Original Research Article

Induction and evaluation of influenza cell-mediated immune responses by HA2 subunit and bio adjuvant as a gene vaccine candidate

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ABSTRACT

The stalk domain of influenza as Hemagglutinin 2 (HA2) is a conserved unit of HA and is a candidate for a vaccine construct. We designed HA2/Mx (Myxovirus Resistance) as bio adjuvant construct for vaccine candidates. After immunization of Razi Balb/c mice in DNA/prime/boost strategy, for determination of the cellular immunity by an *in vivo* assay, the lymphocyte proliferation was evaluated. The result indicated the most stimulation index was by HA2 and Mx in three boosters, and effective stimulation of cellular immunity was obtained. No apparent side effects and histopathology were observed. Due to this result in cellular immunity and appropriate increase in humoral immunity that was obtained in our previous study, as well as the absence of antigen and adjuvant side effects, this construct can be introduced as a suitable candidate for developing an influenza vaccine.

***Keywords*:** Influenza, vaccine, HA2, cellular immunity

INTRODUCTION

Influenza is of considerable health and economic importance due to the disease in humans, birds, and many animals. With the outbreak of Coronavirus and Covid 19 disease, this disease has become doubly important [1]. Influenza virus infection causes both cellular and humoral immune responses. Humoral immune responses are induced by specific antibodies produced by antibody-secreting plasma cells. This process is supported by a CD4 + T helper. Cellular immune responses begin after the

detection of viral antigens delivered by MHC class I and II molecules by antigen-presenting cells (APCs) that lead to the activation, proliferation, and differentiation of CD8 + or CD4 + [2].

Due to importance of influenza in communities and also its increasing importance by the prevalence of coronavirus, the emergence of new antigen varieties of these viruses and high costs, the use of commercially inactive vaccines has been limited and the low immunogenicity and, therefore, the need for repeated vaccination and use of adjuvants are the drawbacks of recombinant subunit vaccines, as well as of conventional subunit vaccines [3], efforts are underway to develop a universal vaccine against the disease. One approach to controlling existing circulating strains is to use the DNA vaccines as an alternative vaccine to provide cross-protective immunity and primarily can be used in pandemic events. The basis of these vaccines is the natural process of translating genes into protein in the cells of living organisms to produce immunogenic protein in the body and to induce both cellular and humoral immune systems. The results of studies show that using protected HA epitopes develops neutralizing antibodies against the virus and induces good immunity against the disease [4].

DNA vaccine based on the hemagglutinin gene is an attractive alternative approach to induce broader antibody and Cytotoxic T Lymphocyte (CTL) responses in an animal model. HA plays a vital role in attachment, and by fusion in the virus-host membrane was cleaved of the precursor into HA1 and HA2 subunits. Due to the occurrence of extensive point mutations in the HA1 subunit and the absence of these antigenic changes in the HA2 [5], the idea of using the HA2 subunit, which has multiple conserved antigen loci, to design a cross-immunogenic gene vaccine for the important flu circulating strains were formed in our previous study[6].

Due to the importance of using an appropriate adjuvant in increasing the effectiveness of DNA vaccines and due to the lack of toxicity and side effects in the host [7], the bio adjuvant Mx as a host protein used in the HA2 DNA vaccine.

Mx proteins are interferon-inducing GTPases that are expressed in a wide range of cells, such as macrophages and liver cells, and are involved in regulating the immune system in the cell. This cellular protein is one of the best agents whose role in viral defense has been studied and is

quite specific for viruses of the orthomyxoviridae family [8]. This particular function results from the formation of the Mx multidirectional network and direct interaction between the carbon terminus of the protein and the virus nucleoprotein, which stops the transcription of viral Ribonucleoprotein (vRNP) complexes and the entry of the virus genome into the host cell nucleus. Regardless of the possible antiviral ability of this protein, findings from the Mx sequence similarity of different vertebrate species indicate the presence of highly conserved regions in the interferon induction domain of this protein that is involved in the regulation of the immune system [8].

