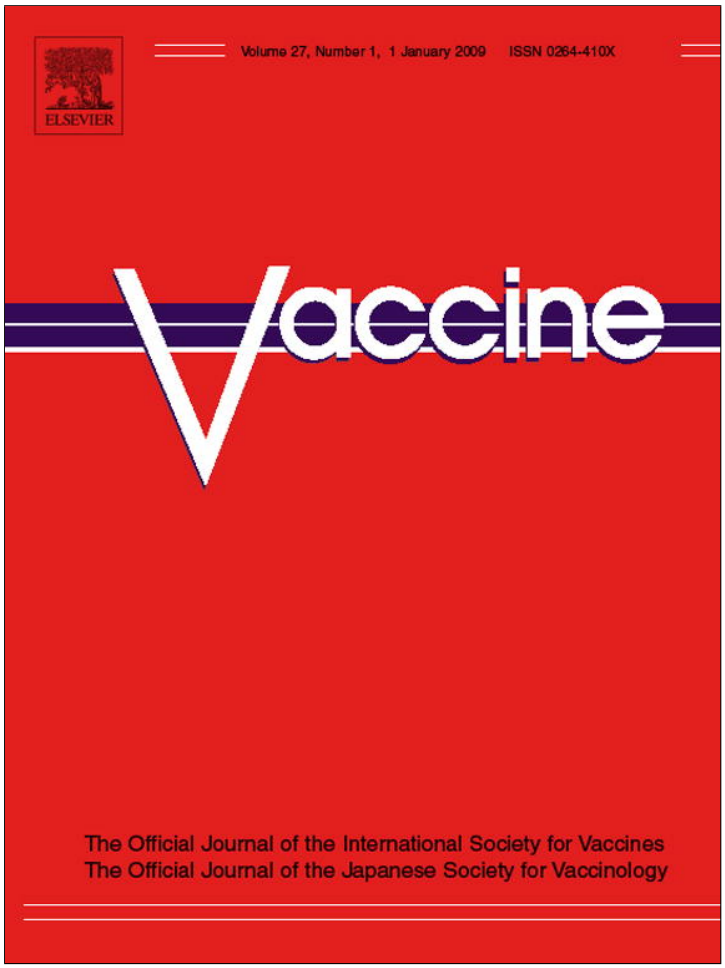

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Evaluation of Japanese encephalitis virus DNA vaccine candidates in rhesus monkeys [*Macaca mulatta*]

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ABSTRACT

We have previously described DNA vaccine candidates against Japanese encephalitis virus (JEV) that were immunogenic in mice. Present study was conducted to evaluate their immunogenicity in rhesus monkeys (*Macaca mulatta*) and compare it with the commercial mouse brain-derived, formalin-inactivated vaccine. Groups of four monkeys were immunized with either pMEa (expressing the anchored form of the envelope protein along with the pre-membrane protein of JEV) or pMEs (expressing the secretory form of the envelope protein along with pre-membrane protein of JEV) by intra-muscular (IM, using needle) or intra-dermal (ID, using gene gun) routes. Following primary immunization with 1 mg plasmid DNA given IM, or 5 μg plasmid DNA given ID, the monkeys were boosted after 1 and 2 months with 0.5 mg DNA given IM or 5 μg DNA given ID, and observed for a period of 6 months. After the second booster, most of the monkeys sero-converted and developed JEV neutralizing antibodies, albeit of low titer. Importantly however, following a sham challenge with the mouse brain-derived inactivated JEV vaccine given 6 months after immunization, the neutralizing antibody titers rose rapidly indicating a vigorous anamnestic response. Based on the JEV neutralizing antibody response following the vaccination and the extent of anamnestic response generated in the immunized monkeys, plasmid pMEa was superior to pMEs. This study indicates that the JEV candidate DNA vaccine is capable of generating protective levels of JEV neutralizing antibodies in rhesus monkeys and prime the immune system effectively against a subsequent exposure to JEV.

1. Introduction

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* family of animal viruses, which includes several viruses of immense medical importance, including those that cause dengue and yellow fever. JEV is responsible for frequent epidemics of Japanese encephalitis fever and it has become endemic in several regions. The virus is distributed through vast geographic areas, including India, China, Japan, and virtually all of South-East Asia. Approximately 3 billion people live in JEV-endemic areas where ~50,000 cases are reported annually, of which approximately 10,000 prove fatal. Moreover, a high proportion of survivors exhibit serious neurologic and psychiatric sequelae [1]. A mouse brain-grown, formalin-inactivated vaccine is manufactured commercially, but it is inherent with certain drawbacks; it is expensive to manufacture, provides immunity of uncertain magnitude and duration, and has been reported to cause allergic reactions, possibly due to the inclusion of murine encephalogenic basic protein or gelatin stabilizer [2,3]. Thus, there is an urgent need to produce an alternate vaccine that may be safer and cheaper. Indeed, several JE vaccine candidates are at various stages of development [4–6].

