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Immunogenicity Evaluation of a DNA Vaccine Expressing the Hepatitis C Virus Non-Structural Protein 2 Gene in C57BL/6 Mice

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ABSTRACT

Backgrounds: Most of the hepatitis C virus (HCV) infections elicit poor immune responses and 75% to 85% of cases become chronic; therefore, the development of an effective vaccine against HCV is of paramount importance. In this study, we aimed to evaluate co-administration of HCV non-Structural Protein 2 and IL-12 DNA vaccines in C57BL/6 mice. **Methods**: A plasmid encoding full-length HCV NS2 protein (non-structural protein 2) was generated and used to vaccinate mice. Negative control (an empty expression vector) was also employed to evaluate the background response. To investigate immune responses against vaccine, C57BL/6 mice received three doses of the vaccine with a two-week interval. Cellular immunity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay for lymphocyte proliferation, lactate dehydrogenase release for cytotoxic T lymphocyte (CTL) activity and cytokine assay. **Results**: The findings demonstrated that immunization of mice with plasmid expressing HCV NS2 induced CTL response, interferon gamma production, and lymphocyte proliferation compared to negative control. The results also demonstrated that co-administration of IL-12 with the HCV NS2 plasmid induced significantly better immune response in C57BL/6 mice. **Conclusion**: DNA vaccine encoding HCV NS2 is an effective candidate that can trigger CTL-based immune response against HCV. In addition, the results suggested that combining the DNA vaccine approach with immune stimulatory cytokines may significantly enhance antigen-specific immune responses. *Iran. Biomed. J. 18 (1): 1-7, 2014*

Keywords: Hepatitis C virus (HCV), NS2 protein, DNA vaccine, IL-12

INTRODUCTION

Approximately 80% of hepatitis C virus (HCV)-infected persons fails to clear the virus and develop persistent infections, which frequently lead to chronic liver disease, fibrosis, cirrhosis, and hepatocellular carcinoma. Current treatments are effective in 50-70% of patients, and it is quite clear that there is an emerging need to develop effective vaccines against HCV [1, 2].

Non-structural protein 2 (NS2) is a 23-kDa) non-glycosylated integral membrane protein (217 amino acids) [3]. The N-terminus of NS2 (residues 1-94) is highly hydrophobic and forms three transmembrane domains, while the C-terminus (residues 94-217) is globular and resides in the cytoplasm [4]. Interactions of NS2 with both structural (E1 and E2) and non-structural (p7, NS3-4A, and NS5A) proteins suggest that NS2 might act as a central mediator in the HCV life-cycle by attracting viral proteins to the replication and assembly sites [5, 6]. Although the exact mode of NS2 action during HCV particle formation is not clear, it has been suggested that it is likely to act at a late step of assembly and maturation when NS2 would attract the envelope glycoproteins to the assembly site and interact with NS proteins during virus assembly [4, 5, 7, 8].

At present, there are approximately 160 million infected individuals living with HCV worldwide, and there is currently no approved vaccine against HCV

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[9]. Therefore, a therapeutic vaccine against HCV would provide significant protection against the infection. DNA vaccines have been used extensively in HCV vaccine research using different HCV genes [10-13]. Among different HCV genes, non-structural antigens present the most genetically conserved viral antigen, which have been employed for induction of cellular immunity in animal models in different vaccine studies [14, 15]. Some vaccine approaches were tested in mice and chimpanzees, but only two DNA vaccine candidates had reached clinical evaluation [16, 17]. CICGB-230 was the first DNA vaccine evaluated in Cuba in Phase I of clinical trial, which combined plasmid expressing HCV structural antigens (core/E1/E2) with recombinant core protein particles Co.120, a protein comprising the first 120 aa of the HCV core antigen [16]. The second HCV DNA vaccine, ChronVac-C, was evaluated in a clinical Phase I/IIa trial in Sweden. The vaccine was based on a plasmid encoding NS3/NS4A under the control of cytomegalovirus (CMV) immediate-early promoter [17]. Therefore, in order to induce a stronger immune response, we administrated IL-12 as a genetic adjuvant. It has been demonstrated that Il-12 as an adjuvant has a critical role in inducing anti-viral and anti-tumor immunity *in vivo* [18, 19].

In the present study, we aimed to determine immunogenicity of NS2 and IL-12 DNA vaccines co-administred in a mouse model. Afterwards, cellular immunity was analyzed against the NS2 recombinant protein in mice.

