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Challenge studies in Rhesus monkeys immunized with candidate hepatitis E vaccines: DNA, DNA-prime-protein-boost and DNA-protein encapsulated in liposomes

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ABSTRACT

Complete ORF2 gene (1983 bp) of hepatitis E virus (HEV) and the 450 bp region within ORF2 containing neutralizing epitope (NE) cloned in pVAX1 and corresponding proteins expressed in baculovirus and prokaryotic systems respectively were evaluated as vaccine candidates. Two doses of liposome encapsulated DNA plus corresponding protein with both ORF2 and NE regions (Lipo-ORF2-DP and Lipo-NE-DP) showed 100% seroconversion and comparable anti-HEV titres in Swiss albino mice. These vaccine candidates were further evaluated as DNA, DNA-prime-protein-boost (DPPB) and liposome formulations in Rhesus monkeys. Monkeys receiving ORF2/NE DNA seroconverted after fourth dose while those immunized employing ORF2-DPPB format seroconverted at 7 weeks post third dose. In view of the delayed weak antibody response, these monkeys were not challenged. Though Lipo-ORF2-DP was immunogenic, 2 of the 4 monkeys developed HEV infection following homologous virus challenge of 100 Monkey Infectious $Dose_{50}$. Both monkeys immunized with Lipo-NE-DP and 1 of the 2 monkeys immunized with NE-DPPB showed complete protection, the second monkey being protected from hepatitis with limited viral replication. Irrespective of the type of immunogen, all challenged monkeys were protected from hepatitis. The results document Lipo-NE-DP to be a promising vaccine candidate needing further evaluation.

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1. Introduction

Hepatitis E, one of the major causes of acute hepatitis in sporadic and epidemic forms in the developing countries, is primarily transmitted by the faecal–oral route. Hepatitis E is usually a self-limiting infection with low mortality. However, in pregnant women, especially in the third trimester, the mortality rate may be as high as 25% [1,2]. In sporadic setting, men and non-pregnant women succumb to fulminant hepatitis E [3]. The disease was earlier thought to be restricted to developing countries. However, hepatitis cases among non-travellers are being increasingly reported in developed countries. Zoonoses is emerging as an important transmission mode [4–8]. The causative agent, hepatitis E virus (HEV) belongs to family *Hepeviridae* and genus *hepevirus*. The virus has special predilection for young adults [9].

HEV is a non-enveloped virus with a single-stranded, positive sense RNA genome of approximately 7.2 kb in length. The genome contains a short 5′ non-coding region (5′ NCR), 3 open reading frames (ORFs), and a short 3′ NCR terminated by a poly-A tract. Non-structural and structural proteins are encoded by ORF1 (approximately 5 kb) and ORF2 (approximately 2 kb) respectively; ORF3 (342 nt) overlaps ORF2 and encodes a small phosphoprotein.

In the absence of a suitable cell culture system or convenient animal model conventional methods cannot be attempted for the vaccine development. The capsid protein (ORF2) is mainly targeted for possible use as candidate vaccine. Recombinant proteins expressed in baculovirus system [10] or bacteria [11] and DNA [12,13] vaccines have been evaluated in primate models. A recombinant protein-based vaccine has undergone successful clinical trial in humans in Nepal [14].

We tried DNA alone and DNA-prime-protein-boost (DPPB) approaches in mice employing either complete ORF2 or the smaller region containing the neutralizing epitope (NE) [15]. Both humoral and cellular immune responses were observed in mice immunized with different immunogens. Subsequently, another approach of encapsulating DNA and corresponding recombinant protein in liposome [16] was tried in mice. Both NE and ORF2 regions were evaluated. These formulations elicited excellent humoral response in terms of early seroconversion and high anti-HEV titres. The

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Table 1
Detailed description of immunogens used in the study.

Vaccine name	Details	Immunization route
ORF2-D	Complete ORF2 gene in pVAX1	By gene gun intradermally (at multiple sites on the abdomen)
ORF2-DPPB	Complete ORF2 gene in pVAX1 and 56 kDa ORF2 protein expressed in baculovirus system	Two doses of DNA by gene gun and one dose of protein intramuscularly on thigh
Lipo-ORF2-DP	Complete ORF2 gene in pVAX1 plus 56 kDa ORF2 protein encapsulated in liposomes	Subcutaneous
NE-D	NE region in pVAX1	By gene gun
NE-DPPB	NE region in pVAX1 and NE protein expressed in bacterial system	Two doses of DNA by gene gun and one dose of protein intramuscularly on thigh
Lipo-NE-DP	NE region in pVAX1 and NE protein encapsulated in liposomes	Subcutaneous
Lipo-pVAX	pVAX1 vector alone encapsulated in liposomes	Subcutaneous

present study reports immunogenicity and efficacy of different formulations in Rhesus monkeys, the preferred animal model for challenge studies. The vaccine candidates under study include DNA alone, DPPB and liposome encapsulated DNA plus protein with either complete ORF2 or NE (Lipo-ORF2-DP or Lipo-NE-DP).

