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Immunogenicity of candidate hepatitis E virus DNA vaccine expressing complete and truncated ORF2 in mice

Tejaswini M. Deshmukh, Kavita S. Lole, Anuradha S. Tripathy, Vidya A. Arankalle*

Hepatitis Division, National Institute of Virology, Microbiological Containment Complex, Sus Road, Pashan, Pune 411021, India

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Abstract

Hepatitis E virus (HEV) is a major cause of enterically transmitted acute hepatitis of adults in developing nations. Our present studies show that, the complete ORF2 gene (1–660 amino acids, a.a.) coding for capsid protein of HEV as candidate DNA vaccine induced significant specific humoral and cellular immune responses in mice. Gene gun based DNA administration led to higher seroconversion rates and HEV-specific antibody titers as against needle-injection method. The region (458–607 a.a.) within ORF2 protein is reported to harbour the predominant neutralization epitope/s (NE) of HEV. The NE DNA also induced HEV-specific immune responses in mice. NE-based DNA-prime-protein boost approach was observed to be superior to NE DNA based approach. Co-administration of plasmid expressing mouse granulocyte macrophage colony stimulating factor (GM-CSF) induced immune response at similar level as that with ORF2/NE plasmid alone. IgG1 was the predominant isotype irrespective of the approach used. HEV-specific antibodies in seroconverted mice sera could bind/neutralize HEV in an *in vitro* ELISA-based assay. In conclusion, efficacy of ORF2 and NE based DNA/DNA-prime-protein-boost approaches are worth exploring in monkey model.

Keywords: Hepatitis E virus; DNA vaccine; Neutralization epitopes

1. Introduction

Hepatitis E virus (HEV) is a major causative agent responsible for sporadic and epidemic acute viral hepatitis in developing countries. HEV is principally transmitted via fecal-oral route, mainly through contaminated water. More than 50% of reported acute viral hepatitis cases from India are attributed to HEV in adult population [1,2]. In the last 5 years, we serologically confirmed 101 outbreaks of hepatitis E in the state of Maharashtra alone. One of the unique features of HEV infection is the high mortality rate of 10–20% observed in pregnant women, especially in the last trimester [3,4]. In sporadic settings, men and non-pregnant women die due to fulminant hepatitis E [5]. HEV has a worldwide distribution and broad host range than previously thought [6]. Swine HEV has been shown to cross-species barrier [7] and a close relationship between swine HEV and some human isolates has been observed [8]. Hepatitis E infection has been transmitted to humans by consumption of undercooked sika deer and wild boar meat, which is suggestive of a possible zoonotic spread of HEV [9,10]. Human HEV cases have been documented in developed nations with no travel history to endemic region.

HEV is classified into five genotypes but to date a single serotype exists. Cross-challenge studies in rhesus monkeys employing several epidemic and sporadic HEV isolates (genotype 1) documented protection from disease up to 5 years after primary infection [11,12], indicating possible utility of vaccine. Administration of normal human immunoglobulins to pregnant women during an epidemic of hepatitis E was shown to be useful suggesting possible role of antibodies in protection [13]. So far, no effective anti-viral treatment for hepatitis E is available and hence there is a need for vaccine against HEV. In the absence of adaptation of HEV to cell culture or laboratory animals,

Corresponding author. Tel.: +91 20 25880982; fax: +91 20 25893640.
E-mail address: varankalle@yahoo.com (V.A. Arankalle).

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conventional methods cannot be tried. Several approaches based on recombinant DNA technology have been tried to develop candidate subunit vaccine [14–21] mainly based on the open reading frame 2 (ORF2) protein or DNA, the most successful being ORF2 protein (56 kDa) expressed in insect cells from baculovirus vector [22–24]. This subunit vaccine protects against genotype 1 (homologous) as well genotypes 2 and 3 HEV challenge [25] and has undergone successful phase II/III clinical trials in Nepal [26].

DNA vaccine is a relatively new tool with several advantages such as expression of concerned gene nearest to its native form, induction of both humoral and cellular immune responses, stability and requirement at very low amounts. This approach has been tried for HEV [14,15]. The method of DNA administration affects the targeting of DNA to appropriate site especially in higher animals. Gene gun offers a good aid to target DNA to skin cells at very low amounts [17,18].

Within ORF2, a region encompassing 6518–6967 nucleotides (nt) (458–607 a.a.) has been reported to express the predominant neutralization epitopes (NE) [27]. The present study evaluated the use of complete ORF2 and NE as candidate DNA vaccines. In addition, utility of gene gun in enhancing immune response and feasibility of GM-CSF gene as genetic adjuvant was also examined. We further assessed the immune responses generated by ORF2 and NE based DNA-prime-protein-boost immunization in out bred mice.

2. Materials and methods

2.1. Construction of recombinant plasmids in pcDNA3.1/pVAX1

2.1.1. Cloning of complete ORF2 gene

Complete ORF2 gene (1983 bp, 5147–7129 nt, 1–660 a.a.) from HEV RNA positive 10% (w/v) human fecal suspension (PM2000, genotype-1, GenBank accession no. DQ459342) collected during an outbreak in Lonawala, Western India (2000) was amplified by reverse transcription-PCR. The resultant PCR product (~2.0 kbp) was TA-cloned in pMos-BlueT vector (Amersham Pharmacia, USA). The complete ORF2 gene was further cloned in pcDNA3.1 vector (Invitrogen, Carlsbad, CA) and the insert was confirmed by sequencing. Complete sequence of the ORF2 insert was determined from both the strands using multiple primers using Big-dye terminator cycle sequencing ready reaction kit (Applied Biosystems, CA, USA). Samples were analysed on automated sequencer (ABI PRISM 310 Genetic analyzer, Applied Biosystems, USA).

2.1.2. Cloning of partial ORF2 gene (NE region)

The NE region (450 bp, 6518–6967 nt, 458–607 a.a.) within ORF2 gene was PCR amplified from the pcDNA3.1 + ORF2 construct. *BamHI* site/start codon and HindIII site/stop codon were incorporated in the forward and reverse primers respectively. The resulting PCR product was cloned into *BamHI* and *HindIII* sites of pcDNA3.1; *BamHI* of pVAX1 respectively to obtain pcDNA3.1 + NE and pVAX1 + NE constructs. The pVAX1 vector, 3.0 kb (Invitrogen, Carlsbad, USA) is FDA approved for human use as DNA vaccine.