Considering the use of Mx protein as a biological adjuvant in influenza DNA vaccine in our previous study and the excellent results, in this study, the induction of the cellular immune response, which is very important in influenza infection, was designed by construct and Mx biological adjuvant has been evaluated.

MATERIALS AND METHODS

Designing and preparation of influenza HA2 / Mx construct

Although this operation was performed in the previous study, due to the high sensitivity of the work and to ensure more cloning of the gene and a more accurate design of the construct, the preparation of the antigen and the adjuvant construct was done again as follows based on the previous method but with corrective changes in some parts as follows: The HA2 sequences of the H9N2 influenza virus were extracted from the GenBank database from their emergence to 2014, and their alignment was performed with ClustalW. Based on our previous study Ribospin™ (GeneAll, South Korea) was used for RNA extraction from H9N2 (JX456181.1), and the fragment was amplified using a set of designed primer; (HA2F: 5′GGATCCCATGGCTGCAGATAGGGATA-3′، HA2R: 5′CCATGGTTATATACAAATGTTGCACCT-3′) (The BamHI and NcoI restriction sites were underlined). DNA was purified using a PCR product extraction kit (GeneAll, Korean) and cloned into a pcDNA3.1 vector by heat shock. The plasmid was extracted using EndoFree® Plasmid Mega Kit (Qiagen, Germany). After evaluating the cloned gene correctness, the concentration was adjusted to 1 µg/µl for immunization trials.

Datasets of Mx sequences from Homo sapiens, Mus musculus, and 24 sequences of Gallus gallus were obtained from Gen Bank and aligned using ClustalW program with default parameters. Based on our in-silico study, a conserved sequence encoding the motif 13SGKSSVLEALSGVALPR30in the GTPase domain (interferon-induced domain) showed better results in inducing T-cell immune responses against influenza viruses. Based on our previous study, the primers MxF: 5′CCATGGGATTGCGGTGATTGGCGA-3′and MxR: 5′-GGGCAGCGGGTCACAATGGAATC-3′were designed for amplification of the Mx fragment (102 bp). The underlines indicate restriction enzyme sites. RNA was extracted from lung and spleen tissues of mice by High Pure RNA Tissue Kit (Roche, Germany), and the PCR product was cloned in pcDNA3.1vector and treated in the same manner mentioned above.

Immunization of mice

A total of 40 female Balb/c mice (Razi Vaccine and Serum Research Institute, Karaj) aged six weeks and weighing an average of 20 g were divided into four groups (each group included ten mice), including one control group and three treatment groups. The mice were housed and treated based on the internal animal ethical committee and institutional guidelines number RVSRI.REC.98.005. The treatment groups include only HA2 in prime and two boosts in group one. HA2 and Mx in prime and boost in group two (one boost). HA2 and Mx in prime and two boosts in group three. To ensure the absence of antibodies against the influenza virus on day -3 (three days before the first injection), blood samples were randomly drawn from the mice, and the antibody titer in the serum samples was evaluated by the HI test. On day 0, the vaccine was administered by DNA/Prime-DNA/Boost strategy at the site of the quadriceps muscle at a rate of 0.1 ml. The first and second boosts were injected 14 and 28 days after the prime. The ethics of keeping the mice in the experimental period were fully observed.

Cell-mediated immune evaluation

Sixty days after injection of the HA2 DNA vaccine, an MTT assay was used to evaluate induced cellular immunity in mice. The cell suspension was prepared in RPMI 1640 from spleen lymphocytes of 106 cells per ml. For each group, 100µl of mitogen Phytohemagglutinin A (PHA) was added to the six microplate wells as a positive control at a concentration of 5 µg/ml at a final concentration. One hundred

microliters of the vaccine were poured into twelve wells in each group. For each group of lymphocytes, six wells were considered a negative control. After adding MTT and incubation, the supernatant of the discharge well and the formed formazan crystals were dissolved by adding 150 µl of Dimethyl Sulfoxide (DMSO) to each well. Then the absorption rate of each well at 630 nm was determined, and the mean with standard deviation was calculated for three experiments. Stimulation Index (SI) was calculated as the mean OD ratio of cells containing cells vaccinated with the gene vaccine to the mean OD of cells containing wells with medium (negative control) [9].

