A viruses undergo genetic reassortments and point mutations (3). Accumulation of point mutation in the surface antigenic proteins, heamagglutinin (HA) and neuraminidase (NA), can lead to annual epidemics. On the other hand, genetic reassortment between human and animal influenza viruses might cause pan-demics (5).

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Vaccination is one of the best ways for protecting human population against influenza virus infection. Inactivated and live attenuated influenza vaccines are constructed based on perpetual surface antigenic changes and need permanent updating. This reconstruction is based on worldwide main circulating strains which are reported by WHO annually (2). Hence, there is a trend to use an alternative method to avoid yearly re-administration and increase efficacy of influenza vaccine.

Hemagglutinin is an integral transmembrane homotrimer protein with crucial role in virus infection. Virus attachment and virus-endosome membrane fusion are intermediated by hemagglutinin (6).

To prepare universal vaccine, scientists have focused on conserved antigenic parts of influenza virus such as hemagglutinin stalk domain which is more conserved compared to its globular head (3). Considering, these recombinant universal vaccines could partially protect against different influenza viruses; they could reduce the sever cases to a milder course of infection (7). Making a universal vaccine is important especially for the elderly, infants or individuals with chronic respiratory illnesses.

Herein, the conserved influenza A/H1N1 hemaglutinin stalk domain including fusion peptide, HA2, was expressed in prokaryotic system and purified with affinity chromatography. The immunogenicity and protection efficacy of recombinant protein was evaluated alone or in combination with Alum or Alum/ CpG in mouse model.

Methods

Protein expression. The expression vector encoding hemagglutinin conserved domain PET-28a/HA2 was constructed previously (8). The expression vector was transformed into the competent E.coli BL21-DE3 cells. The transformants were plated on LB agar containing 50 µg/ml kanamycin. Single colonies were transferred to one liter flask containing 250 ml LB broth (Merck, Darmstadt, Germany) and grown at 37°C in a shaker incubator. At the appropriate optical density at 600 nm (OD 600), bacterial cells were induced by 1 mM IPTG and incubated at 37°C for further six hours, then centrifuged at 4°C for 10 min at 10000 rpm and recombinant protein expression was assessed by electrophoresis.

Protein extraction and purification.

Purification of recombinant protein was performed using Ni-TED 2000 (MN, Germany) columns in following manner. The bacterial pellet was lysed in LEW buffer (300 mM NaCl, 50 mM NaH2PO4, pH 8.0) containing 5 mM Imidazole, then sonicated.

After centrifugation at 10000 rpm, the precipitate was dissolved in LEW buffer plus Triton x-100 and centrifuged. The remaining pellet was re-suspended in LEW buffer plus 8 M urea (LEW-urea) and shaken at 30°C for one hour. The resulting supernatant contained the desired protein. All the samples were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The HA2 protein was eluted using LEW - urea plus 250 mM imidazole. The elution flow rate was decreased to 150 µl/min to increase the efficiency of the column and elutes were analyzed by SDS-PAGE. The purified proteins were desalted by dialysis (Sigma, cut off: 12 KD). The protein concentration was measured by Bradford assay at 595 nm.

Immunization schedule. Six week-old female BALB/c mice were divided randomly into 4 groups (10 mice/group) and immunized subcutaneously with three doses of 15 µg HA2 protein at two-week intervals. One group received HA2 protein alone (HA2) and 2 other groups were injected with protein supplemented with Alum (HA2/Alum) or Alum and CpG (HA2/Alum/CpG). The control group received PBS.

Two weeks after the last injection, the mice were bled through the orbital sinus and the sera were kept at -20°C for ELISA analysis. All experiments were carried out in accordance with the Animal care and use protocol of Institute Pasteur of Iran.

Enzyme-linked immunosorbent assay (ELISA). To evaluate total anti-HA2 IgG, 96-well ELISA plate (Greiner Sigma-Aldrich) was coated overnight at 4°C with 100 µl of 10-4 mg/ml purified protein in phosphate buffered saline (PBS). PBS containing 0.05% Tween 20 (PBST), PBST plus 3% BSA and PBST plus 1% BSA were used as washing, blocking and antibody dilution buffers, respectively. HRP conjugated anti-mouse antibody (1:5000 dilution) was used for assessment of total IgG in mice sera (1:2000 dilution).

Tetramethylbenzidine was used as substrate and the optical density was measured at 450 nm. Controls for this assay comprised of the sera from PBS injected mice and also pre-immunizaed animals.

Lethal challenge. In this study, A/PR/8/34 (H1N1) virus propagated in MDCK cells were used for lethal challenge. Briefly, three weeks after the last immunization, all vaccinated mice, including control animals, were challenged intranasally with one lethal dose 90 (1LD90) of H1N1 virus. Before inoculation, the mice were weighed and lightly anesthetized by IP injection with ketamine/xylazine compound. The mice were kept under class II biosafety cabinet for 2 weeks and monitored daily to assess mortality and morbidity (9).

