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RESEARCH ARTICLE

EVALUATION OF STABILITY, BIO-DISTRIBUTION AND TOXICITY OF FOOT AND MOUTH DISEASE DNA VACCINE DELIVERED BY CALCIUM PHOSPHATE NANOPARTICLES

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ABSTRACT

Intramuscular injection of genetic vaccines elicits high cellular immune response and comparatively less humoral response. To understand the factors contributing for immune response *in vivo* FMDV antigens presented through calcium phosphate nanoparticle as well as naked DNA was injected in guinea pigs and bioavailability was studied. Analysis of mRNA expression in different tissues at various points of time by RT-PCR for a period of 300 days showed that the DNA vaccine presented through calcium phosphate nanoparticle produced transcripts up to 240 days whereas, naked DNA injected animals showed mRNA transcripts up to 120 days. FMDV specific antibodies sustained longer duration in nanoparticle delivered animals than naked DNA injected animals. Also the histopathology analysis of the injected muscle showed no major pathological lesions in both nanoparticle as well as naked DNA injected muscle tissue.

INTRODUCTION

Foot and mouth disease is a highly contagious, viral disease of cloven hoofed animals causing economic loss to livestock production. Even after several decades of research on FMD, following strict quarantine measures along with slaughter of infected animals is the only control measure available today. The conventional oil adjuvant inactivated virus vaccine is able to control the epidemics in most affected countries but does not give sterile immunity. Therefore, alternate vaccines that are safe and more effective should replace the conventional vaccine. A genetic vaccine where the DNA encoding the antigens of virus when injected to animals elicits immune response has been used successfully in various diseases (Donnelly *et al.,* 1997; Smooker *et al.,* 2004). Plasmid DNA vaccines have been demonstrated to be immunogenic and protective in small animals but less effective in larger animals, including non-human primates and humans (Liu *et al.,* 2005; Li *et al.,* 2006). The limitations of the DNA vaccine could be many, of which poor presentation of the DNA in naked form may cause enzymatic removal

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in the body which necessitates large quantities of DNA immunisation in large animals. Therefore the techniques that enhance the DNA delivery may be useful in developing improved DNA vaccines. So, DNA vaccines are presented along with carriers in micro particle or nanoparticle form which deliver the DNA over a period of time from the site of injection and also protect the DNA from nucleases by forming a coat on the surface of the particle thereby facilitating delivery of DNA to nucleus (Welzel *et al.,* 2004).

Calcium phosphate has been used as an adjuvant in Human and animal vaccines (Singh *et al.,* 1998). Calcium phosphate nanoparticles provide a safe and easily manufactured vaccine adjuvant and delivery system, which is used to produce DNA or traditional protein antigen viral vaccines (He *et al.,* 2000, 2002). It has been demonstrated that calcium ions play vital role in endosomal escape, cytosolic stability and enhanced nuclear uptake of DNA through nuclear pore complexes. The efficiency of the transfection can be increased by adding a layer of calcium phosphate and by incorporating DNA/ protein into the particle and preventing its degradation within the cell (Viktoriya *et al.,* 2006; Dechamma *et al.,* 2009). Several reports of successful development of immune response have been reported but detailed study of its *in vivo* distribution is lacking. Hence, it is important to characterise the potential of nanoparticle based calcium phosphate genetic vaccine to determine its pathogenicity and tissue distribution following *in vivo* administration. In our earlier report we showed good immune response against FMD in calcium phosphate nanoparticle DNA vaccine than naked DNA vaccine (Dechamma *et al.,* 2009). In the present work, calcium phosphate nanoparticles entrapping the FMDV`O' P1-3CD DNA vaccine was injected in guinea pigs and mRNA expression of antigen was analysed by RT-PCR for a period of 300 days along with specific antibody detection. Also the stability of the DNA entrapped nanoparticles were analysed at different pH and temperature to know the storing quality *in vitro* as well as in physiological pH and temperature. Calcium phosphate nanoparticles toxic effect if any on the injected muscle tissue was also studied for a defined period of time by histochemical staining.