2.1.3. Cloning of mouse GM-CSF gene

Splenocytes of an adult Balb/c mouse were separated on Ficoll-Hypaque 1077 (Sigma chemicals, St. Louis, MO) gradient using standard protocol. Total RNA was extracted from the cells using TRIzol LS reagent (Invitrogen, Carlsbad, CA). Complete mouse GM-CSF gene (426 bp, 142 a.a. *Mus musculus*; chromosome 11, GenBank accession no. NM009969) was amplified by reverse transcription-PCR. The resultant PCR product was TA-cloned in pGEM-T Easy vector (Promega, Madison, USA) and subsequently in pcDNA3.1 vector.

2.1.4. Endotoxin free bulk plasmid preparations

All above plasmids were purified using endo-free plasmid Maxi-prep columns (Qiagen, Hilden, Germany). Plasmid quality was tested by agarose gel electrophoresis and purity and quantity were assessed by spectrophotometry. For experimental use, the plasmid DNA was reconstituted in endotoxin free water at a concentration of 1.0 μg/μl.

2.2. Preparation of DNA gold micro carriers for gene gun system

Calculated amount of plasmid DNA was coated on to 50 mg of 1 μm gold particles (BioRad, USA) in presence of 100 μl of 0.05 M Spermidine (Sigma chemicals, St. Louis, MO). Plasmid DNA and gold particles were co-precipitated by the addition of 100 μl of 1 M $CaCl_2$, while vortexing. The precipitate was washed thrice with absolute ethanol and resuspended in ethanol solution containing 0.05 mg/ml polyvinylpyrrolidone (BioRad, USA). This gold particle suspension was used to coat inner wall of the Tefzel tubing (BioRad, USA), cut into 0.5 inch pieces and filled in the cartridge holder of Helios gene gun (BioRad, USA). For dose response studies tubes were coated with different amounts of plasmid DNA so that 0.01, 0.1, 1.0, 2.0 μg of DNA would be delivered per shot.

2.3. Adsorption of rORF2p and rNEp to $Al{\cdot}(OH)_3$ and $Al{\cdot}PO_4$

Recombinant ORF2 protein was expressed in baculovirus system described earlier [22] and purified on AKTA BASIC 100 HPLC system (Amersham Pharmacia, USA). rNEp was expressed in bacterial system (pET-15b vector, 5708 bp; Novagen, Germany) and purified using Nickel-chelating resin (Invitrogen, Carlsbad, USA). One μg of rORF2p/rNEp was adsorbed on 3.25 μg of $Al{\cdot}(OH)_3$/$Al{\cdot}PO_4$ (Sigma chemicals, St. Louis, MO) respectively for 1 h at RT on a vortex mixer followed by centrifugation at 5000 rpm for 5 min at

RT. The supernatant was preserved for ELISA and pellet was resuspended in 0.01 M PBS, pH 7.2 with 0.01% Thimerosal (Sigma chemicals, St. Louis, MO). The suspension was refrigerated and diluted to the required dose at the time of immunization.

2.4. Immunization of mice

Six to eight weeks old female Balb/c and Swiss albino mice were used for immunizations. Mice were bled for pre-immune sera before immunization and at regular intervals after receiving the DNA/recombinant protein doses by retro orbital bleeding. Mice groups immunized with the aid of gene gun received shots of plasmid DNA coated gold particles with Helios gene gun at 250 psi Helium pressure on the shaved abdomens. Control mice received 100 μg of pcDNA3.1/pVAX1 DNA/dose in case of intramuscular inoculations or 1.0 μg/dose in case of gene gun inoculations. Groups of Balb/c mice ($n = 10$/group, total two groups including one control group) were injected with 100 μg/dose of pcDNA3.1 + ORF2 DNA dissolved in 100 μl phosphate-buffered saline (0.01 M PBS, pH 7.2) into quadriceps muscles of hind legs via 26.5 gauge needle at 0, 3, 5, 7 weeks interval. A pcDNA3.1 + ORF2 DNA dose response was carried out in both the mice strains using gene gun. Groups of Swiss albino and Balb/c mice ($n = 8$/group, total five groups including one control group of each strain) were immunized at 0, 4, 8 weeks interval with 0.01, 0.1, 1.0, 2.0 μg/dose of pcDNA3.1 + ORF2 DNA. Based on the observations of this experiment, groups of Balb/c mice ($n = 10$/group, total four groups including two control groups) were immunized at 0, 2, 4 weeks and 0, 4, 8 weeks interval with 1.0 μg/dose of pcDNA3.1 + ORF2 DNA. A dose response of pcDNA3.1 + GM-CSF DNA was done in Swiss albino mice ($n = 11$/group, total five groups including one control group) by immunizing them at 0, 4, 8 weeks interval with 1.0 μg/dose of pcDNA3.1 + ORF2 DNA alone or along with 0.01, 0.1, 1.0 μg/dose of pcDNA3.1 + GM-CSF DNA. To assess the effect of pcDNA3.1 + GM-CSF DNA in Balb/c mice ($n = 10$/group, total two groups) were immunized with 1.0 μg/dose of pcDNA3.1 + ORF2 DNA and 1.0 μg/dose of pcDNA3.1 + GM-CSF DNA (0, 2, 4 weeks and 0, 4, 8 weeks interval respectively). Swiss albino mice ($n = 6$/group, total four groups including one control group) were immunized at 0, 4, 8 weeks interval with 1.0 μg/dose of pcDNA3.1 + NE DNA alone or with 1.0 μg/dose of pcDNA3.1 + GM-CSF DNA, to assess the immunogenicity of truncated ORF2 gene. One group was immunized with 1.0 μg/dose of pcDNA3.1 + ORF2 DNA for comparison. Swiss albino mice ($n = 10$/group, total three groups including one control group) were immunized at 0, 4 and 8 weeks interval with 1.0 and 2.0 μg/dose of rORF2p adsorbed to $Al{\cdot}(OH)_3$. Swiss albino mice ($n = 7$/group, total two groups including one control group) were immunized at 0, 4 and 8 weeks interval with 2.0 μg/dose of rNEp adsorbed to $Al{\cdot}PO_4$. Swiss albino mice ($n = 5$/group, total two groups including one control group) were given total two doses of pcDNA3.1 + ORF2 DNA (1.0 μg/dose) at 0 and 4 weeks interval and finally given 2.0 μg of rORF2p with complete Freund's adjuvant (CFA) at 8 weeks (data not shown). In subsequent experiments, CFA/IFA (incomplete Freund's adjuvant) was used with recombinant proteins for mice immunization. Swiss albino mice ($n = 8$/group, total four groups) were primed with 1.0 μg/dose of pcDNA3.1 + ORF2 DNA and boosted with 2.0 μg/dose of recombinant ORF2 protein (rORF2p) in DNA-prime-protein-boost approach. Total three doses were given in short and long dose intervals at 0, 4, 8 weeks (ORF2-S) and 0, 4, 20 weeks (ORF2-L) respectively. Two groups in each type of dose schedule were given two different dose regimens as follows:

