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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

Vidya A. Arankalle[a,*], Kavita S. Lole[a], Tejaswini M. Deshmukh[a], Shubham Srivastava[a], Umesh S. Shaligram[b]

[a] Hepatitis Division, National Institute of Virology, Microbial Containment Complex, Sus Road, Pashan, Pune 411021, India
[b] Serum Institute of India, 212/2 Hadapsar, Pune 411028, India

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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

* Corresponding author. Tel.: +91 20 25871194; fax: +91 20 25871895.
E-mail address: varankalle@yahoo.com (V.A. Arankalle).

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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)

Table 2
Details of immunogens and dose of HEV.

Monkey no.	Dose 1	Dose 2	Dose 3	Dose 4	Challenge/dose (copies/ml)
MM# 201, 202	ORF2-D	ORF2-D	ORF2-D	ORF2-D	NO[a]/NA
MM# 203, 204	ORF2-D	ORF2-D	ORF2 protein	NIL	NO/NA
MM# 205, 206	NE-D	NE-D	NE-D	NE-D	NO[a]/NA
MM# 207, 208	NE-D	NE-D	NE protein	NIL	YES/10^4
MM# 209, 210, 211, 212	NIL	Lipo-ORF2-DP	Lipo-ORF2-DP	NIL	YES/10^4
MM# 213, 214	NIL	Lipo-NE-DP	Lipo-NE-DP	NIL	YES/10^4
MM# 215, 216	NIL	PBS	PBS	NIL	10^4
MM# 217, 218	NIL	NIL	NIL		10^3
MM# 219	NIL	NIL	NIL		10^2
MM# 220	NIL	NIL	NIL		10

NA: not applicable.
[a] Monkeys seroconverted only after 4th DNA dose.

2.4. Titration of challenge virus and challenge of immunized monkeys

The source of the challenge virus (PM2000 strain) was a faecal sample obtained during an epidemic of hepatitis E in 2000 from an acute-phase patient with 10^8 HEV RNA copies/g faeces in Real Time PCR as described below. Two monkeys per group were inoculated intravenously with 1 ml of 10^{-3} (10,000 copies, MM# 215 and 216) and 10^{-4} dilutions (1000 copies, MM# 217 and 218) of the challenge virus. One monkey each was inoculated with 10^{-5} dilution (100 copies, MM# 219) and 10^{-6} dilution (10 copies, MM# 220) of the virus respectively.

The immunized monkeys were challenged 3 weeks after the last dose. MM# 207–214 were challenged intravenously with 1 ml of 10^{-3} dilution of the 10% faecal suspension containing 10^4 copies.

2.5. Monitoring of Rhesus monkeys

Before immunization, all monkeys were bled weekly for 4 weeks for determining base-line levels of serum alanine aminotransferase (ALT) by standard methods. The pre-virus-inoculation ALT value was calculated as the geometric mean of 5 ALT values obtained twice a week just prior to virus challenge. Biochemical evidence of hepatitis was defined as two-fold or greater increase in the post-inoculation/pre-inoculation ratio of ALT. Seroconversion to anti-HEV antibodies/detection of HEV RNA in faeces was considered as evidence of HEV infection. Following HEV challenge all monkeys were bled twice a week for 3 months. Faecal samples were collected on alternate days and stored immediately at −70 °C till used as 10% suspension in PBS.

2.6. Serological assays

For the detection and quantitation of anti-HEV antibodies in mice and monkey serum samples, ELISA was performed as described previously [17], employing ORF2 protein for coating the wells. For the detection and quantitation of anti-NE antibodies, the same protocol was followed except that the coating antigen was replaced with rNE protein. The reciprocal of the highest serum dilution that had an absorbance greater than or equal to the ELISA cut off was taken as the anti-HEV titre. To identify HEV infection in immunized and challenged monkeys, an ELISA using ORF2 protein containing N-terminal 111 amino acids (N-ORF2) was standardized. The immunoreactivity of this protein was shown for the first time by Li et al., [18]. N-terminal segment of ORF2 gene (333 nt, representing N-terminal 111 aa) was cloned in vector pET15b in frame with 5′-His tag, recombinant plasmid was transformed in to *Escherichia coli* strain BL21 (DE3) pLysS and expression of fusion protein was induced by adding 1 mM IPTG for 4 h at 37 °C. The fusion protein was purified from 50 ml bacterial cultures using ProBond resin (Invitrogen, Carlsbad, CA) in buffer system containing 8 M Urea. For coating of the solid phase, 200 ng protein/well was used. Protocol for ELISA was similar to that used for ORF2/NE protein-based assays except that monkey serum was diluted 1:50. Cut off values were calculated as 3 times the mean OD values for 3 pre-inoculation serum samples for all the assays. Samples giving OD values ≥ cut off values were considered reactive for antibodies to the respective antigens.

