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Immunostimulatory CpG Oligonucleotides as an Adjuvant with Recombinant DNA Vaccine Encoding Hepatitis C Virus Core Protein

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ABSTRACT

The immunogenicity and protective efficacy of DNA vaccines have been amply demonstrated in numerous animal models against infectious diseases. In order to increase the potency of DNA vaccines, we have compared the immune response of conventional adjuvants such as aluminum phosphates, Dendrosome, CpG motif and mixture of aluminum phosphate and CpG motif. Female BALB/c mice were immunized with 10, 25 and 50 microgram of HCV core pcDNA3 plasmid mixed with the adjuvants. Each dose of recombinant pcDNA3 and different adjuvant were used as an immunogen in three IM injection periods. Blood samples were collected at four different times. The data indicate that the antibody response achieved following DNA immunization can be enhanced by CpG motif as molecular adjuvant.

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Introduction

The hepatitis C virus (HCV) is the major cause of non a-non B viral hepatitis. More than 50% of those infected will develop chronic hepatitis and some will progress to cirrhosis and perhaps hepatocellular carcinoma [1]. Currently a prophylactic or therapeutic vaccine is not available against Hepatitis C virus.

The vaccines presently licensed for human use can be live attenuated virus or bacteria, inactivated organisms, partially purified preparations of organisms, polysaccharides or recombinant proteins. Vaccines made from inactivated organisms or products derived from them are often formulated with adjuvants to enhance their immunogenicity. Other type of vaccines in development includes peptides, recombinant viral or bacterial vectors expressing heterologous antigens, and DNA vaccines. The potential of plasmid DNA as a vaccine was first suggested by the observation that administration of DNAs encoding hormones or reporter genes could result in their expression [2,3]. Indeed vaccination of mice with plasmid DNA has resulted in the induction of specific antibodies [4,5]. Various means of enhancing immune responses by vaccines have been reported, including co administration of DNA cytokines, sonicated calf thymus-DNA, dendrosome, cationic lipids, aluminum and calcium salts and CpG motif [6-11]. Immunostimulatory DNA sequences (ISS) or CpG motifs are emerging as useful tools for modulating immune responses. ISS are components of the bacterial but not the vertebrate DNA and can be reproduced by certain synthetic oligonuclotides containing CpG motifs [12]. A number of bacterial products, such as lipopolysaccharides, are known to stimulate the mammalian immune responses. One of the major differences between bacterial DNA, which has potent immunostimulator effects, and vertebrate DNA, which does not, is that bacterial DNA contains a higher frequency of unmethylated CpG dinucleotides than that of the vertebrate. Select synthetic oligodeoxynucleotides containing unmethylated CpG motifs have been shown to have immunologic effects and can induce activation of B cell proliferation and immunoglobulin secretion as well as activating the antigen presenting cells. ISS have potent Th1 adjuvant properties when used for immunization with DNA or protein vaccines [13]. We thus decided to demonstrate that some, not all, of these conventional adjuvants are suitable for DNA vaccines and strongly enhance the immune response in animals.

Material and methods

Plasmid construction and purification: HCV cDNA was isolated from an Iranian male individual with chronic hepatitis C and the full length HCV core cDNA was PCR amplified using specific primers designed by DR. Karami and Mrs. N. Rastgoo (National research center for genetic engineering and biotechnology) [14]. The Primer contained restriction endonuclease sites that were used in the subsequent cloning into the DNA vaccine expression vector (pcDNA3). Plasmid DNA was subsequently digested by XbaI and EcoRI. The new recombinant plasmid (HCV core pcDNA3) was amplified in Escherichia Coli (DH5α) and then subcloned to pcDNA3 vector which was digested by these two restriction enzymes. The integrity of cloning was determined by restriction analysis and PCR reactions. The cDNA was amplified

in Escherichia coli (DH5α). Cells were grown under selective pressure with 50 microgram/milliliter ampicillin. Plasmid DNA was subsequently purified and free of endotoxin by using diethyl amino ethyl sephadex (DEAE-sephadex) anion exchange chromatography column in order to increase the supercoiled plasmid and delete the open circular and linear plasmids [15].