JEV genome is a single stranded RNA that encodes three structural (capsid, C; pre-membrane, prM; and envelope, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Of these, E protein is most important as it is involved in a number of important biological functions related to virus infection. These include receptor binding and membrane fusion, besides inducing virus-neutralizing antibodies, which alone are considered sufficient for imparting protective immunity against JEV [7,8]. Thus E protein is very important from the vaccine perspective also.

Plasmid DNA-based vaccination strategies have become an active area of research over the past decade due to their potential to produce safer and cheaper vaccines [9]. These vaccines could be delivered to recipients by intra-muscular (IM) injection or intra-dermally (ID) using a gene gun. IM inoculation of plasmid DNA vaccines to mice induces predominantly T helper

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1 (Th1) immune responses, while T helper 2 (Th2) type immune responses are generated following the ID delivery. The isotypes of antibodies and the types of cytokines induced by the two methods differ and this could have bearing on the efficacy of the vaccine candidate.

We have previously described plasmid DNA vaccine candidates against JEV where plasmids capable of synthesizing JEV prM and E proteins were evaluated in mice [10]. Plasmid pMEa synthesized JEV prM and full-length E protein with intact membrane anchor signal (Ea) while pMEs synthesized prM and E protein devoid of the anchor signal and therefore secretory in nature (Es). In mice, Es protein induced mixed Th1–Th2 immune responses whereas Ea protein induced immune responses that were heavily skewed either towards Th1 or Th2, depending upon the route of inoculation. Both forms of E protein however generated JEV neutralizing antibodies that provided significant protection to mice against lethal JEV challenge.

In pre-clinical studies, after evaluation in rodent models, non-human primates are usually the models of choice as they provide a robust system for evaluation of vaccine candidates. The Old World monkeys have predominantly been used in these types of studies [11] with rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys being most popularly used for evaluation of vaccine candidates against various infectious diseases, including those caused by Flaviviruses. In the present report we have compared the immunogenicity of pMEa and pMEs delivered IM or ID in rhesus monkeys. The DNA immunization induced protective levels of JEV neutralizing antibodies and generated robust memory that led to a rapid and sustained anamnestic response in monkeys sham challenged with mouse brain-grown, formaldehyde inactivated commercial vaccine.

2. Materials and methods

2.1. Animals

Twenty female rhesus monkeys (*Macaca mulatta*), aged between 7 and 8 years and weighing between 5.3 and 8.7 kg, were used in the present study. These were housed at the Primate Research Center, National Institute of Immunology, New Delhi. The monkeys were fed on a commercial high-protein monkey diet supplemented with fresh fruits. Filtered drinking water was provided *ad libitum*. The animals were kept indoors in individual cages with artificial lighting (12 h dark/12 h light cycle) and air-conditioning that maintained the ambient temperature at 21–25 °C and 50% humidity. All procedures were carried out after anesthetizing the animals with ketamine hydrochloride (KETMIN® 50, Thermis Medicare Ltd., Gujarat, India) at a dose of 15 mg/kg body weight. None of the animals had known history of prior flavivirus infection. All animals were routinely examined by trained veterinarians and cared for in accordance with the approved guidelines. All procedures were reviewed and approved by the Animal Ethics Committee of the National Institute of Immunology, New Delhi.

2.2. Plasmid constructs

Construction of the pMEa and pMEs plasmids have been previously described elsewhere [10]. Briefly, pMEa encodes prM and anchored E protein of JEV while pMEs encodes prM and secretory E protein. The plasmid DNA was grown in *Escherichia coli* DH5α cells and purified using a plasmid Maxiprep kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

2.3. Immunizations

Monkeys were immunized with the plasmids given IM or ID. For the IM inoculations, 26G needle and 1 ml syringe was used, whereas for the ID inoculations, a hand-held helium-driven Helios gene gun (Bio-Rad, Hercules, CA) was used. The procedure for preparing the cartridges for the gene gun has previously been described [10]. Briefly, plasmid DNA was precipitated onto 1 μm diameter gold particles using calcium chloride. This DNA-gold slurry was coated on the inner surface of Tefzel® (Bio-Rad, Hercules, CA) tubing that was subsequently cut into 0.5 in. cartridges. The DNA-gold particle ratio was adjusted so that each cartridge contained 1 μg plasmid DNA. Recovery of undetectable quantities of DNA from the spent cartridges indicated that the firing efficiency was close to 100%. For each of the ID inoculations using the gene gun, the monkeys were anesthetized and five non-overlapping 'shots' were fired into the shaved abdomens at a helium pressure of 500 psi.