Safety Evaluation

To investigate the effects of possible tissue damage due to DNA vaccine injection, during daily inspection of mice for local reactions at the injection site, general reaction, and possible weight loss, spleen, and lung samples were taken from mice in different treated and control groups on the third day after injection and at the end of the experiment and tissue sections were stained with hematoxylin and eosin for histopathological evaluation [10].

Statistical analysis

Results based on the mean and standard deviation using SPSS (SPSS, inc., Chicago, IL, USA) software version 22- and One-way ANOVA was examined and statistically processed, and considering the 95 % confidence interval, $p<0.05$ was considered significant.

RESULTS

Cloning of HA2 and Mx genes into pcDNA3.1 vector

The constructed pcDNA3.1/HA2 and pcDNA3.1/Mx were transformed into DH5 alpha Escherichia coli, and the positive clones were screened in the plates using restriction enzyme digestion and sequencing. Digestion confirmed the presence of the genes based on detecting the bands of the expected sizes (Figure 1). The sequencing was checked with the original sequence in the Gene Bank database.

Cell-mediated immune responses evaluation

Results of MTT assay to evaluate cellular immunity as mean Stimulation Index (SI) due to the effect of mitogen (phytohemagglutinin) and antigen (HA2) on peripheral blood lymphocytes isolated from mice in different groups with standard deviation shown in Figure 2. The results show that cellular immunity in treatment groups is significantly higher than in the control group ($p< 0.01$). The results also indicated the best lymphocyte proliferation was seen in group 4 that, injected by the

HA2 and Mx construct in prime and two boosts (The mean lymphocyte stimulation index of 6.768).

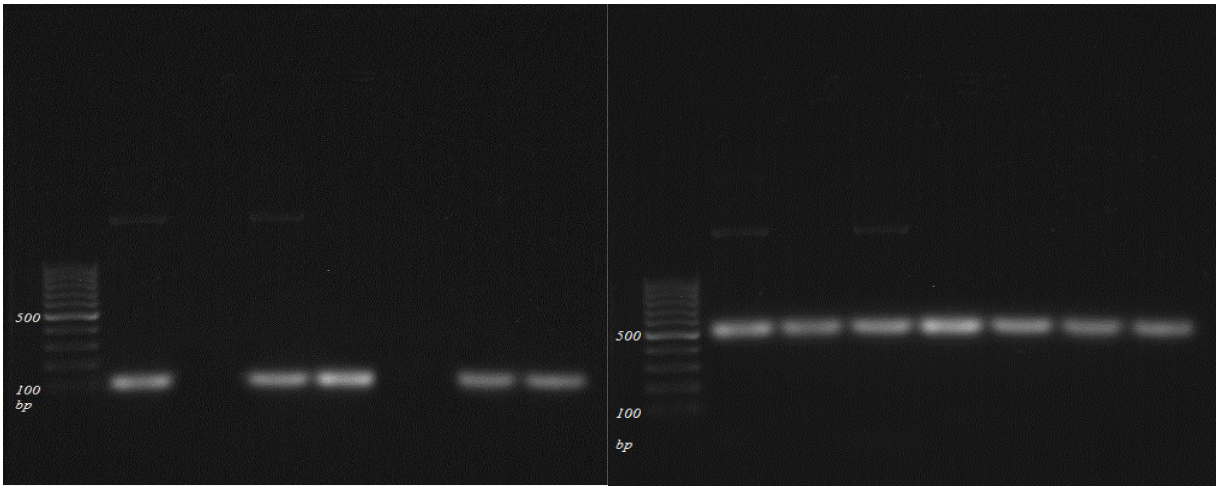

Figure 1. The conformation of Mx (Left) (102 bp) and HA2 (Right) (571 bp) along with molecular weight marker (DNA 100bp) clones by restriction enzyme digestion. Of seven Mx clones, five clones and all of the HA2 clones were confirmed in this assay.