MATERIALS AND METHODS

Construction of plasmids and bacterial transformation. Full-length sequence of NS2 (GenBank® accession no. AB047639) was amplified using standard PCR protocol using NS2 forward primer (5′-CGCCACCATGTATGACGCACCTGT GC-3′) and NS2 reverse primer (5′-TTAAAGGAGCTTCCACCC CT-3′). Amplified segment was then cloned into pcDNA3.1 vector (Invitrogen, USA) under the control of the CMV promoter. Competent cells, *E. coli* DH5α strain, were generated using $CaCl_2$ method and subsequently transformed with empty vector (pcDNA 3.1) or recombinant vector expressing NS2 (pcDNA 3.1-NS2) using heat-shock method [6]. Clones were confirmed by colony PCR, restriction enzyme digestion of the purified DNA, and DNA sequencing using CMV forward primer (5′-GGTCTATATAA GCAGAGCTGGT-3′) and bovine growth hormone reverse primer (5′-TAGAAGGCACA GTCGAGGC-3′). In the previous study [6], the gene expression of pcDNA-FL-NS2 was evaluated and confirmed in eukaryotic cells (Huh-7 and 293T cells). Both monomeric (around 24 kDa) and dimeric (around 48 kDa) forms of the NS2 protein were detected using rabbit anti-NS2 antibody in Western-blotting experiment [6]. Plasmid pcDNA3.1-IL-12 was kindly provided by Dr. T. Sakai (University of Tokushima, Japan).

Purification of plasmid DNA. Transformed bacterial colonies were cultured in 5 ml Luria Bertani broth containing 50 μg/ml ampicillin overnight. DNA plasmids were isolated from bacteria by the alkaline lysis procedure [20]. In brief, 250 ml Luria Bertani broth supplemented with 50 μg/ml ampicillin was inoculated with pcDNA3.1 or pcDNA3.1-NS2, or pcDNA3.1-IL-12. Bacteria were cultured in a shaker incubator at 37°C overnight, pelleted by centrifugation force and resuspended in the resuspension buffer containing 25 mM Tris-HCL (pH 8.0), 10 mM EDTA (pH 8.0), and 50 mM glucose. The bacteria were lysed by lysis buffer (0.2 N NaOH, 1% SDS), neutralized by nneutralization buffer (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml water) and then centrifuged to pellet chromosomal DNA, RNA, and proteins. The supernatant was extracted with phenol/chloroform, followed by ethanol precipitation of the plasmid DNA. The purified DNA was run on 1% agarose gel in TBE buffer (45 mM Tris-boric acid, 1 mM EDTA, pH 8.0), and DNA bands were visualized by ethidium bromide staining.

Immunization of mouse model. In this study, five experimental groups were included (pcDNA3.1, pcDNA3.1-IL-12, pcDNA3.1-IL-12 + pcDNA3.1, pcDNA3.1-NS2, and pcDNA3.1-NS2 + pcDNA3.1-IL-12). Nine female C57BL/6 mice (4 to 6 weeks old) per group were purchased from the Pasteur Institute of Iran (Tehran) and chosen for the experiment. The mice were immunized three times intramuscularly with 90 μg each expression vector with a two-week interval. The constructed DNA vaccine was injected into the muscle cells, where the 'inner machinery' of the host cells 'reads' the DNA and translates it into desired proteins. Since these proteins are recognized as foreign antigens, they are processed by the host cells and presented to the immune system. All of the mice were monitored for two weeks after final inoculation.

Splenocyte preparation. Two weeks after the final immunization, a single-cell suspension of mononuclear cells was obtained from immunized mice. For this purpose, mice were sacrificed, and the spleens were removed. The suspension of isolated spleen cells was treated with lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.2) in order to eliminate red blood cells. The cells were washed and resuspended in RPMI 1640 supplemented with 10%

FBS. Cell numbers were counted using trypan blue exclusion method, and the percentage of cell viability was calculated.

3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay was performed to measure lymphocyte proliferation. The splenocytes were cultured at a concentration of 2×10^5 cells/well in 96-well plates in the presence of 1 µg/ml NS2 antigen as prepared from our previous study [6], 5 µg/ml PHA (Sigma Chemicals, USA), or RPMI-1640 media. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in splenocytes to convert the yellow water-soluble substrate, MTT, into a dark blue formazan product, which is insoluble in water. A solubilization solution was then added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of colored solution was quantified by its measurement at a wavelength of 570 nm by an ELISA reader. All tests were performed in triplicate for each mouse, and results were expressed as stimulation index, which was defined as the ratio of the mean absorbance of antigen-stimulated cells to that of unstimulated ones [21].