Following the primary immunization, the monkeys were given booster doses 1 and 2 months later. For the IM immunizations, 1 mg DNA was used for the primary dose and 0.5 mg DNA for subsequent booster doses. For the ID immunizations, monkeys received 5 shots (5 μg DNA) each time for primary as well as for booster doses. Monkeys immunized with the commercial JEV vaccine (Central Research Institute, Kasauli, India) received one human dose of mouse brain-derived formalin-inactivated vaccine given IM each time. The monkeys were bled 1 day before the primary or booster immunizations to collect sera for the antibody assays. Two weeks after the second booster, the monkeys were bled to isolate peripheral blood mononuclear cells (PBMCs) for carrying out the enzyme-linked immunospot (ELISPOT) assay. The monkeys were subsequently bled every month for collecting sera for determining the antibody titers. Six months after the second booster, monkeys were given a sham challenge with one human dose of the commercial JEV vaccine given IM and bled thereafter every week for up to a month in order to observe the magnitude and duration of the anamnestic antibody response. The sham challenge was used as the intra-nasal live JEV challenge model in monkeys is inconsistent [12], requires high level of biosafety, and is far from the natural virus exposure to humans, that is intradermal.

2.4. Assay for anti-JEV antibody

Anti-JEV antibody titers were assayed by the end-point enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well ELISA plates (Nunc) were coated overnight at 4 °C with porcine stable kidney cell-grown JaOAr strain of JEV (100 μl/well, virus titer 2×10^8 PFU/ml) in carbonate–bicarbonate buffer (45.3 mM $NaHCO_3$, 18.2 mM Na_2CO_3, pH 9.6). The wells were blocked next day by incubating the plate with 1% fat-free milk in wash buffer (PBS + 0.05% Tween-20) at 37 °C for 2 h. The plates were washed thrice with wash buffer before adding 100 μl diluted monkey serum per well. The plates were incubated at 37 °C for 1 h and washed thrice with wash buffer. This was followed by the addition of 100 μl diluted anti-monkey immunoglobulin – horseradish peroxidase conjugate (Sigma) to each well and incubation for 1 h at 37 °C. After washing plates thrice with wash buffer, colour was developed by adding the substrate solution of orthophenylene diamine (0.5 mg/ml in phosphate-citrate buffer containing 0.052 M citric acid, 0.102 M disodium hydrogen orthophosphate, pH 5.0, and 0.1% hydrogen peroxide) at room temperature for 10 min. The reaction was stopped by adding 50 μl 5N H_2SO_4. Absorbance was determined at 492 nm on a microplate reader (SpectraMax, Molecular Devices, USA). A negative control well had all reagents except the serum. Twofold serial