200 ml of LB medium contains bacteria centrifuged 10 minutes in 4000g, Pellets resuspended in 50 ml cold 20% sucrose. Added 10 ml of freshly prepared lysozyme on ice followed by 20 ml cold 0.25 Molar EDTA (PH=8). Spheroblasts centrifuged 10 minutes in 4000g, pellets resuspend in 30 ml 50 mM glucose. 100 microgram/milliliter RNaseA added and kept 15 minutes at 37 ºC. 30 ml solution II (NaOH and SDS) and 30 ml solution III (potassium acetate and glacial acetic acid) added on ice and kept 5 min. Centrifuged at 16000g for 10 min. Added 0.1 V endotoxin removal solution (NaCl , MOPS, Triton X 100 and Isopropanol) and passed through the column. DEAE-Sephadex column was prepared by pouring slurry resin in water passed through the column and equibrated by running a specific buffer (pH=7) (NaCl, MOPS and isopropanol) thought it .The plasmid that has been dissolved in endotoxin removal buffer was passed through DEAE-Sephadex column and the supercoiled plasmid was recovered in the third elution after washing by elution buffer at PH=8.5 (NaCl, MOPS and isopropanol). Plasmids were precipitated by isopropanol and centrifugation 30 min at 12000 rpm. Pellet dissolved in deionized endotoxin free H_2O.

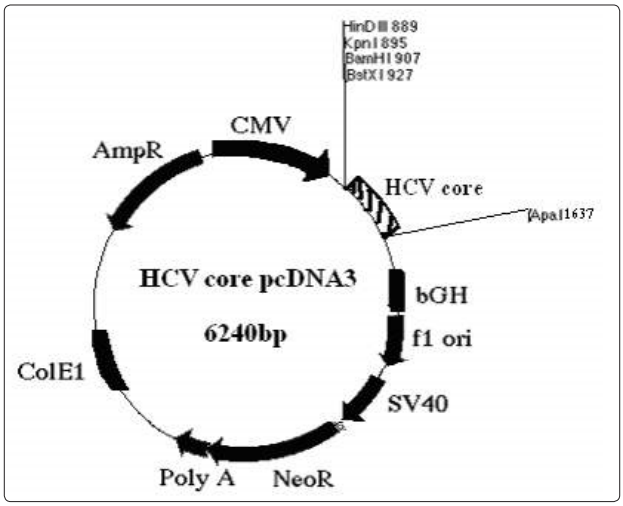

Figure 1: Schematic representation of the HCV core pcDNA3 construct and used in this study

Adjuvants: Four types of adjuvants were used 1) aluminum phosphate (450 µg/ml) with the negative charge that does not physically bind to DNA and greatly enhance antibody response to the construct, 2) CpG motif (5µg/ml) as an immunostimulatory sequence phosphorothioate 5'-TGACTGTGAACGTTCGAGATGA-3' [16,17], 3) 140 KDa dendrosome Den123 with 2.338 x 10^{-12} µg molecular weight (1/150 = Dendrosome/DNA), 4) a mixture of CpG motif (5 µg/ml) and aluminum phosphate (450 µg/ml). The adjuvants were mixed with HCV core pcDNA3 and blended and stirred 1 hour. 100 ul of this mixture was used as immunogen.

Immunization: Five groups of BALB/c female mice (n = 5-7) 6 to 7 weeks old (18-20 gram weight) were immunized by 100 ul of different immunogen. Mice were injected three times after 9, 30 and 50 days by insulin siring in the quadriceps muscle. Blood samples were collected from retro-orbital sinus at 1, 17, 42 and 72 days after injection. These samples were kept at 4° C for 1 hr, serum collected by centrifugation (5min in 15 rpm) and stored in -70° C.