- first DNA dose followed by two protein doses (DPP) and
- first two DNA doses followed by single protein dose (DDP).

Swiss albino mice ($n = 10$/group, total two groups including one control group) were primed with 1.0 μg/dose of NE DNA (pVAX1 + NE construct) and boosted with 2.0 μg/dose of rNEp in DNA-prime-protein-boost-approach. Total three doses were given in short dose interval at 0, 4 and 8 weeks (NE-S) in DDP dose regimen as described above.

All protocols were approved by the Institutional Ethical Committee for the use of animals for experimentation. All mice were bred at parent institute and housing and care met or exceeded all the requirements.

2.5. ELISA for assaying anti-rORF2p and anti-rNEp antibodies

Mice sera were screened for the presence of anti-rORF2p or anti-rNEp antibodies in ELISA employing rORF2p and rNEp as coating antigens according to the method described earlier [28]. Briefly, Sf9 cell lysate containing rORF2p was diluted in 50 mM carbonate buffer (pH 9.5) and used for coating (100 μl/well) 96-well micro-titer plate (Maxisorp, Nunc; Denmark). Following coating at 37 °C for 2 h the wells were blocked with blocking solution at same temperature for half an hour. After washing, mice sera were added to wells at appropriate dilutions (100 μl/well of two-fold serially diluted sera) in blocking solution. Incubation continued at same temperature for half an hour. A 1:10 dilution of pre-immune sera served as negative control. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma Chemicals, St. Louis, MO) was used as detector antibody. The enzymatic reaction with substrate (*O*-phenylenediamine and urea peroxide) was stopped by the addition of 4N H_2SO_4 and the absorbance measured at 492 nm. A serum sample was considered to be reactive when the optical density (OD) value was greater than or equal to thrice mean OD value (ELISA cut-off) of pre-immune sera (negative control).

Purified recombinant partial ORF2 protein (rNEp) expressed in bacterial system was used in ELISA to detect specific antibodies against concerned region. The ELISA procedure remained same as above except rNEp was coated

at 4 °C overnight. Reactivity of mice sera was tested with both recombinant antigens in ELISA. The HEV-specific IgG antibody titers were determined for individual mouse from each group. The reciprocal of the highest dilution that had an absorbance greater than or equal to the ELISA cut-off was taken as the HEV-specific antibody titer. All the analyses were carried out on the geometric mean titers (GMTs) and log-transformed antibody titers with standard errors.

2.6. ELISA based in vitro HEV binding/neutralization assay

In the absence of availability of an *in vitro* or *in vivo* neutralization assay for the detection/titration of anti-HEV neutralizing antibodies in the immunized mice, an ELISA based virus binding/neutralization assay was standardized. Pre- and post-inoculation serum samples from a rhesus monkey experimentally infected with HEV and a 10% (w/v) stool suspension containing 1.3×10^7 RNA copies of HEV/ml were used as the antibody and virus standards respectively. In rORF2p based ELISA, the pre-serum was scored negative whereas the HEV-specific antibody titer of the post-infection serum was 1:6400. This serum was diluted two-fold serially, and equal volumes of the diluted sera were allowed to react with the virus positive stool suspension at 37 °C for 1 h. Presence of specific antibody in the serum-virus mixture was assessed in ELISA as described earlier. Samples showing ≥50% reduction in OD values in ELISA when compared with the respective control (serum + PBS) were considered positive for virus-binding/neutralizing antibodies.

Following standardization of the assay, pre- and post-immunization serum samples from mice immunized employing different protocols were similarly assessed for the presence of virus binding/neutralizing antibodies.

7. Isotype analysis of HEV-specific IgG antibodies

For detecting isotypes, the reaction with test serum was followed by incubation at 37 °C for half an hour with anti-mouse IgG isotype antibodies in goat (IgG1, IgG2a, IgG2b and IgG3) (Sigma chemicals, St. Louis, MO). HRP-conjugated rabbit anti-goat IgG (Sigma Chemicals, St. Louis, MO) was used as detector antibody. Isotyping was done using both rORF2p and rNEp as detecting antigens. The HEV-specific titers for IgG isotypes namely IgG1, 2a and 2b were determined for individual mouse from each group. The reciprocal of the highest dilution that had an absorbance greater than or equal to the ELISA cut-off was taken as the HEV-specific antibody titer. All the analyses were carried out on the log-transformed antibody titers with standard errors.

2.8. Lymphocyte proliferation assay (LPA)

To assess the cell mediated immune response, mice were sacrificed 2–3 weeks after the last dose for spleen cells. Splenocytes were stimulated *in vitro* either with purified rORF2p or rNEp antigen for specific proliferation studies. Briefly, 1×10^5 cells/well were cultured quadripicately in 96-well flat bottom plate (Nunc, Denmark) in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37 °C with 5% CO_2 partial pressure. Cells were stimulated with T cell mitogen phytohaemagglutinin (PHA) (Sigma chemicals, St. Louis, MO) 5 μg/ml as positive control. Twenty and 10 μg/ml of rORF2p and rNEp, respectively were added to cells for specific proliferation. Cells with medium only were treated as negative control. The optimum doses of PHA, rORF2p and rNEp were decided based on previous dose response studies. Cells were pulsed at 96 h with 1 μCi of tritiated thymidine for 24 h. Cells were harvested onto GF/C filter (Whatman, UK) membrane and counts taken on β-counter (LKB Pharmacia, Sweden) using standard protocol. The stimulation index (SI) was determined as follows, SI = experimental counts/spontaneous counts. Mice with SI values ≥3 were considered to be responding.