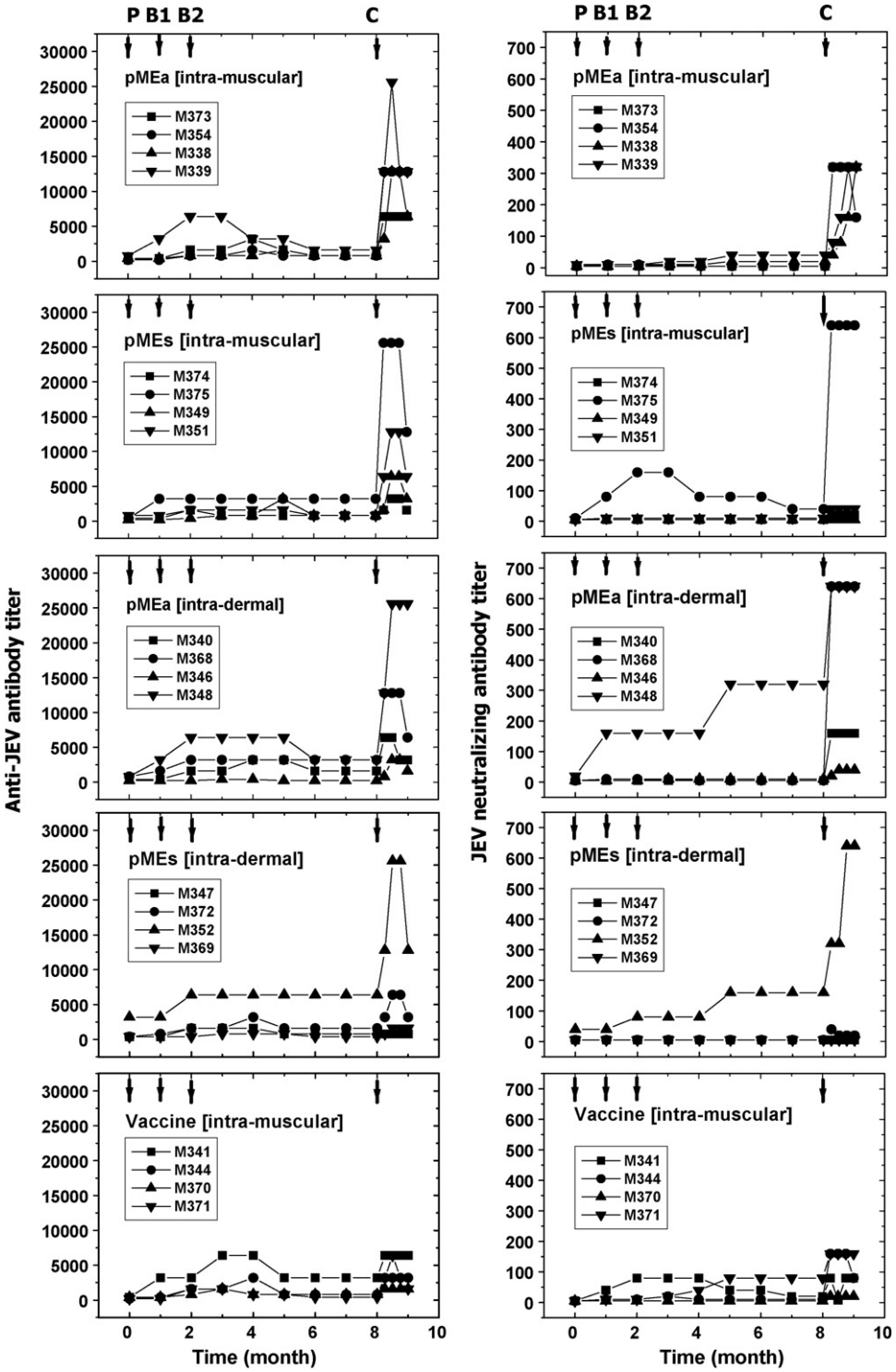

Fig. 1. Antibody response in monkeys immunized with JEV DNA vaccine candidates. Groups of 4 monkeys each were immunized with pMEa or pMEs by IM or ID routes. Another group of 4 monkeys was immunized with formalin-inactivated commercial vaccine. Booster doses, B1 and B2 were given 1 and 2 months after the primary (P) immunization, respectively. Blood samples were collected every month and assayed for JEV antibodies. Monkeys were sham challenged (C) with commercial vaccine 6 months after the second booster dose. Blood samples were collected every week for next 4 weeks and assayed for JEV antibodies. Panels on left show JEV antibody titers determined by ELISA, while that on the right show JEV neutralizing antibody titers. Primary, booster and challenge doses are indicated with arrows. The pre-immunization JEV antibody ELISA titers in all monkeys were ≤800 (base line cut off) except for M352 where it was 3200. The pre-immunization neutralizing antibody titer was ≤10 (base line cut off) in all monkeys except for M352 where it was 40.

dilutions of monkey sera starting at 1:100 were tested. Reciprocal of the highest serum dilution giving an absorbance twice that of the negative control was taken as the anti-JEV antibody titer.

2.5. Assay for JEV neutralizing antibody

Neutralizing antibodies to JEV were measured using the plaque-reduction neutralization assay [13] with a 50% plaque-reduction

endpoint ($PRNT_{50}$). Briefly, the diluted serum samples (100 μl) were heat inactivated and mixed with an equal volume of JEV culture supernatant containing ~100 PFU of the virus. The serum–virus mixture was incubated at 37 °C for 1 h before being added to 6-well tissue culture plates containing ~70% confluent monolayer of porcine stable kidney cells for the plaque development. The percentage of neutralization was calculated from the number of plaques in the presence and in the absence of monkey serum. Twofold serial dilutions of monkey sera starting at 1:10 were tested. The reciprocal of the highest serum dilution that gave at least 50% neutralization was taken as the neutralization titer.

2.6. Assay of IFN-γ and IL-4 secreting cells by ELISPOT

The enzyme-linked immunospot (ELISPOT) assay, originally described for the detection of single B cells secreting antibody [14] was used in the present study for the detection of cells secreting the cytokines interferon gamma (IFN-γ) and interleukin 4 (IL-4). The monkey IFN-γ/IL-4 ELISPOT kits (U-CyTech Biosciences, Utrecht, The Netherlands) were used in the present study in accordance with the manufacturer's instructions. Briefly, two 96-well ELISPOT plates, one each for IFN-γ and IL-4, respectively, were coated with the coating antibodies diluted in PBS, by incubating overnight at 4 °C. The plates were subsequently washed with wash buffer and blocked for 2 h at room temperature with 1% bovine serum albumin. PBMCs were prepared from heparinized blood from the immunized monkeys by Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation and the cell density adjusted to 2×10^6 cells/ml in RPMI-10 (RPMI-1640 supplemented with 10% FCS and antibiotics). The PBMCs were stimulated with *E. coli*-expressed JEV E protein (50 μg/ml). For the positive control, stimulation was carried out by concanavalin A (5 μg/ml) and for the negative control, the cells were simply incubated with RPMI. Stimulation was carried out by incubating for 2 h at 37 °C in round bottom 5 ml snap-cap tubes. The stimulated PBMCs (1×10^5) were added to each well of the coated ELISPOT plates. The plates were covered and incubated at 37 °C/5% CO_2 for 48 h. After incubation, the supernatant from the wells was removed and 200 μl chilled distilled water added to each well. The plates were placed on ice-water mix for 10 min and then washed 10 times with wash buffer. This was followed by the addition of 100 μl diluted biotinylated antibodies to each well and incubation at 37 °C for 1 h. The plates were then washed five times with wash buffer and 50 μl of diluted Φ-labeled anti-biotin antibodies (GABA) were added to each well followed by incubation at 37 °C for 1 h. The plates were washed again five times with wash buffer. This was followed by addition of 30 μl freshly prepared Activator I/II solution to each well and incubation at room temperature in dark for spot development. After the spots had developed, the reaction was stopped by rinsing the plates with water. The plates were air-dried and the spots were counted manually in a KS ELISPOT reader (Carl Zeiss, Göttingen, Germany). All assays were also performed on PBMCs of the same monkey without any stimulation to find out the number of the non-specific spots. These numbers were subtracted from the number of spots obtained in presence of JEV E protein. Each assay was performed in triplicate to calculate the mean.