2.7. Molecular assays

Initially, all the monkey faecal samples were subjected to nested reverse transcription polymerase chain reaction (nested RT-PCR) for the detection of HEV RNA as described earlier [19]. HEV RNA copies in the samples were determined by Taqman RT-PCR assay as follows (7300 Real Time PCR system, Applied Biosystems, CA, USA). A 1067 bp fragment of HEV genome (4632–5698 nt) was PCR amplified and cloned into pGEM-T Easy vector (Promega, Madison, USA). Plasmid was linearised with HindIII, in vitro transcription was done using T7 Riboprobe In Vitro Transcription System (Promega, Madison, USA), quantitated RNA was serially diluted and used as RNA standard. Viral RNA was extracted from samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Primers and probe corresponding to HEV ORF1 genomic region as forward primer 5′ CCGCCTTGCTGTTAGTGACTT 3′; reverse primer 5′ CACACATCTGAGCGACATTCG 3′ and TaqMan minor groove binder (MGB) fluorophore attached probe, FAM 5′ CTCCGCAAGCTC 3′ NFQ, MGB; were used. Standard curve showed linear relationship ($r^2 = 0.99$) from 10 to 10^{10} RNA copies/reaction. The sensitivity of the assay in detecting transcribed HEV RNA was 100 copies/ml.

2.8. Statistical analysis

For comparing anti-HEV titres between groups, *T* test was used. The distribution of viral load was compared by Kolmogorov-Smirnov test. For this, all values for all monkeys in a group were pooled. The statistical analyses were carried out using software SPSS 11.0.

3. Results

3.1. Antibody response in mice

Mice sera from different groups were screened for anti-HEV antibodies by ORF2-based ELISA. The pre-immune sera and the sera of control mice (immunized with Lipo-pVAX) remained antibody negative. Seventy and 90% seroconversion was observed at 2 weeks post 1st dose in Lipo-ORF2-DP and Lipo-NE-DP immunized mice respectively. Reciprocal antibody titres ranged between 40–640 (Lipo-ORF2-DP) and 10–1280 (Lipo-NE-DP) at this time point. Hundred percent mice seroconverted in both the groups