2.9. Statistical analyse

Wilcoxon signed rank test and Mann–Whitney test were used to compare log titers between dependent and independent groups, respectively. Mann–Whitney test was used to compare SI values. The statistical software SPSS 11.0 was used for statistical analyses and P value <0.05 was considered as statistically significant. Non-responders in each group were included in the analyses.

3. Results

1. Construction of recombinant plasmids

Complete ORF2 gene of type 1 HEV (human) was amplified by reverse transcription PCR and the product (~2.0 kb) was TA-cloned. Orientation of the insert was checked with restriction enzymes and the insert was further cloned into vector pcDNA3.1. The clone was sequenced completely and compared with representative sequences from GenBank. It showed 94% nucleotide homology with strain SAR-55 (type-1, human isolate) while at amino acid level the homology was 99.5%. NE region (6518–6967nt, 450 bp) was PCR amplified using above ORF2 clone as a template, cloned in pcDNA3.1 and sequenced for confirmation. The 450 bp NE region sequence matched 100% with the parent ORF2 clone. Further, mouse GM-CSF (426 bp) was amplified by RT-PCR from mouse splenocyte RNA and cloned in pcDNA3.1. Sequence analysis of the cloned GM-CSF gene showed single synonymous change at position 179 (A → G, third position in the codon) while remaining sequence matched with the reference sequence (GenBank accession no. NM009969).

3.2. Humoral immune response in mice

Humoral immune response in mice groups immunized employing different candidate vaccine approaches was analysed by detecting anti-rORF2p or anti-rNEp antibodies in mice sera by ELISA. The HEV-specific antibody titers for IgG, IgG1, IgG2a and IgG2b were determined by ELISA. The pre-immune sera and the sera of control mice of both strains remained HEV-specific antibody negative in ELISA. The average OD values in ELISA (rORF2p detecting antigen) for pre-immune sera of Balb/c and Swiss albino mice were 0.013 ± 0.002 and 0.012 ± 0.002, respectively. The average OD values in ELISA (rORF2p detecting antigen) for all the control groups of Balb/c and Swiss albino mice were 0.034 ± 0.004 and 0.057 ± 0.004, respectively. These values did not differ in ELISA employing rNEp as the detecting antigen. For statistical analysis, the HEV-specific mean antibody titer log(10) for all the mice from control groups was taken as 0.301 ± 0.000.

3.2.1. ORF2 DNA

ORF2 DNA immunization using intramuscular route of administration (total four doses of 100 μg each at 0, 3, 5, 7 weeks interval) resulted in 55% seroconversion with low HEV-specific antibody titers (1:10) (data not shown). Gene gun aided immunization was therefore used to immunize mice with candidate DNA vaccines in all subsequent experiments.

The seroconversion rates observed in Swiss albino mice ($n = 8$/group) at 1 week post dose 2; given total three doses each of different concentrations of ORF2 DNA (0.01, 0.1, 1.0 and 2.0 μg/dose) at 0, 4, 8 weeks interval were 12.5, 62.5, 87.5, 100%, respectively and at 2 weeks post dose 2 were 50, 75, 100, 100% respectively. Thus, the optimum dose, 1.0 μg/dose (total three doses) was used for all subsequent experiments. The seroconversion rates in the Swiss albino mice immunized with optimum dose of ORF2 DNA alone and along with three different dose concentrations of GM-CSF DNA at 4 weeks post dose 1 were 0, 18.1, 18.1, 18.1%, respectively; 1 week after the second dose, were 90, 90.9, 100, 100%, respectively. Antibody titers in Swiss albino mice immunized with ORF2 DNA alone or in combination with GM-CSF DNA did not differ significantly ($P > 0.05$) (Fig. 1). The reciprocal specific antibody titers in Swiss albino mice immunized with 1.0 μg/dose (total three doses) of ORF2 DNA alone ranged between 320 and 1600 and those immunized along with 1.0 μg/dose (total three doses) of GM-CSF DNA also ranged between 320 and 1600.

Seroconversion rates achieved at 1 week post third dose (last dose) in Balb/c mice ($n = 8$/group) immunized with different dose concentrations of ORF2 DNA (0.01, 0.1, 1.0, 2.0 μg/dose) given at 0, 4, 8 weeks interval were 12.5, 50, 12.5, 25%, respectively. Hence, immunization at 2-week interval (0, 2, 4 weeks) of Balb/c mice ($n = 10$/group) with 1.0 μg/dose of ORF2 DNA alone or along with 1.0 μg/dose of GM-CSF DNA was tried and yielded 100% seroconversion 1 week post third dose (last dose) (Table 1). Co-administration of 1.0 μg GM-CSF DNA elevated the specific antibody titers but the rise was not significant ($P > 0.05$). Antibody titers in Balb/c mice immunized with ORF2 DNA alone or with GM-CSF DNA, in 0, 4, 8 weeks dose schedule remained low (Table 1).

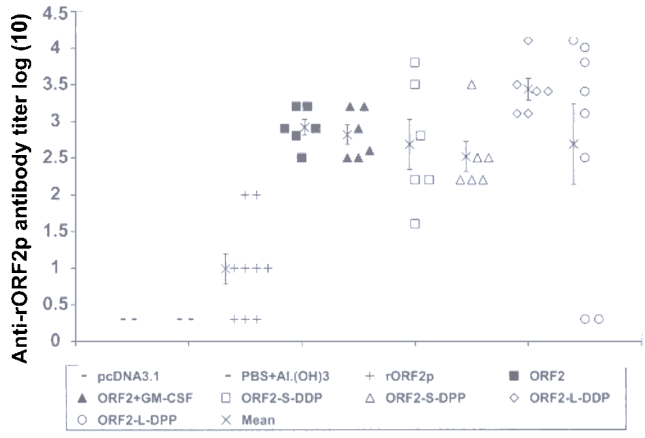

Fig. 1. Serum HEV-specific anti-rORF2p IgG antibody log(10) titers detected in ELISA at 2–3 weeks post last dose (third dose) in Swiss albino mice groups immunized (total three doses, 1 μg/dose/plasmid DNA and 2 μg/dose of rORF2p) with rORF2p (0, 4, 8 weeks), ORF2 DNA (0, 4, 8 weeks), ORF2 DNA with GM-CSF DNA (0, 4, 8 weeks), ORF2 DNA and protein in DDP and DPP regimens of both short (0, 4, 8 weeks) and long (0, 4, 20 weeks) schedules. Error bars represent standard error of the mean log titer.