Lactate dehydrogenase (LDH) cytotoxicity detection activity. HCV-specific cytotoxic T lymphocyte (CTL) activity was measured by LDH release assay using LDH Cytotoxicity Detection Kit (Takara, Japan) according to the manufacturer's instruction. LDH is an intracellular enzyme that is found in the cells of many body tissues. Two weeks after the last inoculation, a single-cell suspension of spleno-cytes was prepared and used as effector cells. A precise number of EL4 cells (4×10^4) in a volume of 100 µl were incubated with effector cells (100 µl) at different effector/target ratios. For preparation of the target cells, EL4 cells were stimulated with 1 µg/ml HCV NS2 antigen. After incubation, the microplates were centrifuged at 250 ×g for 10 min. Supernatant per each well (100 µl) was carefully removed and transferred into corresponding wells of an optically clear 96-well flat bottom microplate. Next, 100 µl Solution C (reaction mixture) was added to each well, and it was allowed 30 min for color development. Optical density was measured at a wavelength of 490 nm by an ELISA reader; the reference wavelength was 630 nm. Data were normalized to maximal (Triton X100 lysis of target cells alone) and minimal (target cells alone) lyses. The percentage of cytotoxicity was then calculated as follows: [(test sample – low control)/(high control – low control)] × 100%. All determinations were performed in triplicate [22].

Cytokine secretion assay. Two weeks after the last immunization, mononuclear cells from immunized mice in 1.5 ml RPMI-1640) were pulsed with NS2 antigen in 5% CO_2 at 37°C. Cell-free supernatants were collected and analyzed for the incidence of IFN-γ and IL-4 using Sandwich-Based ELISA kits (R&D Systems, Minneapolis, USA) following manufacturer's instruction. All tests were performed in triplicate for each mouse.

Statistical analysis. *In vitro* immune response data were collected from five individual experiments and all the experiments were performed in triplicates. The data were analyzed by SPSS 18 using test of homogeneity of variances as well as multiple comparisons and ANOVA tests. A *P* value of 0.05 was considered statistically significant. Each data point represents the mean ± SD of a triplicate.

RESULTS

Plasmid extraction. The bacteria with correct plasmids were cultured in a large scale at 37°C for 16-18 hours. Recombinant plasmids were isolated and confirmed by restriction enzyme digestion and DNA sequencing (Fig. 1). After plasmid extraction, DNA concentration was determined using a Picodrop (PicoDrop, UK) at 260 nm. In addition, purity considered as the relative DNA/protein content was estimated by the same method, and expressed as the ratio of A260 /A280 with a value of 1.8 as expected. The extracted plasmids were also analyzed on 0.8% agarose gel electrophoresis.

Lymphocyte pro-liferation. Groups of C57BL/6 mice were administrated intramuscularly. Two weeks after the last vaccination, mice were euthanized, and the splenocytes were aseptically separated. Splenocytes were re-stimulated *in vitro* and then lymphocyte proliferation was measured by MTT assay. As shown in Figure 2, mice immunization with NS2 + IL-12 (3.44 ± 0.10) and NS2 group control (2.03 ± 0.34) revealed better proliferation response than IL-12 group and negative groups. The results also demonstrated a

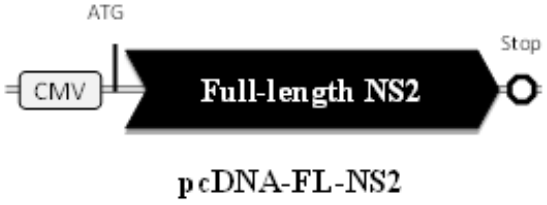

Fig. 1. Schematic representation of the pcDNA-NS2 construct. NS2 gene (GenBank® accession no. AB047639) was under the control of the CMV promoter in the pcDNA3.1 expression plasmid.