2.7. Statistical analysis

Pre-challenge and post-challenge geometrical mean titers (GMTs) were compared to find the fold-difference in titers among different monkey groups. A fourfold or higher difference in titers was considered significant. The statistical significance of different findings between the groups was determined by Student's *t* test.

3. Results

3.1. Safety aspects

There was no obvious manifestation of local or systemic reactions in any of the monkeys inoculated with the plasmid pMEa or pMEs by IM or ID routes during the entire period of study. None of the monkeys exhibited any skin rashes at the injection sites, or any abrupt change in body weight or appetite, indicating safety of the DNA vaccine candidate. Moreover, no deaths occurred during the entire span of the study.

3.2. Anti-JEV antibody titers

Plasmids pMEa and pMEs were given IM or ID as per the protocol described in the methods and immune responses induced by them were compared with those induced by the commercial vaccine (Fig. 1). In each of the immunization groups only 1 out of 4 monkeys seroconverted after the primary dose, except for pMEa ID group where 2 out of 4 monkeys had responded. However, all monkeys seroconverted following the complete immunization protocol that included a primary dose followed by two booster doses.

The geometric mean titer (GMT) of anti-JEV antibodies in monkeys immunized IM with pMEa was 1600 ± 2693 which dipped

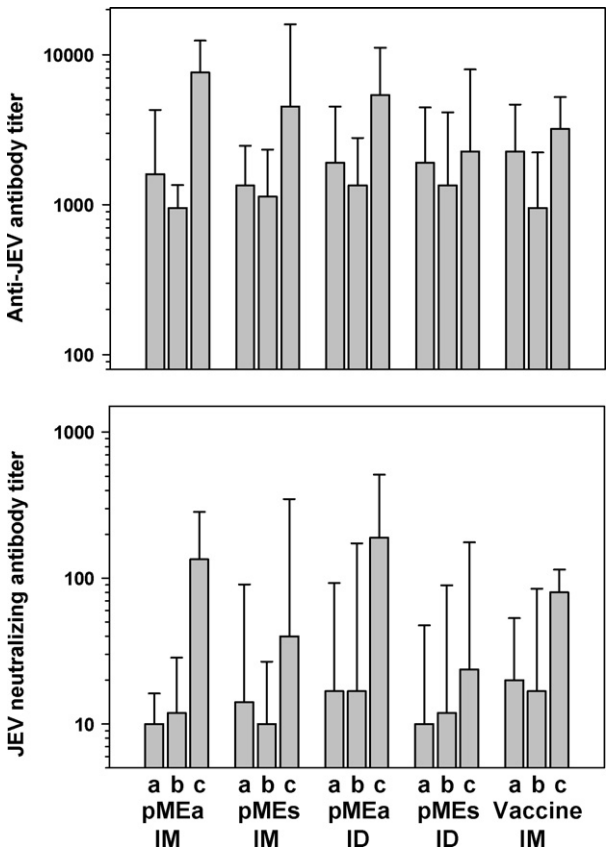

Fig. 2. Anamnestic antibody response in monkeys immunized with JEV DNA vaccine candidates. Shown above are geometric mean titers in various immunization groups of monkeys 1 month after the completion of the immunization protocol (a), 1 day before the challenge (b) and 1 week after the challenge (c). Error bars show the standard deviation. The top panel shows JEV antibody titers determined by ELISA, while the bottom one shows JEV neutralizing antibody titers.

Table 1
Anamnestic antibody response in monkeys following the sham challenge.