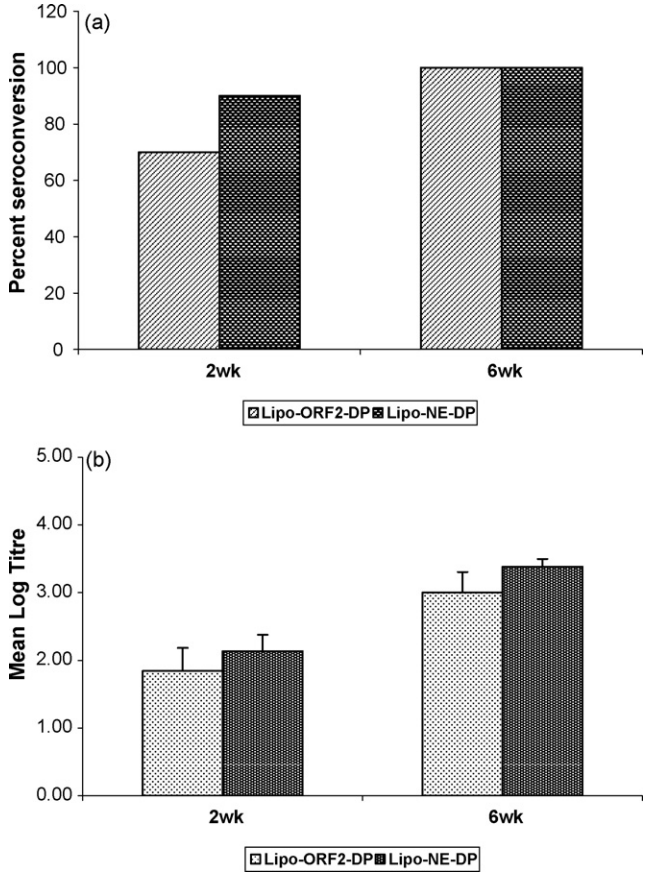

Fig. 1. (a) Percent seroconversion in Swiss albino mice at 2 weeks (2 weeks post dose 1) and 6 weeks (2 weeks post dose 2). Mice groups ($n = 10$/group) were immunized at 0 and 4 weeks with either Lipo-ORF2-DP or Lipo-NE-DP. (b) Serum HEV-specific anti-rORF2p IgG antibody mean log (10) titres detected in ELISA in Swiss albino mice. Mice groups ($n = 10$/group) were immunized at 0 and 4 weeks with either Lipo-ORF2-DP or Lipo-NE-DP. Error bars represent standard error of the mean log titres.

at 2 weeks post 2nd dose (6 weeks) with further increase in the antibody titres to 800–6400 and 800–12,800 respectively (Fig. 1). At both time points anti-HEV titres were comparable in both the groups.

3.2. Titration of the challenge inoculum

As the challenge inoculum was not pre-titrated in Rhesus monkeys, number of HEV RNA copies was determined employing Real Time PCR and was estimated to be 10^7 copies/ml of the 10% stool suspension. Considering Real Time PCR to be ~10-fold more sensitive than infectivity in monkeys, the challenge dose was decided to be 10^{-3}, i.e. 10^4 copies/ml. Fig. 2 depicts dynamics of HEV infection in control, unimmunized monkeys inoculated with different doses of the virus. Both monkeys inoculated with 10^4 (MM# 215 and 216) and 10^3 (MM# 217 and 218) copies each and the only monkey infected with 10^2 copies (MM# 219) showed evidence of HEV infection as indicated by seroconversion to anti-HEV antibodies and excretion of the virus in faeces. Rise in serum ALT (≥two-times the pre-virus-inoculation levels) was recorded for MM# 215, 216, 218 and 219. The monkey (MM# 220) inoculated with 10^{-6} dilution (10 copies/ml) remained IgG-anti-HEV negative. Thus the infectivity titre of the 10% stool suspension was estimated to be 10^6 fifty percent monkey infectious dose (MID_{50}) per gram of faeces and the challenge inoculum contained 100 MID_{50} HEV.

3.3. Anti-HEV response in immunized monkeys

Tables 1 and 2 provide details of the immunogens and different vaccine approaches evaluated. All the pre-immune sera taken prior to the first immunization were negative for IgG-anti-HEV.

3.3.1. Vaccine approaches not yielding encouraging results

None of the monkeys receiving ORF2-DNA, NE-DNA or ORF2-DNA followed by ORF2 protein boost showed the presence of anti-HEV antibodies. As the protocol demanded challenge after the third dose and these monkeys did not seroconvert after the third dose, we took the option of use of fourth DNA dose at 12 weeks for checking seroconversion rather than challenging the monkeys in the absence of anti-HEV antibodies. These monkeys showed weak (anti-HEV titre 1:100) seroconversion at 2 weeks post fourth dose. Thus of the 6 approaches tested in Rhesus monkeys, three approaches including ORF2 and NE DNA alone and ORF2 DNA followed by protein boost were observed to be less effective.

3.3.2. Vaccine approaches evaluated by virus challenge

The monkeys immunized employing NE-DPPB approach did not develop anti-HEV antibodies after two DNA doses. Seroconversion was noted 4 weeks after the protein boost (titres 1:1600 and 800). Of the four monkeys receiving Lipo-ORF2-DP, one seroconverted 3 weeks after the first dose (anti-HEV titre 1:100), all four being anti-HEV positive 1 week after the second dose (anti-HEV titres: 1:100–800). Both the monkeys receiving Lipo-NE-DP seroconverted 3 weeks after the first dose, anti-HEV titres being 800 and 100 respectively. One week after the second dose, the anti-HEV titres rose to 6400 and 800. Thus, for each group, the pattern of serum antibody was similar in all the monkeys, although antibody level was different. As compared to ORF2, anti-HEV titres produced by NE were higher, though statistically insignificant ($p = 0.053$) (Fig. 3).

3.4. Dynamics of HEV infection in control monkeys

Both the monkeys (MM# 215 and 216) infected with 100 MID_{50} challenge virus exhibited moderate rise in serum ALT levels, maximum values being 77 and 42 IU/litre on 38 and 45 days post-inoculation respectively (Fig. 2). Virus excretion as measured by Real Time PCR was evident for 6 weeks, the maximum viral load being 1.5×10^7 and 6.0×10^6 copies/g stool. Seroconversion followed by high titres of anti-HEV antibodies were recorded.