Co-administration of GM-CSF DNA did not enhance the humoral immune response in Balb/c and Swiss albino mice significantly. Balb/c mice required doses at short intervals (2 weeks) to achieve comparable immune response as in Swiss albino mice. Hence, subsequent experiments were carried out in Swiss albino mice only.

3.2.2. NE DNA

Swiss albino mice ($n = 6$/group) immunized with total three doses (1 μg/dose) of the NE DNA resulted in 100% seroconversion 1 week after the last dose (third dose) (Table 1). NE DNA immunization resulted in late seroconversion and low titers as compared to ORF2 DNA ($P < 0.05$) (Table 1). Mice groups receiving NE DNA with GM-CSF DNA showed similar specific antibody titers ($P > 0.05$) (Table 1 and Fig. 2). The reciprocal specific antibody titers in mice immunized with NE DNA alone ranged between 10 and 80 and those immunized along with GM-CSF DNA ranged between 80 and 640 with two mice remaining non-responders in this group.

3.2.3. Recombinant ORF2 and NE proteins

A 55.5 and 77.7% seroconversion was observed at 1 week post third dose (last dose) in Swiss albino mice ($n = 10$) immunized at 0, 4 and 8 weeks interval with 1.0 and 2.0 μg/dose (total three doses) of rORF2p, respectively (Table 1). A 28.6% seroconversion was achieved at 1 week post third dose (last dose) in Swiss albino mice ($n = 7$) immunized at 0, 4 and

Table 1
Seroconversion rates and HEV-specific antibody titers in immunized mice groups detected in rORF2p based ELISA

Mice (n)[a]	Dose and dose schedule	Percent seroconversion			Geometric mean titer ± S.E.M. (n)[b]
		Post dose 1[c] (%)	Post dose 2[d] (%)	Post dose 3[e] (%)	
Balb/c ($n=10$)	ORF2 DNA; 0, 2, 4 weeks		20	100	158.4 ± 1.9 ($n=10$)
Balb/c ($n=10$)	ORF2 and GM-CSF DNA; 0, 2, 4 weeks		40	100	268.2 ± 1.9 ($n=7$)
Balb/c ($n=10$)	ORF2 DNA; 0, 4, 8 weeks		10	20	5.5 ± 1.9 ($n=10$)
Balb/c ($n=10$)	ORF2 and GM-CSF DNA; 0, 4, 8 weeks		0	50	7.1 ± 1.6 ($n=10$)
Swiss albino ($n=11$)	ORF2 DNA; 0, 4, 8 weeks		90	100	833.6 ± 1.3 ($n=6$)
Swiss albino ($n=11$)	ORF2 and GM-CSF DNA; 0, 4, 8 weeks		100	100	661.6 ± 1.4 ($n=6$)
Swiss albino ($n=10$)	rORF2p; 0, 4, 8 weeks		20	77.7	9.8 ± 1.6 ($n=9$)
Swiss albino ($n=8$)	ORF2-S-DDP; 0, 4, 8 weeks		100	100	487.4 ± 2.2 ($n=6$)
Swiss albino ($n=8$)	ORF2-S-DPP; 0, 4, 8 weeks		100	100	332.1 ± 1.6 ($n=6$)
Swiss albino ($n=8$)	ORF2-L-DDP; 0, 4, 20 weeks		100	100	2757.6 ± 1.4 ($n=6$)
Swiss albino ($n=8$)	ORF2-L-DPP; 0, 4, 20 weeks		62.5	62.5	493.3 ± 3.6 ($n=8$)
Swiss albino ($n=6$)	NE DNA; 0, 4, 8 weeks		17	100	40.0 ± 1.6 ($n=4$)
Swiss albino ($n=6$)	NE and GM-CSF DNA; 0, 4, 8 weeks		0	67	43.3 ± 2.7 ($n=4$)
Swiss albino ($n=7$)	rNEp; 0, 4, 8 weeks		14.2	28.6	4.0 ± 1.4 ($n=7$)
Swiss albino ($n=10$)	NE-S-DDP; 0, 4, 8 weeks		0	60	174.0 ± 3.0 ($n=8$)

[a] Number of mice taken in each group.
[b] HEV-specific IgG antibody geometric mean titers determined at 2–3 weeks post last dose (third dose) for immunized mice groups. Number in parentheses denotes the number of mice taken from each group for titer determination.
[c] Percent seroconversion at 2 or 4 weeks post dose 1.
[d] Percent seroconversion at 2 or 4 or 16 weeks post dose 2.
[e] Percent seroconversion at 1 week after the third dose (last dose).

8 weeks interval with the 2.0 μg/dose (total three doses) of rNEp (Table 1). The HEV-specific antibody titers in both these groups were low (Figs. 1 and 2).

3.2.4. DNA-prime-protein-boost

Swiss albino mice ($n=8$) were primed with 1.0 μg of ORF2 DNA and subsequently boosted with 2.0 μg of rORF2p in two different dose schedules (short and long) and two different dose regimens (DDP and DPP) in DNA-prime-protein-boost approach. Seroconversion rates in these mice groups are given in Table 1. HEV-specific antibody titers of

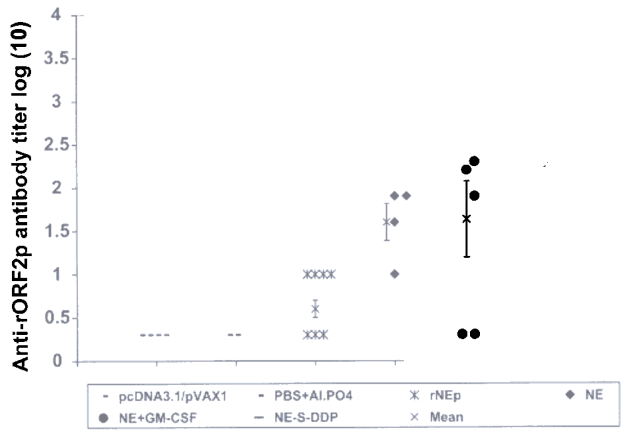

Fig. 2. Serum HEV-specific anti-rORF2p IgG antibody log(10) titers detected in ELISA at 2–3 weeks post last dose (third dose) in Swiss albino mice groups immunized (total three doses, 1 μg/dose/plasmid DNA and 2 μg/dose rNEp) with rNEp (0, 4, 8 weeks), NE DNA (0, 4, 8 weeks), NE DNA with GM-CSF DNA (0, 4, 8 weeks) and NE DNA and protein in DDP regimen of short (0, 4, 8 weeks) schedule. Error bars represent standard error of the mean log titer.