Results

HA2 recombinant protein production. The protein of interest was expressed in BL21 (DE3) E.coli and harvested at 6 hour post-inoculation. Gel electrophoresis revealed high expression level of a 27 kD HA2 recombinant protein (Figure 1a). Due to the formation of protein aggregates, the extraction was performed by denaturation technique using 8M urea and sonication procedure. All results were analyzed by SDS-PAGE. As illustrated in Figure 1b, the majority of undesired proteins were removed. Since the recombinant protein had histidine tag fused to its both ends, nickel affinity chromatography column was used to purify HA2 protein with at least 95% purity. As the purified protein was insoluble in alkaline condition, salt and urea removal was carried out using urea gradient dialysis against

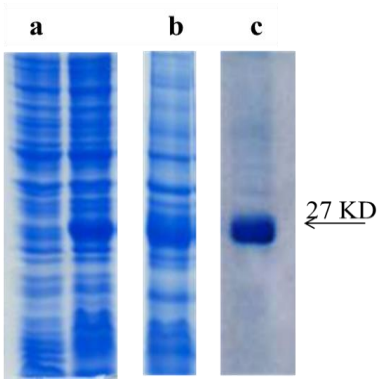

Fig. 1. Analysis of expression and purification of recombinant protein by SDS-PAGE a) Expression of PET-28a/ HA2 plasmid in (DE3) BL21 E.coli strain; left column: before induction, right column: six hours after induction by IPTG. b) Final extraction product, c) purified HA2 protein after dialysis.

phosphate buffer to prepare suitable protein for injection (Figure 1c). This led to decrease in protein precipitation and avoided protein structure denaturation.

The HA2 solubility was studied in pH 4-9 and it was mostly soluble in pH ~ 8.5. Subsequently, this pH was applied for HA2 dialysis. Final concentration of purified protein was measured by Bradford protein assay as 0.55 mg/ml.

Measurement of specific antibodies. Two weeks after the last vaccination, total specific anti-HA2 IgG was measured in mice sera samples by ELISA. As indicated in Figure 2, specific anti-HA2 IgG was produced in all groups receiving recombinant protein.

Results analysis by One way ANOVA statistical technique revealed significant increase ($p<0.001$) in IgG antibody production in all groups as compared to PBS control group.

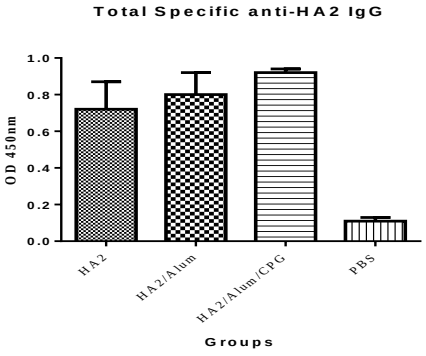

Fig. 2. Measurement of anti-HA2 antibody levels by ELISA assay. Data are the averages of triplicates. The differences between cases and control group were statistically significant ($P<0.001$).

Figure 2 illustrates more specific antibody production in mice immunized with different adjuvants in comparison with mice that received only HA2, however, this difference was not significant.

Viral challenge. Two weeks after the last vaccination, the mice of each group received one LD90 of PR8 (H1N1) intranasally. The mice were monitored for two weeks regarding weight loss and mortality rate.

The results showed that only 20% of mice that received HA2, HA2/Alum, or HA2/Alum/CpG survived in challenge with PR8 virus. All mice in control group received PBS died in 7 days after H1N1challenge. (figure 3)

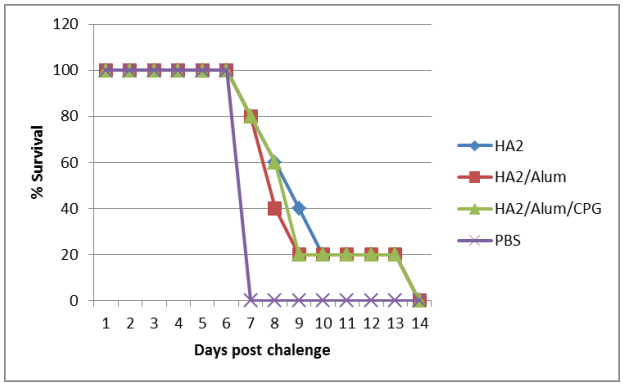

Fig. 3. Survival rate in mice challenged intranasally with one LD90 of A/PR/8/34 (H1N1) two weeks after the last immunization. Survival data are presented as the percentages of animals surviving among the total number monitored.

Discussion

For more than 50 years, vaccination with inactivated virus has been used to control influenza infection. Annually vaccines against specific influenza circulating strains are produced under the supervision of WHO or CDC (2, 3, 10).