3.5. Assessment of HEV infection in challenged monkeys

Monkeys from NE-DPPB, Lipo-NE-DP and Lipo-ORF2-DP groups were evaluated for protection following challenge. Irrespective of type of immunogen, none of the challenged monkeys exhibited raised ALT levels and were protected from hepatitis (Table 3). Complete protection from infection was offered by Lipo-NE-DP, both animals not excreting the virus. Similarly, one of the monkeys immunized with NE-DPPB (MM# 207) also did not show any evidence of virus replication, HEV RNA being absent in all the faecal samples screened. The other animal (MM# 208) showed reduced excretion for a shorter time. Lipo-ORF2-DP was least effective, all the 4 monkeys excreting the virus for extended period of time. As compared to the control monkeys, overall viral load in faeces was significantly less in monkeys immunized with NE-DPPB ($p < 0.001$); no difference was noted in Lipo-ORF2-DP immunized monkeys ($p > 0.4$). Among NE-immunized groups the difference was non-significant ($p > 0.4$).

Comparison of IgG-anti-HEV titres in the challenged monkeys showed that except two monkeys (MM# 209 and 211) immunized with Lipo-ORF2-DP, all other monkeys exhibited either same or declining antibody titres. Anti-HEV titres in MM# 209 and 211

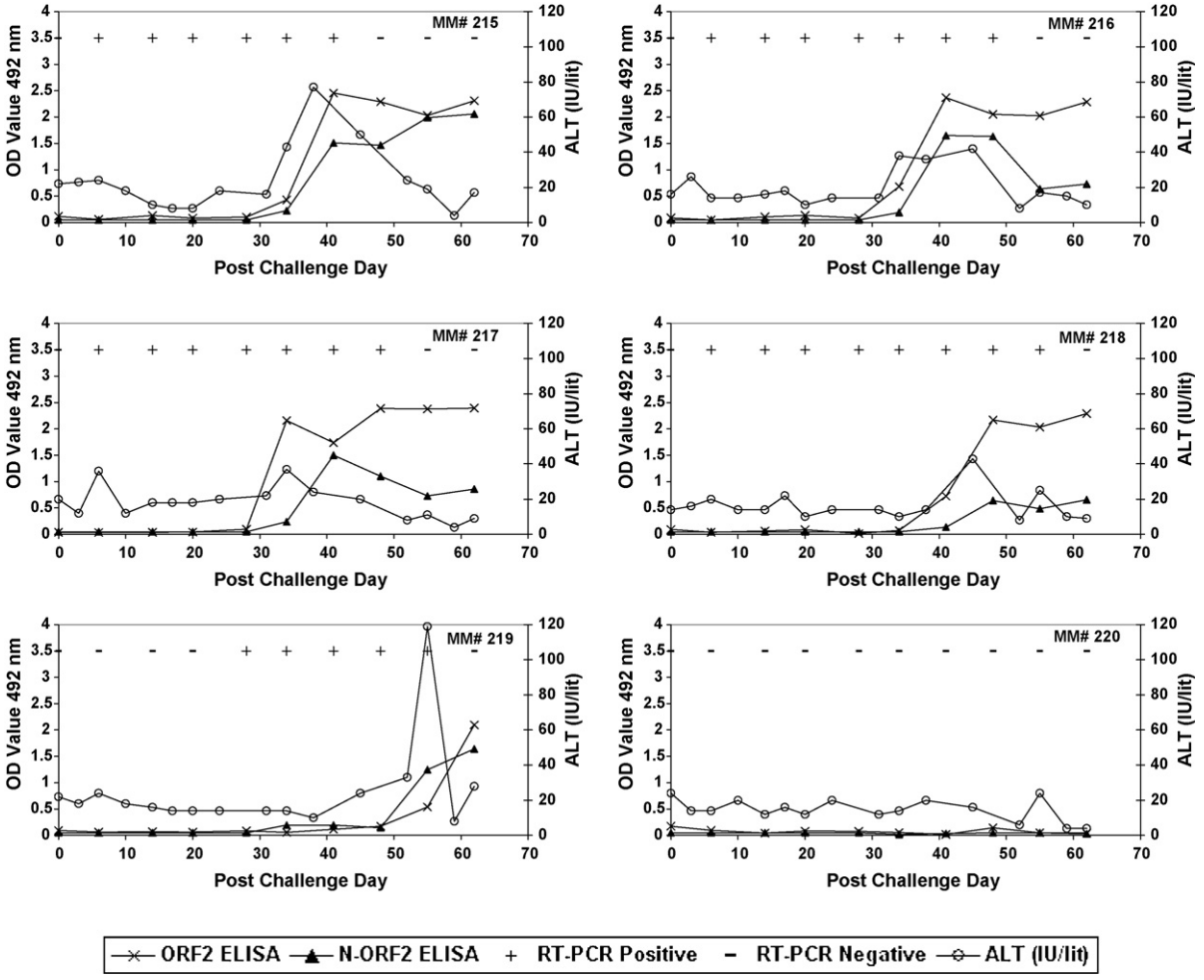