Swiss albino mice groups immunized employing different ORF2 based approaches are showed in Fig. 1. The reciprocal specific antibody titers in ORF2-S-DDP, ORF2-S-DPP, ORF2-L-DDP and ORF2-L-DPP groups ranged between 40 and 6400, 160–3200, 1280–12,800 and 320–12,800, respectively with two mice remaining non-responders in the last group. The specific antibody titers in ORF2-L-DDP group were significantly higher than ORF2 DNA group ($P<0.05$) while in the remaining three groups the antibody titers did not differ significantly.

Fig. 3 depicts dose-wise anti-HEV response in different groups of mice. The specific antibody titer increased after every dose (total three doses) in ORF2-S-DDP, ORF2-L-DDP and ORF2-L-DPP groups. The antibody titer after dose 3 (last dose) increased by ~2-fold in mice immunized in DDP regimen of short interval dose schedule (ORF2-S) whereas titers increased by ~60- and ~13-fold after third dose (last dose) in mice immunized in DDP and DPP regimens respectively in long interval dose schedule (ORF2-L).

The seroconversion rates in Swiss albino mice ($n=10$) primed with 1.0 μg of NE DNA and subsequently boosted with 2.0 μg of rNEp in DDP dose regimen in short interval dose schedule (0, 4 and 8 weeks; NE-S) are given in Table 1. The reciprocal specific antibody titers in this group ranged from 400 to 3200 with two mice remaining non-responders. The geometric mean HEV-specific antibody titer (174.0 ± 3.0) in these mice was higher than those observed in mice immunized with total three doses of NE DNA alone (40.0 ± 1.6), NE DNA in combination with GM-CSF DNA (43.3 ± 2.7) and rNEp alone (4.0 ± 1.4) (Fig. 2).

HEV-specific antibody responses were also detected in seroconverted mice from all groups described above against rNEp as detecting antigen in ELISA. Seroconverted

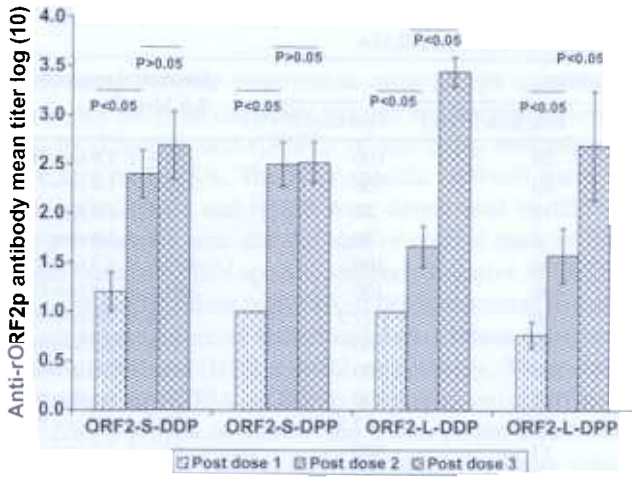

Fig. 3. Serum HEV-specific anti-rORF2p IgG antibody mean log(10) titers detected in ELISA just before doses 2 and 3, and 2–3 weeks post dose 3 in Swiss albino mice groups immunized with ORF2 DNA and protein in DDP and DPP regimens of both short (0, 4, 8 weeks) and long (0, 4, 20 weeks) schedules (total three doses, 1 μg/dose/plasmid of ORF2 DNA and 2 μg/dose of rORF2p). Error bars represent standard error of the mean log titer.

mice of both strains remained ELISA reactive up to 24 weeks.

3.2.5. *ELISA based in vitro HEV binding/neutralization assay*

For the standardization of virus binding/neutralization assay, pre- and post-inoculation serum samples from an experimentally infected monkey were used as anti-HEV negative and positive controls and HEV RNA positive stool sample (1.3×10^8 RNA copies/g stool) as the source of the virus. A clear binding of HEV with the anti-HEV antibodies present in the post-inoculation serum was evident as shown by dilution dependent inhibition of binding of the anti-HEV antibodies to the recombinant ORF2 protein coated on the well (Table 2). At lowest dilution (antibody excess), the amount of virus was not sufficient for neutralization and percent reduction in OD value was less than 50%. When mice sera representing different immunization approaches were tested similarly, complete inhibition of the ELISA reactivity was noted for every specimen. These results clearly demonstrated that similar to natural infection, the antibodies produced by immunization with ORF2 or NE candidate vaccines could bind to the virus.

3.2.6. *Anti-HEV IgG isotype analysis*

Anti-HEV antibodies in the mice sera were reactive to all the IgG subclasses (1, 2a, 2b and 3) (data not shown). Since, IgG3 isotype was low in all the groups, titers for IgG1, 2a and 2b were determined for Swiss albino mice. The IgG2b titers were significantly lower than the 1 and 2a in all the mice groups (data not shown). For mice immunized employing different approaches the predominant isotype detected was IgG1 (Fig. 4). Pre-immune and control mice sera were non-reactive for the HEV-specific IgG and all its isotypes. This IgG1 predominance is suggestive of Th2 type of immune response.