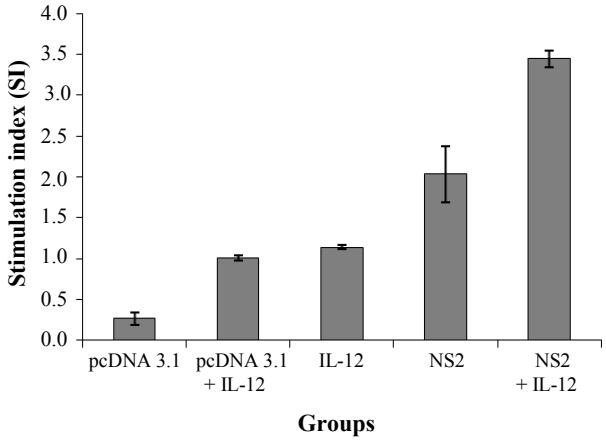

Fig. 2. HCV NS2-specific lymphocyte proliferation. Two weeks after the last administration, the spleens were removed and lymphocyte proliferation was evaluated by the MTT assay Data were expressed as means ± SD (n = 9). Data was considered statistically significant at $P<0.05$.

significant difference in the lymphocyte proliferation index in the NS2 + IL-12 group compared with the NS2 group alone ($P>0.05$) (Fig. 2).

Cytotoxic T lymphocyte responses. To evaluate the specific CTL in the various groups of immunized mice, the LDH release was measured. Splenocytes from vaccinated mice were re-stimulated *in vitro*. As seen in Figure 3, LDH activity was significantly increased in pcDNA3.1-NS2 group as compared to the control group ($P<0.05$). The results showed no significant difference in LDH leakage between NS2 and NS2 + IL-12; therefore, IL-12 did not significantly augment CTL activities. These results indicated that mice immunized with NS2 or NS2 + IL-12 developed strong specific CTL responses.

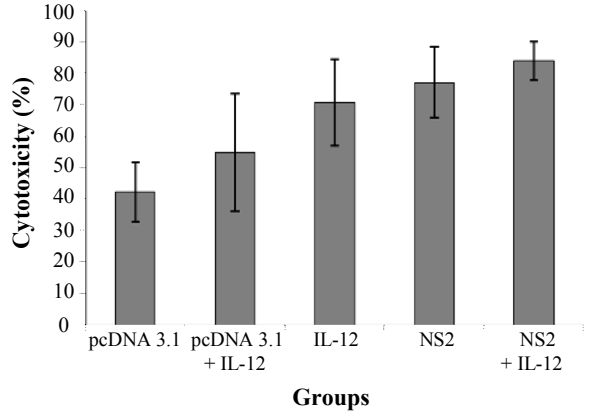

Fig. 3. CTL activity. CTL assays to demonstrate lymphocytes in mice vaccinated with different DNA vaccines using quantitative measurement of LDH release. Data were collected from LDH results at various E/T ratios and expressed as percent cytotoxicity ± SD (n = 9). Data was considered statistically significant at $P<0.05$.

Cytokine assay. Splenocytes from all the five groups of mice were assayed for their IFN-γ and IL-4 cytokine levels in the supernatants. The spleen cells of mice vaccinated with pcDNA3.1-NS2 plasmid showed higher levels of cytokines compared with those of mice administered with empty vector (Fig. 4). Compared with high levels of IFN-γ secretion, spleen cells from the pcDNA3.1-NS2 group produced relatively lower levels of IL-4, and booster immunization did not increase IL-4 secretion (Fig. 4).

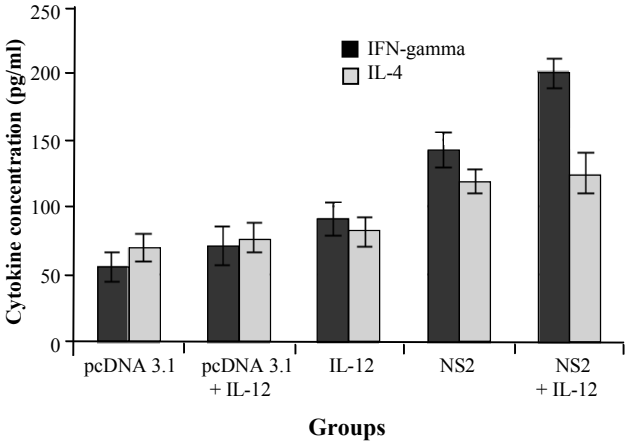

Fig. 4. The concentrations (pg/ml) of Th1-type cytokine of IFN-Gamma and Th2-type cytokine of IL-4 in the supernatants. Collected supernatants were evaluated for the IFN-γ and IL-4 release using ELISA. The concentration of the cytokines was determined by comparison to a standard curve of serially diluted positive control samples. Bars = means SD. Each sample was examined in triplicate and the results were representative of two independent experiments ($P<0.05$).