Fig. 2. Determination of MID_{50} titre of the challenge inoculum. On day zero, all the monkeys received different dilutions of HEV intravenously as MM# 215, 216 (10^{-3} dilution), MM# 217, 218 (10^{-4} dilution), MM# 219 (10^{-5} dilution) and MM# 220 (10^{-6} dilution). Open circle (○) shows serum ALT levels. Presence or absence of HEV RNA in faeces is marked by + or − signs. ELISA reactivity of the serum samples is shown as OD values; cross (x) and closed triangle (▲) represent anti-ORF2 and anti-N-ORF2 antibodies respectively.

increased to levels similar to control monkeys strongly suggesting replication of the virus leading to the boosting effect (Fig. 4).

3.6. Anti-N-ORF2 antibodies as indicator of HEV infection in immunized monkeys

Development of anti-N-ORF2 antibodies was used as a marker to differentiate between immunization and infection. Control monkeys experimentally infected with HEV exhibited seroconversion to ORF2 (112–607/660) and N-ORF2 proteins on the same day (Fig. 2). None of the monkeys immunized with NE in different formats developed anti-N-ORF2 antibodies either after immunization or challenge suggesting the absence of infection in these animals. It is interesting to note that all the 4 monkeys immunized with the complete ORF2 DNA and 56 kDa protein encapsulated in liposomes did not develop anti-N-ORF2 antibodies. Immune response

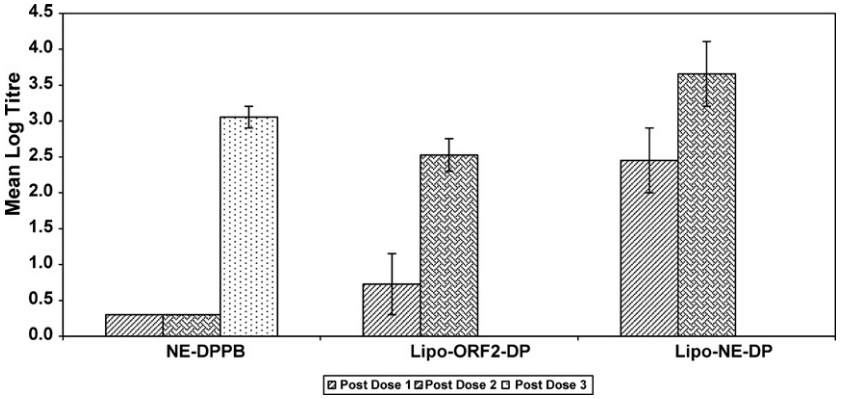

Fig. 3. Anti-HEV titres in monkeys immunized with NE-DPPB (2 monkeys), Lipo-ORF2-DP (4 monkeys), Lipo-NE-DP (2 monkeys). Lipo-ORF2-DP and Lipo-NE-DP groups were immunized with 2 doses while NE-DPPB group with 3 doses. Antibody titres were determined 4 weeks after each dose.