On the other hand, constant changes in influenza type A surface antigens due to unpredictable mutagenesis or reassortment lead to emergence of new variants that could escape from the immune system. As a result, virus could become unresponsive to the preventive actions and could cause epidemics or worldwide pandemics that would threaten human population (7, 11).

Present inactived vaccines failed to offer complete protection in elderly and infants (12). These vaccines mostly induce antibody production against seasonal strains that share similarities in their antigenic regions but are not efficient against new variants. Therefore, researches seek to produce a single vaccine against virus strains that confer immunity to different HA subtypes and would be effective in prevention of any probable epidemics or pandemics (3, 10, 13, 14).

An ideal vaccine would be safe and confer long-lasting efficiency and protectiveness against different strains of virus (4). In this regard, small subunit of HA (HA2) which is conserved compared to globular head of the molecule, could be a promising candidate in preparation of a universal vaccine. This region of HA sequence has 85% homology among different influenza virus subtypes, and 95% homology between various strains of a single subtype, which could induce cross-reactive immune responses (15).

Three different antigenic regions have been identified in HA2. These regions comprise fusion peptide, HA2 ectodomain and the most immunogenic region in the internal part (125-175 amino acid residues) (7, 16). Protective potential of these regions have been studied by different research groups and challenge results showed milder illness and fewer deaths in mice (17, 18, 19).

Researches have shown that; although the HA2 protein is coated by HA1 in intact viruses, anti HA2 antibodies are also produced during human, rabbit and mouse influenza infections (20, 15). In 2010, Steel et al. showed that by removing HA1 globular head, HA2 glycoprotein immunogenicity is increased, as vaccination of mice with hemagglutinin with no head particles, caused proliferation of multi HA subtypes cross-reactive antibodies which could confer immunity against influenza infection (21).

Accordingly, in the current study, subunit vaccine design was conducted based on conserved area in hemagglutinin protein. Our construct, HA2, comprises the conserved influenza A/H1N1 hemagglutinin stalk domain, launch with fusion peptide. This protein was expressed in bacterial expression host and approved by Western blotting in our lab

previously. In this research, HA2 protein was expressed, extracted and purified in large-scale.

Immunogenicity of the purified protein was assessed using mice model. BALB/c mice immunization results exhibited that recombinant HA2 protein alone could stimulate immune system strongly and induce specific antibodies production. These animals demonstrated acceptable and meaningful antibody responses in comparison with the control groups. Herein, in the aim of increasing recombinant protein immunigenicity, different adjuvants were applied (Alum and Alum/ CpG).

Aluminum hydroxide as called Alum has been extensively used as human vaccines adjuvant. This adjuvant could increase immunogenicity by forming depot, Ag trapping at injection site and continuously releasing of antigen to antigen presenting cells and could lower antigen removal by the liver. Activation of complement, eosinophil and macrophage could be other possible mechanisms of action of aluminum salts. It is assumed that aluminum hydroxide mostly increases humoral immune responses by induction of Th2 cells (22).

The group that received HA2 vaccine supplemented with Alum demonstrated more, although not significant, immunogenicity as compared to group which received protein without adjuvant. To improve vaccine efficacy, CpG motif was added to Alum.

These oligonucleotides are similar to bacterial DNA motif and constitute a long chain of DNA deoxynucleotides with central CpG dinucleotides. It is an immune system modulator which can stimulate antibody production. It has been shown that CpG motif could shift immune pathways toward Th1 cells and balance both humoral and cellular immune responses (23). Teloni et al. demonstrated that application of CpG as adjuvant would lead to increase in IgG2a/IgG1 ratio, and is able to increase systemic and mucosal immunity (24).

In the present study the group that received HA2 recombinant protein with Alum/CpG demonstrated more but not significant immunogenicity regarding total IgG, as compared to group that received HA2 only or supplemented with Alum.

Therefore, it can be concluded that HA2 recombinant protein could provoke immunogenicity in BALA/c mice and this immune response could be elevated with Alum and Alum/CpG.

Although the immune responses were promising, none of the immunized sera, even when used at a low 2-fold dilution, neutralized or reduced infectivity of either virus (H1N1 or H3N2) in a virus neutralization test (data not shown). Moreover, there was insignificant protection of HA2-immunized mice when challenged with the mouse-adapted strain A/Puerto Rico/8/34.

These results are in agreement with findings from previous studies showing that HA2 immunization conferred only minimal protection (20%) against virus challenge (25).

It has been shown that humoral immunity is not individually sufficient to produce the perfect immunity against influenza A virus and both antibody and cell-mediated immune responses are involved in the protection against influenza viruses (26). Therefore, HA2 protein alongside with other conserved proteins of influenza virus should be studied to reach a suitable vaccine formulation for broad spectrum cross-reactive immune responses.

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