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Calcium phosphate nanoparticle prepared with foot and mouth disease virus P1-3CD gene construct protects mice and guinea pigs against the challenge virus

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ABSTRACT

Calcium phosphate nanoparticles provide safe and easily manufactured vaccine adjuvant and delivery system for DNA vaccines. In the present study FMDV "O" P1-3CD DNA vaccine was encapsulated in calcium phosphate nanoparticles of size 50–100 nm diameters. The maximum loading and entrapment efficiency of nanoparticles were studied by spectrophotometer, as well as agarose gel electrophoresis. *In vitro* transfection efficiency of these calcium phosphate nanoparticles was found to be as good as commercial transfecting reagent lipofectamine. *In vivo* analysis of the calcium phosphate nanoparticle P1-3CD (CaPNP1-3CD) FMDV "O" vaccine in mice and guinea pigs could induce significant cell mediated and humoral immune response. Also, immunized mice and guinea pigs were protected against the challenge virus.

1. Introduction

Calcium phosphate has been used for over 30 years to deliver genetic material to mammalian cells. This method has additional advantage as an adjuvant over other transfection procedures such as viral vectors and dendrimers in respect of superior biocompatibility and reduced non-specific immune response (Su et al., 2000). However, as adjuvants for DNA vaccines these are to be further improved to achieve better gene delivery, depot effects, targeting of antigen presenting cells and activation of the desired type of immune response. The exact mechanisms of such delivery systems are not yet fully understood and need further investigations to develop more effective vaccines.

Foot and mouth disease (FMD) is a highly contagious viral disease, which is endemic in Asia, Africa and South America (Parida et al., 2006). It is an economically significant disease of cattle, sheep, goats, pigs and cloven-hoofed wildlife species. It causes production losses, high mortality in young animals and is a major constraint to international trade in live animals and their products (Kitching et al., 2007). Conventional vaccines against FMD consist of chemically inactivated whole virus antigen combined with either an oil, or aluminium hydroxide and saponin adjuvant, depending on the intended target species (Doel, 2003). Although effective at preventing clinical signs of the disease, the current FMD vaccines, even with a potency of $\geq$3 PD50 may not induce sterile immunity, and depending on the level of challenge infection, virus may persist, giving rise to carrier animals (Doel et al., 1994).

Alternate to conventional vaccine, DNA vaccines containing analogs of virus genes capable of expressing viral antigens in host cells may be the choice (Beard et al., 1999). However, naked DNA when used as vaccines in human trials needed substantially higher doses of DNA to induce protective immune response (MacGregor et al., 1998; Wang et al., 2001) which may be due to the ineffective delivery strategy or rapid degradation of the injected DNA.

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Therefore, safe and effective DNA delivery strategies and DNA protection methods are needed to overcome these limitations and enhance the potency of DNA vaccines. Besides other optimization strategies reviewed (Gurunathan et al., 2000), the use of nanoparticles as DNA delivery vehicles has emerged as a promising new approach to DNA vaccination (Ragusa et al., 2007). Particularly inorganic calcium phosphate nanoparticles have been developed as delivery systems as well as adjuvant for DNA vaccines (He et al., 2000, 2002; Bisht et al., 2005). In this study, we investigated whether a calcium phosphate nanoparticle which is also proven adjuvant (Su et al., 2000) could be used to deliver the DNA vaccine for FMD. The FMD virion is composed of four structural proteins, termed VP1, VP2, VP3 and VP4, which are encoded by the P1 gene (Domingo et al., 2002) and a serine protease encoded by 3C gene (Grubman and Baxt, 2004) is required to catalyze the cleavage of the polyprotein to generate capsid proteins (Saiz et al., 2002). The nonstructural protein 3D is a potent stimulator of cellular and humoral specific response (Foster et al., 1998). So all the structural and nonstructural genes P1, 2A, 3C and 3D which are required for FMD viral capsid assembly (Beard et al., 1999) from FMDV O-R2/75 was chosen as a candidate vaccine.