DISCUSSION

In present study, we have demonstrated that co-administration of DNA vaccine encoding HCV NS2 gene with plasmid containing IL-12 adjuvant is able to induce a significant cellular immune response in C57BL/6 mice. With the high global disease burden and public health impact of hepatitis C, development of an effective vaccine is of major significance. An efficient vaccine against HCV infection requires vigorous and focused $CD8^+$ T-cell responses against viral antigens [23]. However, many challenging obstacles lie ahead of this goal. Given the importance of the NS2 protein in the HCV life-cycle, we chose NS2 as a vaccine candidate against HCV. For this purpose, the immunogenicity of HCV-NS2 DNA vaccine with or without IL-12 adjuvant in the induction of cellular immune responses was evaluated in C57BL/6 mice. Our results showed that IL-12 could boost the MTT and LDH responses as compared with control groups. In addition, NS2 + IL-12 significantly

induced greater lymphocyte proliferation and cytotoxicity (P<0.05). As shown in Figure 2, the NS2 + IL-12 group experienced the greatest lymphocyte proliferation, indicating that vaccination with NS2 + IL-12 induces the proliferation of specific lymphocytes. As illustrated in Figures 2-4, cellular immune responses including the amount of released LDH, IFN-γ production, and lymphocyte proliferation have been increased by the adjuvant.

DNA vaccines have a number of advantages over other vaccines, including the ease to generate and manipulate as well as its potency in stimulating Th1-biased immune responses against an encoded antigen. Kwon *et al.* [24] have demonstrated that co-injection of plasmid expressing IL-12 and DNA vaccine encoding antigen genes resulted in enhanced Th1 and CTL responses and a decrease in antibody production. Therefore, in present study, we evaluated immunogenicity of DNA vaccine encoding NS2 in combination with plasmid encoding IL-12 as a genetic adjuvant.

DNA-based immunization can induce lympho-proliferative responses against the structural proteins core [25-27], E1 [28], E2 [28], and the non-structural proteins NS3, NS4, and NS5 in mice [11] and buffalo rats [29]. Various methods have been used to augment the cellular immune responses [24, 26]. It has been shown that IL-12 drives strong TH1-based cellular immune responses in small animal models [30] and non-human primate models of vaccination when used as genetic adjuvant [31].

Immunization against HCV will involve the generation of cell-mediated immunity to mediate killing or down-regulation of infected cells [32]. One of approaches that enhance DNA-based immune responses is the co-administration of cytokine expressing plasmids with DNA vaccine expressing the gene [25]. In this respect, IL-12-encoding plasmids are particularly promising, since they stimulate TH1 responses [18, 33] and increase the induction of antigen-specific CD8 + CTL [33, 34]. In several studies, co-delivery of plasmids encoding IL-12 with DNA vaccines induced enhanced protection against viral infections and tumors [18, 34].

TH2 and TH1 cytokines have different roles in polarization of immune responses. TH2 cytokines preferentially drive humoral immunity, whereas TH1 cytokines drive induction of cellular immunity. To understand the role of IL-12 in development of immune responses, we examined the effect of co-injection of NS2 DNA vaccine with and without IL-12 on Th1/Th2 ratio. As shown in Figure 4, co-inoculation of IL-12 significantly augmented the production of IFN-γ (TH1-type) compared with NS2 DNA vaccine only. In contrast, the IL-4 (TH2-type) level was not significantly increased by IL-12 co-inoculation. The production of IFN-γ has a dominant effect in enhancing the ability of phagocytic cells to produce IL-12. IL-12 also increases the production of IFN-γ, creating a positive reinforcement loop. Early induction of IFN-γ by IL-12 expression is also the key to the start the innate immune responses [35].

Our results indicate that pcDNA3.1-NS2 vaccination significantly induces cellular immune responses (cytokine secretion, CTL, and T-helper lymphocyte responses) in C57BL/6 mice. It seems that the HCV-NS2 could be an effective candidate gene to induce cellular immune responses against HCV.

In conclusion, results presented in this study suggest that co-immunization of IL-12 with the NS2 DNA vaccine enhances the cellular immune response of the NS2 DNA vaccine and augments the NS2 vaccine ability to protect efficiently against HCV. These outcomes have noticeable concepts for prophylactic and/or therapeutic vaccines.

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