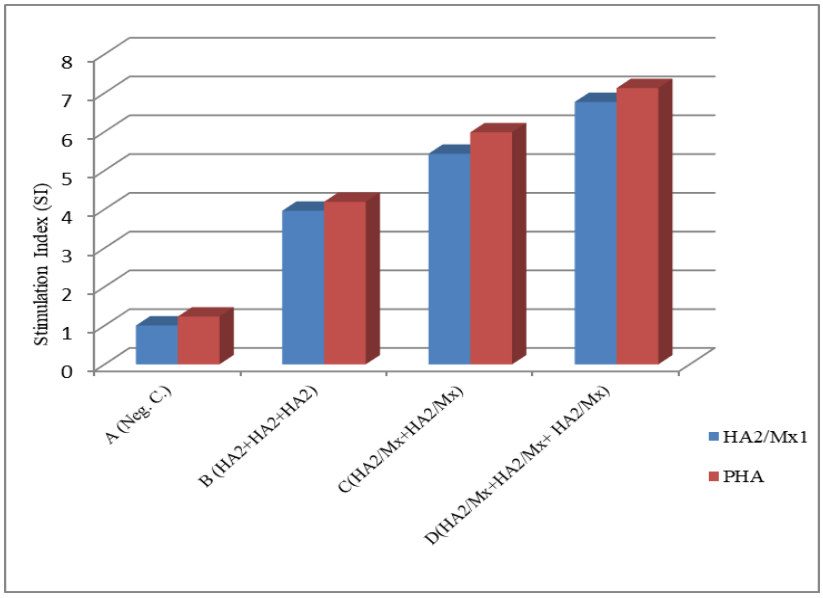

Figure 2. The Stimulation Index (SI) of lymphocyte proliferation assay in immunized mice by the Mx adjuvanted-HA2 influenza vaccine. The results indicated the adjuvanted vaccine stimulates the lymphocytes in the MTT assay similar to PHA and at a high level.

Safety evaluation

Daily examination of the tested mice showed no local reactions such as swelling, redness, inflammation, injury at the injection site, or general reactions or death. The mean weight of mice did not differ in all control and treatment groups and increased from 20 g to 32.8 g in the experimental period. In histological examination, no unwanted inflammatory reaction, hyperemia, or cell deformity due to injections were observed in the lung and spleen samples of mice (Figure3).

DISCUSSION

Influenza is a very important infectious disease in humans and many animals and birds. The importance of this disease, apart from its effects on public health, goes back to the biology of the virus and the antigenic changes that occur in this virus and make it difficult to deal with this disease. Studies have shown that if a change in amino acids responsible for identifying and regulating the specificity of virus binding to the receptor occurs in an animal host from α2,6 to α2,3 in hemagglutinin protein or the presence of glutamic acid at site 92 of NS1 protein, it causes the virus to escape host cytokine antiviral responses or mutations occur at loci 627 and 701 of the PB2 protein. Then the virus acquires a new receptor-binding property that causes the virus to diversify, adapt to the new host, and spread the infection [11]. Thus, the rapid spread of emerging and re-emerging strains of influenza virus and the recording of evidence of transmission of some strains from avian species to humans and other mammals have led global attention to improve and optimize influenza control and prevention methods [12].

A various point mutations in the HA1 domain of influenza viruses [13-15] made us in previous studies, focused on the HA2 subunit because the stalk domain of influenza virus (HA2) is a conserved part of the HA [16-18]. Khana et al. indicated the antibodies against HA2 can neutralize a broad spectrum of influenza virus strains and subtypes [14]. Fan et al. also showed that immune response against the HA2 subunit could potentially elicit broadly inhibitory antibodies [19].

Despite the many advantages of gen vaccines, they need an appropriate adjuvant to provide adequate immunogenicity [20]. Bio adjuvant, especially the Mx introduced in our

previous study, induced immune responses in gen vaccine against the influenza disease. So, in an earlier study, we constructed the HA2 subunit influenza DNA vaccine for protection against influenza disease and Mx bio adjuvant for influenza DNA vaccines. Based on comparative modeling analysis, the best Mx coding sequences were placed in the GTP use domain as selected [6].