Measurement of antibody response: To determine the anti-core antibodies, 96 microplates (HCV Core kit, by BIOKIT, S.A. Spain) coated with core antigen were used. 100 ul of serial dilutions of individual mouse sera sample diluents were added and incubated at 37° C for 1 hr. Plates were washed five times with pushing buffer, 100 ul of enzyme conjugate, 1/15 diluted with conjugate was added at 37° C for 1 hr. Plates were washed five times with washing buffer. 100 ul of substrate chromogen (1ml substrate + 20 µl chromogen) was added and incubated 30 minutes at room temperature. 100 ul of stopping buffer was added. Optical density (OD) measurement at 450 nm was carried out. The cut-off value to consider a positive mouse anti-core antibody response was calculated [18].

Results

Plasmid: To enhance purity and concentration of the supercoiled HCV core pcDNA3 plasmids DEAE-Sephadex chromatography was used after endoxine removal. The purity of extracted supercoiled plasmids was checked by agarose gel electrophoresis (Figure 2). As it scan be seen, the HCV core pcDNA3 plasmid passed through DEAE-Sephadex column chromatography is much more pure (lane 2, 4 and 5) than the plasmid extracted by endotoxin free kit (lane 1) alone with the and the open circular and linear form of plasmids are removed.

The concentration of extracted plasmid was calculated by UV spectrophotometer and absorbance in 260 nm. The ratio of absorbance in 260 nm to the 280 nm proves the absence of protein contamination in the extracted HCV core pcDNA3. After the concentration of plasmids were determined every adjuvants were mixed with the required concentrations of the DNA.

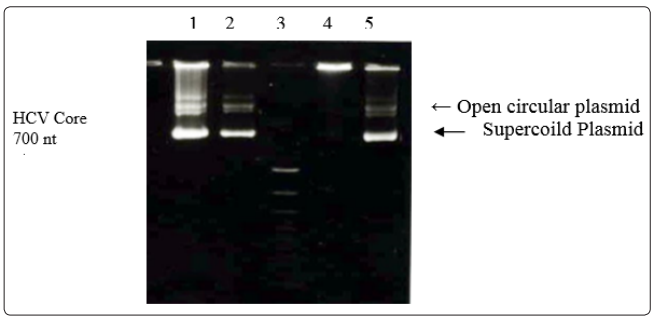

Figure 2: Purification of HCV core Plasmid by DEAE-Sephadex chromatography,
Line 1: HCV core pcDNA3 by free endotoxin method, before chromatography.
Lines 2 and 5: HCV core pcDNA3 plasmid after chromatography.

Effect of different immunogens on antibody response:
To investigate the effect of adjuvants in DNA vaccine, 10, 25 and 50 ug of HCV core pcDNA3 in PBS, aluminium phosphate (450 µg/ml), Dendrosome (1/150 = dendrosome/DNA), CpG motif (5µg) and CpG motif + aluminum phosphate (450 µg/ml) in a total volume of 100 ul have been injected in to the female BALB/c muscle. The antibody response after 17, 42 and 72 days was determined by using HCV core Elisa kit (Figure3).