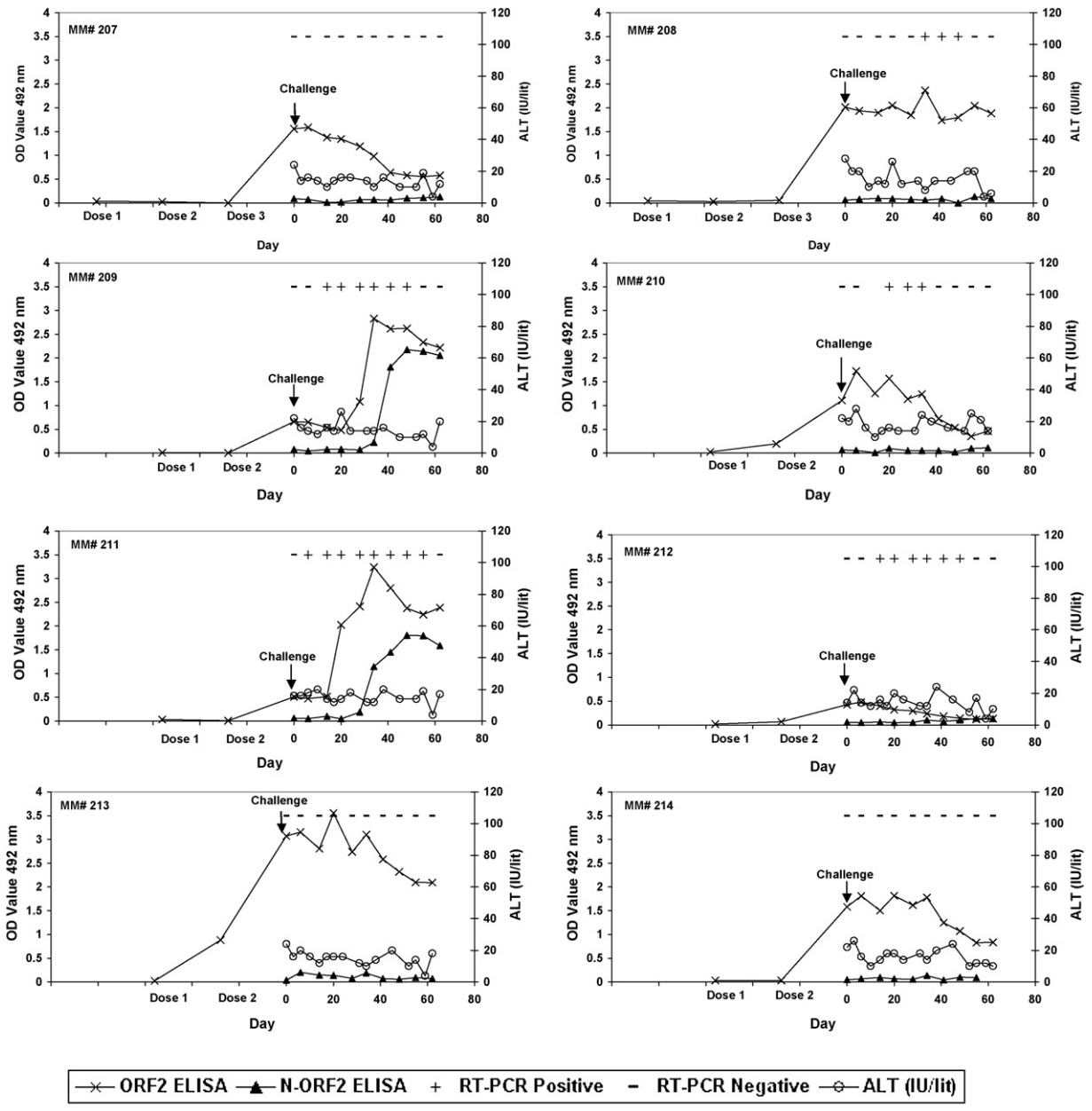

Fig. 4. HEV challenge of monkeys immunized with different preparations. Dose-1, 2 and 3 show schedule of immunizations. Arrow indicates viral challenge of 10^2 MID_{50}. Open circle (○) shows serum ALT levels. Presence or absence of HEV RNA in faeces is marked by + or − signs. ELISA reactivity of the serum samples is shown as OD values; cross (x) and closed triangle (▲) represents anti-ORF2 and anti-N-ORF2 antibodies respectively. MM# 207, 208 were immunized with NE DNA-prime-protein-boost, MM# 209–212 were immunized with ORF2-liposome and MM# 213, 214 received NE-liposome. The dynamics of HEV infection in control monkeys (MM# 215 and 216) inoculated with the same dose of the virus (10^2 MID_{50}) is depicted in Fig. 2.

Table 3
Summary of challenge experiment.