Immunogen and Route	Monkey number	Anti-JEV antibody titer					JEV neutralizing antibody titer				
		Pre-challenge	Post-challenge				Pre-challenge	Post-challenge			
			Week 1	Week 2	Week 3	Week 4		Week 1	Week 2	Week 3	Week 4
	373	800	6,400	6,400	6,400	6,400	<10	320	320	320	320
	354	800	12,800	12,800	12,800	12,800	<10	320	320	320	160
pMEa [IM]	338	800	3,200	12,800	12,800	6,400	20	40	80	160	320
	339	1600	12,800	25,600	12,800	12,800	40	80	160	320	320
	GMT	951	7,610	12,800	10,763	9,050	11.8	134.5	190.2	269.0	269.0
	374	800	1,600	3,200	3,200	1,600	<10	20	20	20	20
	375	3200	25,600	25,600	25,600	1,2800	40	640	640	640	640
pMEs [IM]	349	800	1,600	6,400	6,400	3,200	<10	<10	<10	<10	<10
	351	800	6,400	12,800	12,800	6,400	10	40	40	40	40
	GMT	1131	4,525	5,381	9,050	4,525	10.0	40.0	40.0	40.0	40.0
	340	1600	6,400	6,400	3,200	3,200	<10	160	160	160	160
	368	3200	12,800	12,800	12,800	6,400	<10	640	640	640	640
pMEa [ID]	346	200	800	3,200	3,200	1,600	10	20	40	40	40
	348	3200	12,800	25,600	25,600	25,600	320	640	640	640	640
	GMT	1345	5,381	9,050	7,610	5,381	16.8	190.2	226.2	226.2	226.2
	347	800	800	800	800	800	<10	<10	<10	<10	<10
	372	1600	3,200	6,400	6,400	3,200	<10	40	20	20	20
pMEs [ID]	352	6400	12,800	25,600	25,600	1,2800	160	320	320	640	640
	369	400	800	1,600	1,600	1,600	<10	<10	<10	<10	<10
	GMT	1345	2,262	3,805	3,805	2,690	11.8	23.7	20	23.7	23.7
	341	3200	6,400	6,400	6,400	6,400	20	80	80	80	80
	344	800	3,200	3,200	3,200	3,200	10	160	160	160	80
Vaccine [IM]	370	800	1,600	1,600	1,600	1,600	<10	20	20	20	20
	371	400	3,200	6,400	3,200	1,600	80	160	160	160	160
	GMT	951	3,200	3,805	3,200	2,690	16.8	80.0	80.0	80.0	67.2

Immunized monkeys were sham challenged with formalin-inactivated commercial vaccine given IM. Blood was collected a day before the challenge and every week following the challenge for 4 weeks, and assayed for anti-JEV antibody titers by ELISA and JEV neutralizing antibody titers. Titers in individual monkeys as well as geometric mean titers (GMTs) of all immunization groups have been given. For the GMT calculation, titer of <10 was taken as 5.

to 951 ± 400 over the next 6 months. The sham challenge at this stage raised the anti-JEV antibody titers in these monkeys to 7610 ± 4800 within a week (Fig. 2, Table 1). This resulted in eightfold increase in antibody titers ($p = 0.01$). The GMT of pMEs IM-immunized monkeys after the two booster doses was 1345 ± 1131 which dropped marginally to 1131 ± 1200 over the 6-month period before challenge. Following the challenge the antibody titer rose to 4525 ± 11426 within a week, resulting in a fourfold enhancement of antibody titer ($p = 0.25$). The antibody titer in pMEa ID-immunized monkeys following the two booster doses was 1902 ± 2600. This dropped to 1345 ± 1445 at the time of challenge, following which titers rose fourfold to 5381 ± 5782 ($p = 0.08$). The pMEs ID-immunized monkeys had a GMT of 1902 ± 2561 following the complete immunization, and these dropped to 1345 ± 2778 over the 6-month period. The challenge led to only a marginal increase of 1.7-fold in titer ($p = 0.53$). In the case of the vaccine-immunized monkeys, the GMT following the two booster doses was 2262 ± 2400 which dropped to 951 ± 1280 over the 6-month period. The challenge at this stage enhanced the anti-JEV antibody titers by 3.4-fold to 3200 ± 2013 ($p = 0.10$).

Thus, following the challenge, pMEa IM- or ID-immunized, and pMEs IM-immunized monkeys showed significantly enhanced JEV antibody titers whereas this difference was not significant in case of pMEs ID-immunized or commercial vaccine-immunized monkeys.

3.3. Neutralizing antibody titers

Following the complete immunization schedule, 3 out of 4 pMEa IM-immunized, 2 out of 4 pMEs IM-immunized, 3 out of 4 pMEa ID-immunized, 1 out of 4 pMEs ID-immunized and 3 out of 4 vaccine-immunized monkeys developed JEV neutralizing antibodies. Most of the plasmid DNA immunized monkeys, whether by IM or ID route, developed low titers of JEV neutralizing antibodies. However, following the sham challenge with mouse brain-derived formalin-inactivated commercial vaccine, the majority of the monkeys responded remarkably well, exhibiting a rapid and sustained anamnestic neutralizing antibody response (Fig. 1, Table 1). Thus pMEa IM-immunized monkeys had a GMT of 11.8 ± 16.5 before the challenge. The JEV neutralization titer rose by ~11-fold to a GMT of 134.5 ± 151.0 in 1 week following the challenge ($p = 0.06$). In the case of the pMEs IM-immunized monkeys the pre-challenge GMT of 10.0 ± 16.8 was enhanced by fourfold to 40.0 ± 309.5 within a week of challenge ($p = 0.34$). In case of pMEa ID-immunized monkeys, the JEV neutralizing antibody GMT went up by ~11-fold, thus a pre-challenge GMT of 16.8 ± 75.8 rose to post-challenge GMT of 190.2 ± 322.6 within a week ($p = 0.17$). There was very little enhancement (~2-fold) of JEV neutralizing antibody titers following the challenge in pMEs ID-immunized monkeys ($p = 0.59$). In the case of the vaccine-immunized monkeys, the pre-challenge GMT of 16.8 ± 34.7 rose to 80 ± 68.0 post-challenge, resulting in ~5-fold enhancement in titers ($p = 0.09$).