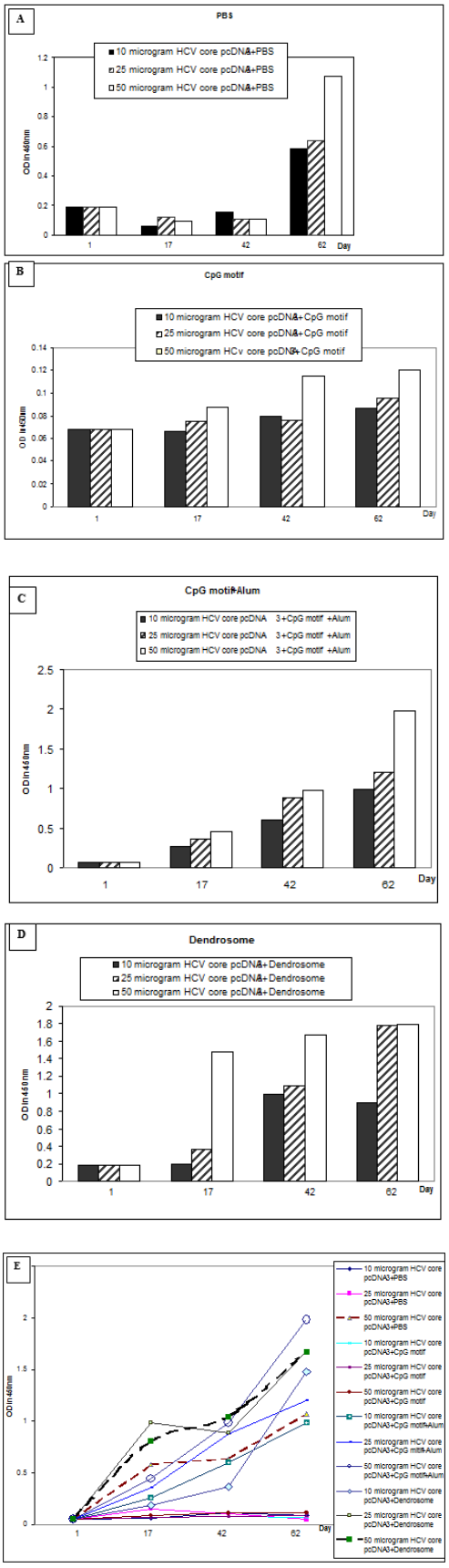

Figure 3: Core-specific antibody measurement by Elisa test elicited in BALB/c mice by injection with 10, 25 and 50 ug HCV core pcDNA3 and (A) PBS, (B) CpG motif, (C) CpG motif + aluminum phosphate and (D) Dendrosome as an adjuvant. (E) All adjuvant in a comparative chart

PBS and aluminum phosphate dos not improve the humoral immune response and immune response increased proportional to the dose of HCV core pcDNA3 and the times of injection not to the presence or type of adjuvants. After the third injection with 50 ug DNA, the antibody titer was increased slightly. This increase was seen in every case, but not considerable improvement in antibody titers.

Dendrosome improved the antibody response especially after third injection. Using the CpG motif as an adjuvant also improve the antibody response after third injection with of 50 ug HCV core pcDNA3 plasmid. But the mixture of CpG motif and aluminum phosphate as an adjuvant was the best adjuvant and improves the antibody response more than others. In fact 50 ug HCV core pcDNA3 after third injection by mixture of CpG motif and aluminum phosphate had the highest antibody titer because of synergistic effect of CpG motif and phosphate aluminum. However one can not ignore the possible side effects may accompany the use of aluminum ion at such concentration.

Discussion

One of the problems of DNA immunization is the degradation of plasmids by extra cellular nuclease. A way to prevent this from happening is to protect the DNA from these enzymes. For this purpose, in this experiment the dendrosome was employed as protectant carrier to diminish the nuclease specific degradation of the plasmid vaccine. Aluminum salts have been used to increase antibody response in a formulation of DNA vaccine [19].

Efforts to improve the immunogenicity of DNA vaccines against hepatitis C have focused on manipulation of the antigens, antigen delivery, route of administration, or the schedule, increasing the quantity of antigen, adding additional factors like cytokines and other viral antigens such as E2, E4, expressing core antigen in different recombinant expression systems (e.g., yeast vs. mammalian cells), administering vaccine subcutaneously, and giving additional doses.

This study demonstrated that ALK81 adjuvant can enhance the immunogenicity of the DNA vaccine mediated core antigen compared to other conventional aluminum adjuvants.

However the mixture of aluminium phosphate with HCV core pcDNA3 stimulated the anti-core antibody response. CpG motif is an immunostimulatory sequence, which is used as an adjuvant in DNA vaccine [13]. It has been used to increase the efficiency of HCV core pcDNA3 immunization with success.

Interestingly sera from mice immunized with recombinant plasmid combined with CpG motif and aluminum phosphate as adjuvant showed the highest immunostimulatory effect. Sera from mice immunized with 50 ug HCV core pcDNA3 and CpG motif + aluminium phosphate could be an effective method in inducing humoral response to HCV core. Moreover, our data indicate that the magnitude of the anti-HCV core humoral immune response upon the DNA immunization could be modulated by plasmid dose, times of injection and type of adjuvant.

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