Vaccine type	Monkey no.	Reciprocal anti-HEV titre at challenge	Peak/pre-challenge ratio of ALT values (weeks elevated)	HEV RNA copies in faeces, peak titre/ml in 10% stool suspension (duration in weeks)
NE DPPB	MM# 207	1600	1.0 (0)	Not detected
	MM# 208	800	1.7 (0)	2.2×10^4 (3)
Lipo-ORF2-DP	MM# 209	200	1.1 (0)	2.9×10^6 (6)
	MM# 210	1600	1.09 (0)	1.0×10^4 (3)
	MM# 211	200	1.09 (0)	2.6×10^6 (5)
	MM# 212	200	1.35 (0)	1.0×10^4 (6)
Lipo-NE-DP	MM# 213	12,800	1.06 (0)	Not detected
	MM# 214	1600	1.06 (0)	Not detected
Placebo	MM# 215	<10	3.6 (2)	1.5×10^7 (6)
	MM# 216	<10	2.1 (1)	6.0×10^6 (6)

was mainly targeted against the 112+ protein component. However, two monkeys (MM# 209 and 211) showing >10-fold rise in anti-ORF2 antibodies after challenge (evidence of infection) showed the presence of anti-N-ORF2 antibodies (Fig. 4). Rise in anti-HEV titres and the detection of anti-N-ORF2 antibodies were simultaneous. Anti-N-ORF2 antibodies were not detected in the other two monkeys (MM# 210 and 212) immunized with Lipo-ORF2-DP and not exhibiting rise in anti-ORF2 titres after challenge and all the 4 monkeys immunized with NE in different formats. These results clearly demonstrated that two of the four monkeys immunized with Lipo-ORF2-DP developed HEV infection following challenge whereas all the four monkeys immunized with NE were protected.

3.7. Comparison of anti-HEV titres employing rORF2p and rNEp for ELISA

As anti-NE antibodies were proposed to be the neutralizing antibodies, we compared antibody titres following immunization and infection employing rORF2p and rNEp as coating antigens in ELISA. The titres were similar using both antigens. The anti-NE titres at the time of challenge in NE-immunized monkeys and protected against hepatitis E were 800, 1600, 1600 and 12,800. In ORF2-immunized monkeys, two with anti-NE titres of 1600 and 200 were protected while the other two with the titres of 200 each were infected, though protected from disease. Overall, all the monkeys with anti-NE titres of $\geq$200 were protected from hepatitis. Two of the three monkeys with titres of 200 each showed evidence of infection and extensive virus replication. Of the five monkeys with anti-NE titres >200 ($\geq$800) three exhibited sterilizing immunity while two showed evidence of infection with reduced viral load. Both the control monkeys developed high anti-NE titres (>12,800).

4. Discussion

Open reading frame 2 encoding a protein of 660 amino acids has been the target for vaccine development for hepatitis E employing different approaches, the most successful being the 56 kDa (112–607 aa) recombinant protein expressed in baculovirus expression system [10,14]. Present study documents the utility of the smaller (458–607, 150 aa) NE region containing the putative neutralization epitope [20] as a vaccine candidate. In continuation of mice experiments [15] evaluation of a different concept led to the striking observation of early seroconversion and high anti-HEV titres in mice immunized with two doses of Lipo-ORF2-DP or Lipo-NE-DP (Fig. 1) and prompted us to evaluate this approach in macaques.

We tried both ORF2 and NE regions in three formats, i.e., DNA alone, DNA-prime-protein-boost and encapsulation of DNA and the corresponding protein in liposomes. As DNA vaccine, both ORF2 and NE were not able to mount antibody response when used in 3 doses of 20 μg each with gene gun. An additional dose was required to elicit the antibody response in these monkeys questioning utility of HEV DNA vaccine. As the protocol demanded challenge after the third dose and the monkeys remained anti-HEV negative, we did not challenge them. Therefore, role of cell-mediated immunity in protection against HEV infection could not be evaluated. Kamili et al. [13] have described complete protection of cynomolgus monkeys immunized with 4 doses of full-length ORF2 DNA with the aid of gene gun after challenge with 10,000 MID_{50} of the heterologous challenge. We did observe superiority of gene gun in inducing immune response to the candidate DNA vaccines in mice, though the results were not reproduced in Rhesus monkeys [15].

In the prime-boost approach, ORF2 showed late seroconversion (7 weeks post third dose) and therefore this group was not challenged. For NE, as against 3 DNA doses, DPPB approach was found to induce high titres of anti-HEV antibodies and protection indicating important role of the NE protein in mounting immune response. We did not evaluate NE protein alone as an immunogen as the response was poor in mice [15].