Thus, following the challenge, pMEa IM- or ID-immunized, and pMEs IM- or commercial vaccine-immunized monkeys showed significantly enhanced JEV neutralizing antibody titers whereas this difference was not significant in case of pMEs ID-immunized monkeys.

3.4. Secretion of IFN-γ and IL-4 by monkey PBMCs

ELISPOT assay was carried out to enumerate monkey PBMCs that secreted either IFN-γ or IL-4, following stimulation with *E. coli*-synthesized JEV E protein. Concanavalin A was used as the positive control for stimulating the PBMCs. The results are expressed as the number of spot forming cells (SFC) for every 1×10^5 input cells per

Table 2
Cytokine profile of monkeys immunized with JEV DNA vaccine candidates.

Immunogen and route	Monkey number	IFN-γ		IL-4		IL-4/IFN-γ SFC ratio	Neutralizing antibody titer		
		Mean SFC/100,000 cells		Mean SFC/100,000 cells			Pre-challenge	Post-challenge	Fold increase
		Con A	E protein	Con A	E protein				
pMEa [IM]	373	1643	239	639	406	1.69	<10	320	64
	354	786	2	321	30	15.0	<10	320	64
pMEa [ID]	340	1278	9	489	28	3.11	<10	160	32
	368	1535	4	690	62	15.5	<10	640	64
pMEs [IM]	374	1114	8	524	11	1.37	<10	20	4
	375	380	8	628	170	21.2	40	640	16
pMEs [ID]	347	1850	107	762	52	0.48	<10	<10	1
	372	1259	14	717	22	1.57	<10	20	4
Vaccine [IM]	341	654	114	368	58	0.50	20	80	4
	344	951	12	395	9	0.75	10	160	16

IFN-γ and IL-4 secreting cells from monkey PBMCs were enumerated by ELISPOT assays after incubation with *E. coli*-synthesized JEV E protein. Mean of spot forming cells (SFCs) from three assays was obtained after subtracting the number of background SFCs in the absence of stimulation. Based on the preponderance of IFN-γ or IL-4 secreting cells, immune response was considered as Th1 or Th2, respectively. For calculating the fold increase of JEV neutralizing antibody titer following the challenge, titer of <10 was taken as 5.

well in Table 2. These results show that vaccine-immunized monkeys had a higher number of IFN-γ secreting PBMCs than those secreting IL-4, suggesting a Th1 dominated immune response. However, majority of plasmid-immunized monkeys showed Th2 dominated immune response except monkey M347 where immune response was of Th1 kind. Consistent with these observations, monkeys with Th2 response showed generally a greater enhancement of antibody titers following the challenge.

4. Discussion

Several JEV proteins have been evaluated in the DNA vaccine modality for their ability to induce protective immunity in the murine system. These include plasmids expressing NS1 [15], E [16–18] or prM and E [10,19–21] proteins of JEV. These have shown variable degree of efficacy in murine model systems with plasmids expressing prM and E conferring high degree of protection of mice against lethal JEV challenge, largely due to their ability to induce JEV neutralizing antibodies. We previously described two plasmids which expressed prM protein along with full-length anchored E or truncated secretory E protein. While both plasmids induced JEV neutralizing antibodies in mice, the quality of immune response in terms of Th1 or Th2 immune responses was different for the two proteins. We found that secretory E protein (Es) induced mixed Th1–Th2 immune responses while anchored protein (Ea) induced immune responses that were heavily skewed either towards Th1 or Th2, depending upon the route of inoculation. Thus, Ea induced distinctly Th2 response when plasmid was delivered ID using gene gun whereas it was Th1 following the IM immunization [10]. Based on the ratio of IFN-γ and IL-4 secreting cells in ELISPOT assays, predominant Th2 immune response was observed in all plasmid DNA-immunized monkeys in the present study, except monkey M347. This was consistent with the observation that both plasmids induced JEV neutralizing antibodies in all monkeys except M347. The monkey M347 showing Th1 immune response in ELISPOT assay, failed to show any increase in antibody titers following the sham challenge. While JEV neutralizing antibodies alone are considered sufficient for protection, Th1 immune responses against JEV may also be protective [22]. Indeed, plasmid pCMXEN-immunized mice showing Th1 immune responses were protected against JEV challenge in the absence of any detectable virus neutralizing antibodies [16]. It is thus possible that plasmid immunization inducing Th1 and/or Th2 immune responses in monkeys against JEV have protective value.