2. Materials and methods

2.1. Animals

2.1.1. Mice

Six to eight weeks old female Swiss albino mice were immunized subcutaneously with different liposome formulations at 0 and 4 weeks interval. Blood samples were collected by retroorbital bleeding for pre-immune sera before immunization and at regular intervals after giving the doses. All the protocols were approved by the ethical committee of the institute for the use of animals for experimentations.

2.1.2. Primates

Twenty anti-HEV negative female Rhesus monkeys (*M. mullata*) of about two years of age were used in this study. The institutional and national ethical committees approved the use of these monkeys. The housing, maintenance, and care of the Rhesus monkeys complied with the guidelines and requirements of the relevant national animal ethical committee.

2.2. Candidate vaccines and immunizations

Preparation of full-length ORF2 DNA, NE DNA and the expression and purification of the corresponding proteins is described earlier [15]. Complete ORF2 gene (1983 bp, 5147 nt–7129 nt, corresponding to 660 aa) and the NE region (450 bp, 6518 nt–6967 nt, encoding for 458–607 aa of ORF2 protein) cloned in pVAX1; 56 kDa ORF2 protein (rORF2p) expressed in baculovirus system (56 kDa, 112–607 aa, is the truncated form of ORF2 protein resulting due to processing in Sf9 cells) and 150 aa NE protein expressed in prokaryotic system (rNEp) were used.

2.2.1. Preparation of DNA gold micro carriers and DNA immunization

Two hundred microgram of plasmid DNA was coated on to 50 mg of 1 μm gold particles (BioRad, USA) in the presence of 100 μl of 0.05 M spermidine (Sigma chemicals, St. Louis, MO). DNA and gold particles were co-precipitated by the addition of 100 μl of 1 M $CaCl_2$ and the precipitate was washed thrice with absolute ethanol. The suspension of gold particles in ethanol containing 0.05 mg/ml polyvinylpyrrolidone (PVP) was used to coat inner wall of Tefzel tubing (BioRad, USA). Tube was cut into 0.5 in. pieces and filled in the cartridge holders of Helios gene gun (BioRad, USA). Each Rhesus monkey received 10 shots on shaved abdomen (2 μg plasmid DNA/shot, total 20 μg DNA) with gene gun at 400 psi Helium pressure.

2.2.2. DPPB

Monkeys received two doses (20 μg each) of either ORF2 or NE DNA with gene gun and third dose of corresponding protein (20 μg) adsorbed onto either $Al(OH)_3$ (for rORF2p) or $AlPO_4$ (for rNEp) (total 65 μg Al gel/20 μg protein) by intramuscular injection on thigh.

2.2.3. Lipo-ORF2-DP/Lipo-NE-DP

The DNA and corresponding protein (either complete ORF2 or NE) were co-entrapped into liposomes by dehydration and rehydration method [16]. Phosphatidyl Choline (PC), Dioleoyl Phosphatidyl Ethanolamine (DOPE) and Dioleoyloxy Trimethyl Ammonium Propane (DOTAP) were mixed in the molar ratio of 4:2:1 in chloroform and dried completely in a vacuum rotary evaporator (Rota-Vapor R-205). The lipid mixture was hydrated with endotoxin free water; shaken vigorously to get multilammellar large vesicles, sonicated to reduce the size to small unilamellar vesicles (SUV). DNA and protein were mixed together and added to the SUV suspension in the mass ratio of 1:200. The mixture was freeze-dried and rehydrated with PBS. Rhesus monkeys were inoculated subcutaneously with 500 μl liposome suspension containing 20 μg DNA + 20 μg protein/dose.

2.3. Immunization schedules

Table 1 provides the detailed description of candidate vaccines used in the present study.

2.3.1. Mice immunization

Three groups of mice (n = 10/group) (50 μl/dose) were immunized with two doses of liposome encapsulated formulations as follows:

(a) Lipo-pVAX (pVAX1 vector alone): 1 μg/dose
(b) Lipo-ORF2-DP: 1 μg each of DNA and protein/dose
(c) Lipo-NE-DP: 1 μg each of DNA and protein/dose

2.3.2. Monkey immunization

Table 2 provides details of the immunogens used and immunization schedules for the monkeys. The interval between two doses for all protocols was 4 weeks. Three approaches each were tried with the full-length HEV ORF2 and the truncated NE region as follows:

(1) ORF2-D: Three ORF2 DNA doses (20 μg/dose) (MM# 201, 202)
(2) ORF-2 DPPB: Two ORF2 DNA doses (20 μg/dose) and one rORF2p dose (20 μg/dose) (MM# 203, 204)
(3) NE-D: Three NE DNA doses (20 μg/dose) (MM# 205, 206)
(4) NE-DPPB: Two NE DNA doses (20 μg/dose) and one rNEp dose (20 μg/dose) (MM# 207, 208)
(5) Lipo-ORF2-DP: Two Lipo-ORF2-DP doses (20 μg each of DNA and protein/dose) (MM# 209, 210, 211, 212)
(6) Lipo-NE-DP: Two Lipo-NE-DP doses (20 μg each of DNA and protein/dose) (MM# 213, 214)

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