Our previous study showed that the designed construct as Mx bio adjuvant HA2 DNA vaccine exhibited high functional humoral antibody titers. During influenza infections, both CD4 and CD8 memory cells subsets respond to and mediate the control of an influenza virus infection [11,21]. CTLs recognize epitopes from HA or internal proteins presented on MHC class I molecules [22] and play an important role in the induction of cell-mediated immunity [23,24]. So, in this study, we evaluated the cell-mediated immune responses by this construct against influenza. Due to the advantages of in vivo assay for the evaluation of cells and since the results of this method are more accurate than in vitro methods, and to increase the reliability of the results, a lymphocyte proliferation assay method was used to evaluate cellular immunity in this study. The result of the proliferation assay indicated a significant increase in the stimulation index of the lymphocyte induced by the Mx bio adjuvanted HA2 DNA construct ($p< 0.01$).

In the different prime- boost regimens, the best lymphocyte proliferation was seen in group 4 that injected by HA2 and Mx in prime and two boosts. The results also show that the higher the number of booster doses, the higher the cellular immune response.

Use of commercially inactive vaccines has been limited due to antigenic shift and drift of influenza virus and the low immunogenicity. Also the need for repeated vaccination and use of adjuvants are the drawbacks of recombinant subunit vaccines, as well as of conventional subunit vaccines [3], DNA vaccine is the best choice against influenza provided that the conserve and effective gene and the safe and effective adjuvant are selected. The Result of this study showed these two factors are correctly selected. Therefore, this structure can be introduced as an effective vaccine against influenza.

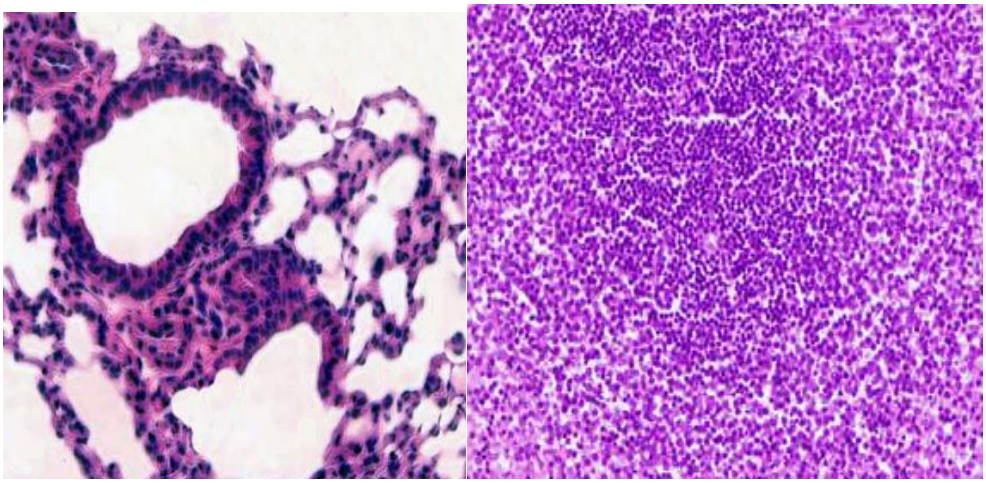

Figure 3. Histopathology of lung (Right) and spleen (Left) of treated mice by HA2 and Mx Adjuvant. There is not any specific tissue change and inflammatory reaction.

CONCLUSION

Recently much research focused on bio adjuvants such as cytokines, interferon-induced peptides, and immune system regulator proteins such as Mx, due to their roles in promoting chemotaxis of immune cells, limiting inflammation and sepsis, regulating metabolism, and enhancing vaccine responses [25]. Post-vaccination adverse reactions frequently occur in recipients [26], but immunization by HA2 DNA vaccine and Mx bio adjuvant in this research had no side effects such as inflammation or tissue degeneration in the spleen and lungs of treated mice.

Considering the data of cell-mediated immune response of our construct in this study and due to suitable humoral immunity in the previous research, and the absence of any side effects in both studies, this construct can be introduced as a vaccine candidate for immunization against influenza virus.

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