MATERIAL AND METHODS

Cells, animals and viruses

BHK-21 $clone_{13}$ cells at passage number 84 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat inactivated foetal bovine serum (FBS). Cultures were incubated at 37 ^{o}C with 5% CO_2. *E.coli* DH5α cells were used for amplification of plasmids. Animal experiments were carried out as per the approved norms of the Animal Ethics committee of the Institute. Guinea pigs bred and maintained at Animal Experiment Station, Indian Veterinary Research Institute; were used for studies. FMDV O- R2/75 vaccine strain adapted to cell culture was titrated in 96 well cell culture plates. Fifty percent endpoint was calculated by Reed –Muench method (1938) and expressed as $TCID_{50}$.

Preparation of FMDV'O'P1-3CD Calcium phosphate nanoparticles and characterisation

FMD vaccine construct pCDNA 3.1+ (Invitrogen) plasmid encoding P1-3CD of FMDV 'O' serotype under the CMV promoter was available in the FMD Research laboratory, was purified by endotoxin free maxi Kit (Sigma). Calcium phosphate nanoparticles entrapping FMDV 'O' P1-3CD vaccine construct was prepared as reported (Dechamma *et al.,* 2009) , characterised and stored at 4 ^{o}C until use.

Animal immunization

Seventy five guinea pigs (half male and half female) were fed on standard diet. Twenty five guinea pigs received intramuscular administration in gluteus muscle of 100 µg of naked plasmid DNA FMDV'O'P1-3CD; similarly twenty five guinea pigs received intramuscular administration in gluteus muscle of 100 µg of FMDV 'O' CaPN P1-3CD. Remaining twenty five were kept as control animals.

Samples collection

Guinea pigs were sacrificed at various time points till 300 days. In sterile condition approximately 10

mg of different tissues were collected rinsed in PBS followed by triturating in Trizol (Invitrogen). The following organs were harvested from guinea pig and tested for the presence of FMDV gene RNA: heart, liver, lung, kidney, spleen, thymus, muscle (injected as well as distal opposite), and lymph nodes (inguinal, auxiliary, mesenteric). Each time blood was collected and serum was separated for antibody analysis. Same time injected muscle and distal muscle was collected in 10 % formalin in neutral saline for histochemical staining.

RNA Extraction from tissues

Trizol method of RNA extraction was done as per manufacturer's (Invitrogen) instruction. The method involves triturating tissues using triturate in 0.5 ml trizol and incubating for 5 minutes at room temperature. Equal volume of chloroform is added and vortex thoroughly and then centrifuging at 12000 rpm for 10 minutes. Aqueous layer is collected and precipitated with 0.5 volume of isopropanol by keeping at -70 ^{o}C for half an hour. RNA was pelleted at 12000 rpm for 10 minutes and washed in 80 % Diethyl Pyrocarbonate **(**DEPC**)** alcohol and vacuum dried. The pellet was resuspended in RNase free water and RNA concentration was determined using spectrophotometer at 260 nm.

c-DNA Synthesis and PCR Amplification

c-DNA for cellular RNA was synthesized using oligod(T) primer. RNA (2µg) was heat denatured at 65 ^{o}C for 5 minutes and snap cooled in ice. Subsequently reverse transcription buffer containing 25 mM Tris HCl, pH 8.3, 50 mM KCl, 6 mM $MgCl_2$ and 200 µM dNTPs was added along with 100 µM of oligod(T) primer to the final reaction 20 µl. To the reaction mixture 200 units of MMLV reverse transcriptase (Superscript III Invitrogen) were added and c-DNA synthesis was allowed to proceed at 50 ^{o}C for 1 h, followed by 65 ^{o}C for 5 minutes heat inactivation. Synthesized c-DNA was used for amplification of FMDV specific gene using specific primers yielding 650 bp of VP1 gene fragment. Reaction mixture containing 4 µl of purified c-DNA, 20 pM of each primer VP1 (O) L-[GGCGGTACCATGACCACCTCCCCGGGTGA G] and 2AR- [GCGGGAATTCGCGAAGGGCC CAGGGTTGGACTC], 25 mM Tris HCl of pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM of dNTPs and one unit of Taq DNA polymerase in a total volume of 30 µl. Reaction was carried at 94 ^{o}C for 3 minutes followed by 35 cycles of 1 minute at 94 ^{o}C, 1 minute at 60 ^{o}C, primer extension at 72 ^{o}C for 1 minute and final extension of 72 ^{o}C for 10 minutes. PCR amplified product was analyzed in a 1.2 % agarose gel along with marker.