All monkeys that received plasmid DNA or the commercial vaccine made anti-JEV antibodies. Thus, after the second booster the mean anti-JEV antibody titers in plasmid-immunized monkeys ranged between 1345 and 1902. Although these were lower than the mean titer of 2262 in vaccine-immunized monkeys, the differences were statistically not significant (p values ranged between 0.40 and 0.96). Importantly, most of the monkeys developed JEV neutralizing antibodies, albeit of low titers. The GMTs of JEV neutralizing antibodies in plasmid-immunized monkeys ranged between 10 and 17 and these did not differ significantly from the GMT of 20 reached in vaccine-immunized monkeys (p values ranged between 0.23 and 0.77). Although low in titers, JEV neutralization antibodies induced in monkeys by plasmid immunization may be protective as neutralizing titer of 1:10 or more is accepted as evidence of protection against JEV according to World Health Organization [1,23]. Significantly, following the sham challenge, there was a rapid rise in both the total as well as the neutralizing antibody titers in most of the monkeys. The mean anti-JEV antibody titers in plasmid-immunized monkeys ranged between 2262 and 7610 compared to the mean titer of 3200 in vaccine-immunized monkeys. The differences, however, were not statistically significant (p values ranged between 0.09 and 0.80). The mean neutralizing antibody titers also, that ranged between 23.7 and 190.2 in plasmid-immunized monkeys, were statistically not different from the mean titer of 80.0 in vaccine-immunized monkeys (p values ranged between 0.17 and 0.67). Importantly, there was vigorous anamnestic neutralizing antibody response following the sham challenge. Thus, pMEa-immunized monkeys showed ~11-fold enhancement in JEV neutralization titer compared to ~5-fold enhancement in the case of vaccine-immunized monkeys. However, differences in the post-challenge titers in the two groups of monkeys were statistically not significant ($p = 0.34$).

Following the primary immunization with the commercial vaccine, only 1 out of 4 animals showed seroconversion whereas 13 out of 16 plasmid-immunized monkeys showed vigorous antibody rise following the sham challenge with the commercial vaccine. Importantly, the rise in titers following the challenge was recorded within the first week and it was sustained at least till 4 weeks post-challenge when the experiment was terminated. These findings show the generation of a robust anamnestic neutralizing antibody response and clearly indicate that JEV DNA vaccine was able to efficiently prime the immune system, which led to generation of long-lived memory B cells. This is significant since earlier studies have documented that anamnestic neutralizing antibody response is critical for conferring protection against a lethal JEV challenge in the mouse model [24].

Compared with our previous studies in mice, wide individual differences in the immune responsiveness of the monkeys within

each group were evident. This was however anticipated as monkeys used in these studies were outbred and had varying genetic background. Others have made similar observations on monkey immunogenicity using different candidate vaccines. For example, out of the 3 cynomolgus monkeys that were immunized with 3 doses of pNJEME construct, only 2 developed neutralizing antibodies detectable in a $PRNT_{70}$ assay but none in a $PRNT_{90}$ assay [12]. In another study where a dengue type 1 DNA vaccine candidate was being evaluated [25], three Aotus monkeys immunized three times IM with plasmid DNA, developed neutralizing antibody titers of <10, 40 and 160 in a $PRNT_{50}$ assay. In yet another dengue type 1 DNA vaccine study [26], two of eight rhesus monkeys immunized three times IM with plasmid DNA did not develop any neutralizing antibodies, although the remaining six monkeys developed neutralizing antibodies with $PRNT_{50}$ titers in the range of 20–320.

In the present study, 3 out of 4 pMEa IM- or ID-immunized, 2 out of 4 pMEs IM-immunized, 1 out of 4 pMEs ID-immunized and 3 out of 4 vaccine-immunized monkeys made detectable JEV neutralizing antibodies. However, all monkeys immunized with pMEa IM or ID, or with commercial vaccine showed vigorous anamnestic response, whereas only 3 out of 4 pMEs IM-immunized and 2 out of 4 pMEs ID-immunized monkeys had anamnestic response. These data thus indicate that pMEa is superior to pMEs for inducing anti JEV immune response in monkeys and the difference in immune responses induced by pMEa and the commercial vaccine were not statistically significant.

A 3-dose regimen for the mouse brain JE vaccine is currently recommended [27] as the 2-dose regimen failed to provide complete immunization in ~20% of subjects [28]. The JE DNA vaccine candidates tested here generated JEV neutralizing antibody titers in monkeys following 3 doses. A safe and cheap JE vaccine requiring fewer doses that could be given at the same time as other paediatric vaccines would be most desirable. To achieve this, efforts are underway to further enhance the immunogenicity of JE DNA candidate vaccine.

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