3.3. *Cell mediated immune response in mice*

Results of the LPA are depicted in Tables 3 and 4. Splenocytes from control group mice of both strains did not respond to both the recombinant antigens (SI value range 0.5–2.4 with rORF2p and 0.008–2.6 with rNEp). The SI values in PHA stimulated cells ranged from 0.3 to 132.3. The SI values in the immunized Swiss albino mice groups are shown in Table 4. Percent recall response was less in Balb/c mice compared to Swiss albino mice. Thus, immunization with ORF2

Table 2
Binding/neutralization of HEV with anti-HEV antibodies in a monkey experimentally infected with the virus and mice immunized with vaccine candidates

Species and details	Serum dilution	OD values (492 nm) in rORF2p based ELISA	
		Serum + PBS	Serum + virus
Rhesus monkey post-inoculation	1:100	1.738	1.213
	1:200	1.778	0.708
	1:400	1.521	0.372
	1:800	1.151	0.085
	1:1600	0.634	0.055
	1:3200	0.475	0.06
	1:6400	0.195	0.022
Rhesus monkey pre-inoculation	1:100	0.083	0.099
Mice immunized with			
ORF2 DNA	1:100	0.711	0.075
ORF2 DNA + GM-CSF DNA	1:80	0.800	0.122
ORF2-S-DDP	1:400	0.914	0.021
ORF2-L-DPP	1:200	0.977	0.081
NE DNA	1:20	0.249	0.107
NE DNA + GM-CSF DNA	1:100	0.447	0.045
NE-S-DDP	1:200	0.826	0.092
Pre-immune	1:10	0.073	0.077

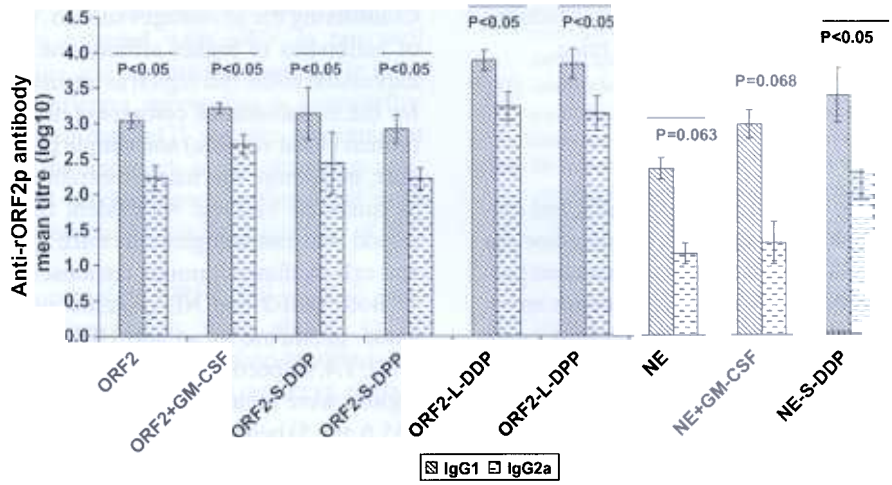

Fig. 4. Serum HEV-specific anti-rORF2p IgG1 and 2a antibody mean log(10) titers detected in ELISA at 2–3 weeks post last dose (third dose) in Swiss albino mice groups immunized (total three doses, 1 μg/dose/plasmid DNA and 2 μg/dose of rORF2p or rNEp) with ORF2 DNA (0, 4, 8 weeks), ORF2 DNA with GM-CSF DNA (0, 4, 8 weeks), ORF2 DNA and protein in DDP and DPP regimens of both short (0, 4, 8 weeks) and long (0, 4, 20 weeks) schedules, NE DNA (0, 4, 8 weeks), NE DNA with GM-CSF DNA (0, 4, 8 weeks) and NE DNA and protein in DDP regimen of short (0, 4, 8 weeks) schedule. Error bars represent standard error of the mean log titer.

Table 3
Cell-mediated immune response in Swiss albino and Balb/c mice

Approach[a]	Responder/total tested (%recall response)							
	Swiss albino (recall antigen)				Balb/c (recall antigen)			
	Control mice		Test mice		Control mice		Test mice	
	rORF2p	rNEp	rORF2p	rNEp	rORF2p	rNEp	rORF2p	rNEp
ORF2 DNA	0/5 (0%)	0/5 (0%)	6/11 (54.5%)	ND[b]	0/5 (0%)	ND[b]	1/12 (8.3%)	ND[b]
ORF2 + GM-CSF DNA[c]	0/5 (0%)	0/5 (0%)	2/11 (18.2%)	ND[b]	0/5 (0%)	ND[b]	0/14 (0%)	ND[b]
rORF2p	0/5 (0%)	0/5 (0%)	3/9 (33.3%)	4/9 (44.4%)	ND[b]	ND[b]	ND[b]	ND[b]
ORF2-S-DDP[d]	0/5 (0%)	0/5 (0%)	2/7 (28.6%)	2/7 (28.6%)	0/5 (0%)	ND[b]	ND[b]	ND[b]
ORF2-S-DPP[d]	0/5 (0%)	0/5 (0%)	3/6 (50%)	2/5 (40%)	0/5 (0%)	ND[b]	ND[b]	ND[b]
NE DNA	0/5 (0%)	0/5 (0%)	3/9 (33.3%)	5/9 (55.6%)	0/5 (0%)	ND[b]	ND[b]	ND[b]
NE-S-DDP[d]	0/5 (0%)	0/5 (0%)	7/8 87.5%	6/8 75.0%	ND[b]	ND[b]	ND[b]	ND[b]

[a] All DNA doses were given by gene gun and protein by needle-injection
[b] Not done.
[c] Mice co-immunized with 1.0 μg/dose of GM-CSF DNA.
[d] DNA prime protein boost.

Table 4
The stimulation indices (SI) in immunized Swiss albino mice groups as assessed in LPA

Approach[a]	SI value range, median value, *P* value (recall antigen)	
	rORF2p	rNEp
ORF2 DNA	3.0–6.1, 3.0, $P<0.01$	ND[b]
ORF2 DNA + GM-CSF DNA[c]	0.03–3.8, 1.5, $P>0.05$	ND[b]
rORF2p	0.9–5.4, 1.7, $P>0.05$	1.0–7.2, 1.5, $P>0.05$
ORF2-S-DDP[d]	0.3–4.6, 1.9, $P=0.051$	0.9–6.0, 1.7, $P<0.01$
ORF2-S-DPP[d]	1.9–10.5, 3.3, $P<0.01$	2.2–4.9, 2.6, $P<0.01$
NE DNA	1.4–4.5, 2.6, $P<0.01$	1.2–6.1, 3.4, $P<0.01$
NE-S-DDP[d]	0.2–19.4, 7.5, $P<0.05$	1.2–16.9, 6.7, $P<0.01$

[a] All DNA doses were given by gene gun and protein by needle-injection.
[b] Not done.
[c] Mice co-immunized with 1.0 μg/dose of GM-CSF DNA.
[d] DNA prime protein boost.