Stability

FMDV 'O' CaPN P1-3CD nanoparticles (10 µg DNA) was pelleted and resuspended in 100 µl of PBS of varying pH(5, 6, 7, 7.5, 8 and 9) and stored at 4 ^{o}C, 24 ^{o}C and 37 °C. At different points of time samples were treated with 100 mM EDTA and analysed in 0.8 % agarose gel.

Histopathology

Muscle tissue at the site of injection and distal muscle tissue were collected at specified time and preserved in 10 % neutral buffered formalin. Fixed tissues were processed by routine paraffin embedding technique (Singh and Sulochana, 1996). The paraffin sections of 5-7 µm were stained with hematoxylin and eosin to observe routine histomorphology which was analysed in Olympus BX51 inverted microscope.

Detection of anti FMDV antibodies

ELISA plates were used for coating with FMDV O-R2/75 vaccine strain 1:2 diluted in PBS pH 7.6. These were subsequently blocked with 5 % skimmed milk powder (SMP)-PBST for 1h at 37 ^{o}C and reacted with 1:100 diluted guinea pig sera for 1h. After three washes, 1:5000 diluted rabbit antiguinea pig IgG conjugated with horse radish peroxidase (Sigma) was added to wells and the plates were incubated at 37 ^{o}C for 1h. The reaction was developed by adding 50 µl of OPD substrate (20 mM *O*- phenyl diamine, 0.01 % hydrogen peroxide in phosphate/citrate buffer 0.1M, pH 5.6). Reaction was stopped by adding 2M H_2SO_4, results were read at 492 nm and corrected for background with control group sera.

Serum neutralization tests

Two fold dilution of antiserum in DMEM without FCS was carried out in a 96 well flat bottomed tissue culture plates (Nunc, USA). Virus suspension at 100 $TCID_{50}$ 50 µl was added to all antisera wells and the mixture was incubated for 1 h at 37 ^{o}C. BHK-21 cell suspension 50 ul (2x 10^{6} ml^{-1}) was added to each well and incubated for 72 h. appropriate serum, virus and cell control were included in the test (Golding *et al.,* 1976). The end-point titres were calculated as the reciprocal of the serum dilution at which 50 % of the cell monolayer showed CPE.

RESULTS

FMDV 'O' CaPN P1-3CD nanoparticle characterization

The size distribution was determined with a zetasizer 3000 HSA (Malvern Wortcesershire, UK). The nanoparticle has an average size of 80 nm and zeta potential in PBS (pH 7.2) was -22 mV (Fig.1).

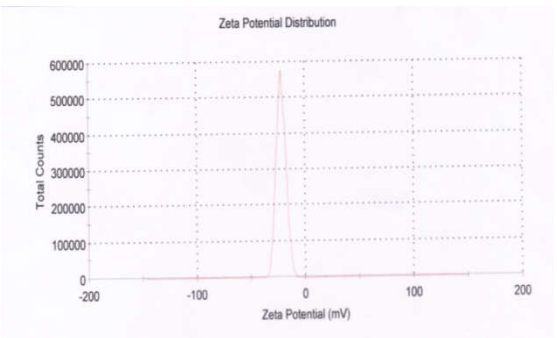

Fig. 1. Zeta potential of FMDV'O'CaPN P1-3CD (DNA 0.5 µg/µl in PBS pH 7.2)

In vivo distribution of FMDV 'O' CaPN P1-3CD nanoparticle

We followed the fate of the DNA vaccine in different tissues of the animal after the immunization in calcium phosphate nanoparticle and naked DNA presentation. DNA vaccine presented in calcium phosphate nanoparticle delivery system was available at the injected muscle for 270 days, draining lymph nodes and spleen and kidney up to 240 days. However in heart was available up to 180 days (Table 1a). Naked DNA vaccine was available at the site of injection and draining lymph nodes, spleen up to 120 days. In the vital organs heart, kidney naked DNA was available up to 60 days (Table 1b).