The DNA was entrapped with calcium phosphate to produce nanoparticles which was used to induce cellular and humoral immunity *in vivo*. CaPNP1-3CD DNA vaccine injected mice and guinea pigs were challenge immunized to determine the protective efficacy of DNA/nanoparticle vaccine formulations *in vivo*.

2. Materials and methods

2.1. 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 fetal bovine serum (FBS). Cultures were incubated at 37 °C with 5% CO_2. *Escherichia coli* DH5α cells were used for cloning and 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 protection studies. Albino Swiss mice were procured from Veterinary College Hebbal, Bangalore.

FMDV O-R2/75 vaccine strain adapted to cell culture was titrated in 96-well cell culture plates and guinea pig adapted virus was passaged in guinea pig foot pads and used in the studies. Fifty percent endpoint was calculated by Reed–Muench method (1938) and expressed as $TCID_{50}$ or $GPID_{50}$ as the case may be.

2.2. Plasmid and plasmid purification

The pCDNA3.1+ (Invitrogen) plasmid encoding P1-3CD of FMDV "O" serotype under the CMV promoter and for *in vitro* transfection experiments the green fluorescent protein (GFP) encoding plasmid pHUGG (transfer vector carrying UTR-GFP under the HR promoter) was used. Both the plasmids were provided by Dr. V.V.S. Suryanarayana, FMD Research Lab, Indian Veterinary Research Institute. Plasmid DNA was maintained and purified from *E. coli* DH5α using Qiagen plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions.

2.3. Preparation of FMDV P1-3CD calcium phosphate nanoparticles and characterization

The basic method of preparation of pDNA loaded nanoparticles of calcium phosphate were similar to that reported (He et al., 2002) with reduced stirring time of 3 h. The plasmid DNA (pP1-3CD) 500 μg in 7.5 ml of 12.5 mM calcium chloride was mixed with 7.5 ml of 12.5 mM dibasic sodium phosphate and 10 mM sodium citrate slowly in dropwise to control the pH 7.2. The solution was stirred for 3 h when the particle size of less than 100 nm could be formed. The nanoparticles were pelleted at 800 × *g* for 15 min and washed once with PBS (pH 7.2) and stored at 4 °C until use.

2.3.1. Transmission electron microscopy (TEM)

One drop (30 μl) of the aqueous dispersion of nanoparticles and one drop (30 μl) of 1% phosphotungstic acid were put on a formavar coated copper grid (1% solution of formavar was prepared in spectroscopic grade chloroform) and air dried in a vacuum desiccator. The dried grid was then examined under an electron microscope (Hitachi-800 TEM).

2.3.2. Entrapment efficiency

The DNA loaded nanoparticles were washed repeatedly in sterile PBS (pH 7.2) by pelleting at 800 × *g* for 5 min. Each wash was checked for the presence of DNA by agarose gel electrophoresis or spectrophotometrically. The nanoparticles were finally washed with 70% alcohol and air dried. Known quantity of (mg) nanoparticles was treated with 100 mM EDTA at room temperature for 30 min. The amount of DNA released from the nanoparticles was measured spectrophotometrically at 260 nm. The percentage efficiency of entrapment was then calculated from the amount of DNA originally added in the preparation (DNA_O) using the equation $E\% = DNA_R/DNA_O \times 100$ where DNA_R is the amount of DNA released (Bisht et al., 2005).

2.3.3. DNA loading capacity

In order to determine the maximum DNA loading capacity of calcium phosphate nanoparticles, different amounts of DNA (100–1000 μg) were added during the preparation of nanoparticles, keeping the total amount of calcium and phosphate ion concentrations constant. The nanoparticles dispersed in aqueous solution during the preparation were then subjected to agarose gel electrophoresis. The excess DNA remained as free DNA after saturation was visible as distinct band in the gel, which was also measured by spectrophotometer at 260 nm.

2.3.4. Stability

The DNA entrapped nanoparticles were treated with 10 units of DNase (Sigma) for 1 h at 37 °C. After the