DNA/rORF2p and NE DNA/rNEp based approaches induced cell mediated immune response in Swiss albino mice.

4. Discussion

A vaccine against HEV could limit the sporadic and epidemic cases occurring in developing countries, decrease the high mortality observed in pregnant female patients and protect travellers from developed nations visiting endemic areas. The observed worldwide distribution and broad host range of HEV, compels it to be labelled as 'emerging pathogen' with a potential to affect developed nations in near future (http://www.who.int/inf-fs/en/fact097.html). In the absence of a cell culture system or laboratory animal model, efforts to develop anti-viral drugs against HEV have been negligible and subunit vaccines based on recombinant DNA technology presently remains the only alternative.

To date, all the epidemiological, animal transmission and vaccine studies of HEV affirmatively indicate ORF2 gene as the appropriate region for vaccine development. Though mouse is not a challenge model for HEV, it offers a very quick system to test any new vaccine approach before conducting experiments in susceptible monkey requiring convincing ethical and scientific explanations for their use. The present study demonstrates that, the plasmid construct pcDNA3.1 + ORF2 induces humoral and cell mediated immune responses in Swiss albino and Balb/c mice by gene gun method. In agreement with studies done before, intramuscular DNA administration in Balb/c mice resulted in poor rate of seroconversion and low specific titers [14,15,19]. The success of naked DNA immunizations is largely dependant on the method of administration, especially in case of higher animals [17]. Our study established the gene gun based DNA immunization to be superior to intramuscular injection in mice. As Swiss albino mice were observed to be a better detection system over Balb/c mice, subsequent experiments were carried out in Swiss albino mice only. Seroconversion rates were dose dependent and with gene gun, dose as low as 10 ng resulted in 62.5% seroconversion 1 week after third dose of ORF2 DNA. Gene gun based administration required 1.0 μg/dose (total three doses) as against 100 μg/dose for intramuscular injection (total four doses).

GM-CSF has been used as a genetic adjuvant with viral DNA vaccines to enhance humoral and cellular immune responses [29]. Co-administration of mouse GM-CSF DNA with ORF2/NE DNA did not offer any significant advantage such as increase in HEV-specific antibody titers or *in vitro* proliferation of splenocytes. Thus, with gene gun method, co-administration of GM-CSF DNA seems to be dispensable in mice

While the experiments with complete ORF2 were under progress, the identification of neutralization epitope/s in a smaller region (150 a.a.) of ORF2 (660 a.a.) [27] was reported. This neutralization epitope/s was observed to be cross-reactive with all mammalian HEV genotypes [30,31]. Considering the advantages such as, possibility of generation of antibodies of higher affinity, use of bacterial system for expression of the NE region as against the baculovirus system for the expression of complete ORF2 (current recombinant protein based vaccine) and simple protein purification procedure, an attempt was made to explore the utility of this region as candidate vaccine. As evident from Tables 1 and 3, NE region was immunogenic in mice generating both humoral and cell mediated immune responses.

Both ORF2 and NE proteins alone were less immunogenic, geometric mean anti-HEV titres being 9.8 ± 1.6 and 4.0 ± 1.4, respectively, whereas in the DNA alone format both regions were found to be better immunogens, ORF2 (GMT = 855.6 ± 1.5) being superior to NE (GMT = 40.0 ± 1.6). However, when the mice were immunized with two DNA and one corresponding protein dose at 0, 4, 8 weeks, the boosting effect was prominent with NE region (GMT = 174.0 ± 3.0), while titres with ORF2 (487.4 ± 2.2) remained comparable to 3 ORF2 DNA doses (833.6 ± 1.3). These results document for the first time possibility of use of a smaller neutralizing epitope containing region as a candidate hepatitis E vaccine and stress evaluation in rhesus monkeys, the challenge model.

The DNA-prime-protein-boost approach has been successfully employed for several viral pathogens [32,33] and was observed to induce memory response. Our study tested two dose regimens (DDP and DPP) for ORF2 in each of short and long dose schedules in mice. The specific antibody titer increased after every dose (total three doses) in ORF2-S-DDP, ORF2-L-DDP and ORF2-L-DPP groups. Immunization of mice with ORF2 DNA at 0, 4, 20 weeks was not tried. A single dose regimen (DDP) for NE in short dose schedule was tested in mice. The NE based DNA-prime-protein-boost approach was observed to be the best as mentioned above. The percent recall response in this mice group was highest over all the other groups assessed for cell mediated immune response. The HEV-specific antibody response generated in mice by all candidate vaccines was detected up to 24 weeks.

Presently, a practical cell culture system or small animal model for HEV replication is not available. Hence, direct evidence for neutralizing capacity of the antibodies generated in immunized mice was not possible. We therefore assessed the binding potential of HEV-specific antibodies in immunized mice sera to HEV in an ELISA based *in vitro* virus binding/neutralization test. Binding of the antibodies generated in mice employing every approach with both ORF2 and NE regions with Hepatitis E virus was clearly shown. The virus-antibody binding completely inhibited the capacity of the antibodies to attach to the capsid ORF2 protein in ELISA. Though simultaneous loss of infectivity could not be shown as rhesus monkeys need to be inoculated with the antibody-virus mixture, the data provides best possible evidence for generation of antibodies being able to bind to the virus. Similar indirect evidences for neutralizing capacity of HEV-specific antibodies generated in previous studies have been reported [34–36].

Though the role of cell mediated immune response in recovery of patients infected with HEV is not well defined, generation of this arm of immune response in mice immunized employing different approaches is noteworthy. Isotyping studies indicated that IgG1 was the predominant isotype irrespective of the approach or the detecting antigen. The observed IgG1 predominance is typical of gene gun based DNA immunization [37] and is suggestive of Th2 pathway [38].

In conclusion, both complete ORF2 and a small neutralizing epitope-containing region were shown to be immunogenic in mice as DNA and DNA-prime-protein-boost vaccines and need to be evaluated in rhesus monkeys, the best available challenge model for HEV.

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