Both ORF2 and NE were immunogenic when administered as Lipo-DP formulations. NE appeared to be a better immunogen, both immunized animals seroconverting after one dose as compared to 1 of the 4 animals immunized with Lipo-ORF2-DP. Though both ORF2 and NE produced comparable anti-HEV titres, their ability to protect following HEV challenge was different. Excellent protection was offered by Lipo-NE-DP as both the animals were protected from the disease as well as infection. Absence of excretion of the virus and anti-N-ORF2 antibodies strongly suggests development of sterilizing immunity in these animals. Though Lipo-ORF2-DP was immunogenic and protected all the animals from hepatitis, 2 of the 4 challenged monkeys showed evidence of HEV infection as indicated by rise in anti-ORF2 antibodies, extensive replication of the virus and development of anti-N-ORF2 antibodies. This study also confirms the findings of Zhou et al. [21] that anti-N-ORF2 antibodies represent a useful serological marker for diagnosis of HEV infection in individuals as well as animals immunized with ORF2-based vaccines and warrants evaluation in an endemic setting.

Though anti-N-ORF2 antibodies were detected during infection with the virus, these antibodies were not detected in monkeys immunized with complete ORF2 DNA in Lipo-ORF2-DP. Whether this reflects modulation of the immune response by the 112–607 protein component of the vaccine remains to be seen.

We would like to point out here that NE was also shown to be a good immunogen in the DPPB format. MM# 207 developed sterilizing immunity whereas MM# 208 showed significantly low viral load, both monkeys being negative for anti-N-ORF2 antibodies.

We used homologous virus (genotype 1) for challenge. However, based on the cross-genotype protection reported so far [22] we tend to believe that the candidate vaccines will offer protection against all the HEV genotypes. Low virus dose (100 MID_{50}) used for challenge reflecting natural exposure led to moderate (77) and marginally high (42) rise in ALT levels in the control animals. Demonstration of protection against severe hepatitis following higher challenge virus dose is essential in subsequent experiments. Our results clearly demonstrate utility of NE in protecting animals against infection, the most important finding of the study.

This study confirms the observations by Zhou et al. [20] that the anti-HEV profiles in experimentally infected monkeys were almost identical with either 112–607 aa or 458–607 aa ORF2 proteins as coating antigens in ELISA. A striking finding was, despite substantial replication of the virus, no ALT rise was observed in two of the challenged monkeys (MM# 209 and 211). We agree with the possibility proposed by Zhang et al. [23] that the extent of liver damage in hepatitis E may not be a direct reflection of virus titre.

In our earlier experiments in mice, ORF2 DNA when administered with gene gun was found to be better immunogen than NE DNA whereas in the DPPB format, boosting effect was prominent with NE protein [15]. Absence of anti-HEV antibodies after 3 ORF2/NE DNA doses, late serocoversion with ORF2 DPPB approach and better boosting effect of NE protein demonstrate partial agreement between results obtained in mice and monkeys.

Zhou et al. [21] have proposed the monitoring of anti-NE antibodies in assessing neutralizing antibody response following immunization. From our study it appears that anti-NE titre as low as 200 may indicate protection against disease. However, these antibodies do not seem to reflect protection from infection. Considering the fact that NE offers complete protection of monkeys, the possibility of non-anti-NE antibody mediated immune response in protection needs to be evaluated.

Though 56 kDa protein vaccine has undergone a successful clinical trial, attempts for the development of better vaccines must

continue. Our experiments provide the possibility of use of a small, easy to purify protein employing bacterial expression system with obvious advantages over the baculovirus expression with multiple purification steps [24]. In the DNA/protein liposome encapsulation format, NE was distinctly superior to ORF2. Comparison of liposome-NE protein with liposome-NE-protein-DNA (shown to be the best formulation in this study using 2 animals) in a larger number of Rhesus monkeys would be the next logical step. Liposome-based candidate vaccines are already in different phases of clinical trial (Clinical Trial Identifier No. NCT00001042 and No. NCT00197301, http://www.ClinicalTrials.gov). The technology used in encapsulation does not need sophisticated instrumentation/protocols and could be easily produced by a vaccine manufacturing company of international standards from a developing country where the disease is endemic.

In conclusion, our results document that both ORF2 and NE DNA were poorly immunogenic in macaques. NE region was highly immunogenic in DPPB as well as DP-liposome formats, 3 of the 4 immunized monkeys developing sterilizing immunity. The formulation generated by the encapsulation of NE DNA and protein in liposome offered best protection and merits further in-depth evaluation.

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