Effects of distribution on DNA vaccine immunogenicity

The DNA vaccine immunogenicity was compared between naked DNA and calcium phosphate nanoparticle modulating the delivery of DNA by measuring the FMDV specific serum antibody by indirect ELISA (Fig. 2) and SNT using FMDV'O' (Fig.3). Animals injected with the naked DNA vaccine showed serum antibody specific to FMDV detecting from 30 days, reaching maximum titre by 45 days, stable till 60 days and decline was observed. Conversely injection of the same concentration of DNA in calcium phosphate nanoparticles resulted in higher antibody level on 25 dpi and magnitude of the response increased by 3 fold by 35th day remained in the peak till 120 days and gradual decline was observed. Similarly the SN titres of 1.5 were observed in calcium phosphate DNA vaccines at about 28 days to 150 days where as naked DNA SN titres of 1.5 were observed for very short period of 30 days to 90 days.

Stability

FMDV'O'CaPNP1-3CD stored at pH 7.5 was stable at 37 ^{o}C and 24 ^{o}C up to 270 days. And showed part of DNA released into the solution at 37 ^{o}C. On EDTA treatment the encapsulated DNA was released and found to be intact (Fig. 4). The same pH stored particles at 4 ^{o}C were stable for only 150 days. Particles stored at pH 4, 5, 6, 8 and 9 showed degradation of DNA as early as 24 h (data not shown).

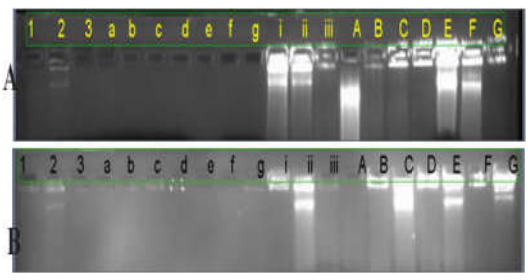

Fig. 4. Neutralization indices of serum samples of immunized guinea pigs. Serum samples were collected from individuals before sacrifice. Virus suspension at 100 $TCID_{50}$ (10^{-6}) was used. SN titers were calculated as the reciprocal of the serum dilution at which 50% of the cell monolayer showed CPE.

Histopathology

FMDV'O'P1-3CD DNA injected muscle in H&E stain showed no appreciable pathological changes. Mild hyperaemia with closely packed muscle fibres was seen on 72 h (Fig.5) which were normal on 7th day (Fig. 5).

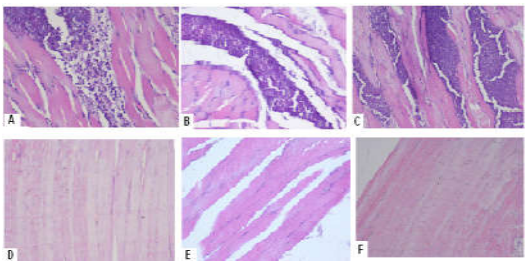

Fig 5. Photomicrograph (200X in Olympus BX51) of gluteus muscle of injected guinea pig. The paraffin sections of 5-7µm were stained with hematoxylin and eosin to observe routine histomorphology.

A. Section of gluteus muscle injected with FMDV 'O' CaPN P1-3CD after 24 h showing , oedema and infiltration of Neutrophils.
B. Section of gluteus muscle injected with FMDV 'O' CaPN P1-3CD after 48 h showing trapping of nanoprtcles.
C. Section of gluteus muscle injected with FMDV 'O' CaPN P1-3CD after 72 h showing inflammation, muscle necrosis
D. Section of gluteus muscle injected with FMDV 'O' CaPN P1-3CD after 7 days showing normal muscle fibers.
E. Section of gluteus muscle injected with naked FMDV 'O' P1-3CD after72 h shows Mild hyperaemia.
F. Healthy section of gluteus muscle injected with PBS (pH 7.2) control.

FMDV'O'CaPNP1-3CD injected muscle tissue showed mild inflammation, oedema and infiltration of neutrophils on 72 h. Also granular material deposited between the muscle fibres was observed. In some area muscle necrosis with loss of normal architecture was observed which was recovered by 7 days (Fig 5).

DISCUSSION

DNA vaccination technology provides alternative means for the development of safe and effective vaccines for infectious diseases. However, multiple doses of high amounts of naked plasmid DNA are required to elicit the desired protective response. Consequently, to optimise the vaccine and reduce the amount of DNA, different methods of delivery, such as gene gun, liposome, nano and micro particles have been used. The encapsulation of the DNA in calcium phosphate nanoparticle represents a potential method to target both cell mediated and humoral immune response. However understanding the factors like duration of bioavailability of the antigen in various organs and stability of the particles in different pH and temperature is crucial to achieve effective immunisation.

The nanoparticle size and charge plays crucial role in the entry of the nucleic acids into the cell. The prepared particles were in the average range of 80 nm (data not shown) which forms a stable colloidal solution (Mathew Epple *et al.,* 2008). Zeta potential distribution of the FMDV'O'CaPNP1-3CD nanoparticles showed net negative charge, indicating binding capacity with negatively charged plasmids. Zeta potential varies from 6 to 50 mV, strength of electrostatic interaction (ZPXu *et al.,* 2006). We found average -22mV which is a medium zeta potentials driving force leading to adherence on to cell surface and nanoparticle biomolecules delivery into the cell. In the cell the nanoparticles should withstand temperature/pH for the retained period and release the DNA. This is confirmed from the stability studies where nanoparticle stored at 37°C and pH 7.5 show intact DNA for a period of 270 days. The same particles stored at higher and lower pH showed degradation of DNA which is shown in Fig. 3 where DNA is showing smear or not present as it moved off from the well.

It is been reported biocompatible inorganic calcium phosphate nanoparticles are chemically stable (Zhi Ping Xu., 2006). And their physiochemical properties can be kept unchanged during the whole delivery process. They are not generally subject to microbial attack, having low toxicity and they exhibit good storage stability (V. Sokolova *et al.,* 2008). But most nanoparticles are believed to accumulate in the cells once they are endocytosed. Therefore cytotoxicity was evaluated by histopathological observation since more than 50 % of antigen is released within 24 h of immunisation. Our studies showed nanoparticle injected muscle samples at 72 h with mild hyperaemia and inflammatory changes which recovered by 7th day which could be due to immediate burst of antigen into cytoplasm in low pH of endosomes. The dissolution of calcium phosphate could increase the pH and thus slow down further dissolution. In such a way, DNA could be released in a controlled manner into the

cells and show no toxic effect on the cells. Nanoparticles like other colloidal drug carriers are able to pass through all capillaries after injection into the muscle tissue (Kreuter, 1994) and rapidly taken up by the macrophages of the reticulo endothelial system. So gene expression was observed as early as 24 h in all the vital and immune organs. Immediately after the injection because of hydrostatic pressure caused by injection DNA enters the cells through circulation, reaches other immune organs leading to transgene expression. Part of the injected materials remains in the interstitial space (Dupuis *et al.,* 2000), which was observed in histochemical staining of the injected muscle tissue. To examine the period of antigen availability, we studied the expression of the gene at mRNA level till the following period of 300 days. After the injection antigen expression was seen in injected muscle, all the organs collected for 120 days in both naked, as well as nanoparticle DNA. Due to the localisation of immune cells to lymph nodes and spleen, gene was available in these organs longer time than vital organs with respect to both naked and nanoparticle DNA. However, nanoparticle DNA was available longer period of 240 days in injected muscle and immune organs like spleen, lymph node clearly indicating coating of nanoparticles alters the uptake of the particles into blood macrophages (Schafer *et al.,* 1992) and antigen may be released slowly because of encapsulation in calcium phosphate. Corroborating these findings antibody level of the nanoparticle injected animals showed three fold increases on 45th day, which sustained till 120 days, thereafter declined gradually whereas, naked DNA injected animals antibody remained for very short period of 35 dpi to 45 dpi. Although the biological relevance of these antibodies has not been tested in vivo, study has demonstrated that there was a good correlation between serum and anti FMDV neutralising antibodies and protection (Wang *et al.,* 2002.).These findings are in a good agreement proving calcium phosphate nanoparticle a based delivery of DNA vaccines induces better and longer duration of immune response.

Conclusion

The study clearly reports calcium phosphate nanoparticles entrapping FMDV vaccine DNA may be a better delivery system for DNA vaccines as it confirms availability of the antigen and persistence of antibody for longer duration than naked DNA. The stable physicochemical property of the calcium phosphate nanoparticles and biocompatible calcium phosphate has no interfering toxic effect on the muscle tissue indicating its application as vaccine delivery.

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