Effect of plasmid size on the immune response to Japanese encephalitis virus DNA vaccine in mice

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ABSTRACT

Background: The envelope (E) protein, along with the pre-membrane (prM) protein of the Japanese encephalitis virus (JEV), are important mediators of humoral immunity against the virus. *Objective:* To establish whether the plasmid size could influence the immunogenicity of this DNA vaccine candidate in terms of antibody inductive capacity. *Methods:* The prM and E genes of JEV were cloned in pCEP4 and pCDNA to produce the constructs pMEa and pcMEa, respectively. The former construct was approximately double the size of the latter. The plasmid constructs were used to immunize mice, both in terms of equal weight, as well as equal number of molecules. *Results:* The antibody-inductive capacity (both anti-JEV and neutralizing antibodies) of pCDNA was superior to that of the pCEP4. Importantly, the mice challenge studies indicated that the higher titer of neutralizing antibodies of the pCDNA group lead to higher survival in this group. *Conclusion:* A smaller plasmid would be better for preparing a DNA vaccine construct.

Keywords: Plasmids, Japanese encephalitis virus, DNA vaccine.

INTRODUCTION

Plasmid DNA-based vaccination approaches have emerged as a promising strategy over traditional modes of immunization.[1] A DNA vaccine candidate has previously been evaluated against JEV in mice[2] and rhesus monkeys.[3] Since DNA vaccines need to be endocytosed by antigen-presenting cells (APCs) before processing, the size of the plasmid used in the construct could influence the efficiency of uptake, thereby affecting the magnitude of the immune response. It is likely that the size of the plasmid could also influence the number of recombinant plasmid molecules being administered in a single shot, thereby underscoring its importance from a process-economics standpoint.

The plasmids pCDNA3.1 and pCEP4 (Invitrogen, Carlsbad, California, USA) were used for making the constructs. pCDNA3.1 (5.4 kb) is roughly half the size of pCEP4 (10.2 kb). Both plasmids have similar features with respect to the multiple cloning site (MCS), the mammalian CMV promoter, and the ampicillin resistance gene. The plasmids pCDNA[4] and pCEP4[5] have been widely used in various DNA vaccine formulations. The same plasmid (pSL301; Invitrogen, Carlsbad, California, USA) expressing different antigens has been compared for efficacy in a single DNA vaccine formulation.[6] Interestingly, here, two different plasmids (pCDNA and pCEP4) have been evaluated that differ in size but incorporate the same antigen for antibody-inductive potency.

MATERIALS AND METHODS

Plasmid Constructs

The plasmid constructs were prepared from cDNA synthesized by reverse transcription of viral genomic RNA isolated from the culture supernatant of JEV GP78-infected porcine stable kidney (PS) cells using synthetic oligonucleotides,

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as described elsewhere.[7] The resulting polymerase chain reaction (PCR) product, containing engineered *Bam*H1 and *Kpn*1 sites was cloned into the eukaryotic expression vectors, pCDNA and pCEP4 (Invitrogen, Carlsbad, California, USA) to yield the plasmid constructs pcMEa and pMEa, respectively (capable of synthesizing prM and Ea proteins). The plasmid DNA was purified from transformed *Escherichia coli* DH5α cells by using the plasmid Maxiprep kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Mouse Immunization and Challenge with JEV

The study was approved by the Institutional Animal Ethics Committee. All immunizations were carried out on 4 week-old inbred Balb/c mice, divided into 6 groups (A-F) (n=6 per group). Six mice were taken in the control group. Groups A-E were injected with plasmid DNA. Group A and B received 100 and 58 μg of pMEa, respectively. Groups C-E received 100, 58, and 38 μg of pcMEa, respectively. Therefore, on the one hand, groups (A and C), and (B and D) received equal amounts (100 and 58 μg) of pMEa and pcMEa, respectively. On the other, groups (A and D), and (B and E) received equal number of molecules of the two plasmids, *i.e.,* 100 μg pMEa as against 58 μg pcMEa in the former case, and 58 μg pMEa as against 38 μg pcMEa in the latter. Group F received one-tenth of the adult human dose of a commercially available JEV vaccine (Central Research Institute, Kasauli, India). The control group did not receive any treatment (Table 1). Two boosters were

Table 1: Comparison of pMEa and pcMEa constructs used for immunization

Group	pMEa (µg)	Group	pcMEa (µg)	Comments
A	100	C	100	Equal weight
B	58	D	58	Equal weight
A	100	D	58	Equal molecules
B	58	E	38	Equal molecules

given 3 weeks and 5 weeks after the primary immunization. At 6 weeks after the primary immunization, the mice were challenged intracerebrally (IC) with a suprarenal dose (100 LD_{50}) of the Vellore strain JEV and observed for 4 weeks.

Assay for Anti-JEV Antibody

The mice were bled one day before the primary or boosters to collect sera for the antibody assays. Two-fold serial dilutions of sera samples (starting at 1:25) were assayed for anti-JEV antibody titers by the end-point enzyme-linked immunosorbent assay (ELISA) using JEV JaOAr as the capture antigen.[8] Briefly, after coating overnight at 4°C with JEV in carbonate-bicarbonate buffer (45.3 mM $NaHCO_3$, 18.2 mM Na_2CO_3 [pH 9.6]), the wells were blocked by incubating the plates with 1% fat-free milk in wash buffer (PBS containing 0.05% Tween-20) at 37°C x 2 hours. The plates were washed 3 times with wash buffer before adding 100 µL of diluted mouse serum per well. The plates were incubated at 37°C for 1-hour and washed three times with wash buffer. Diluted anti-mouse 100 mL Ig-horseradish peroxidase (HRP) conjugate (Dako, Glostrup, Denmark) was added to each well and the plates were incubated for 1-hour at 37°C. After washing plates 3 times with wash buffer, the color was developed by adding the substrate solution of orthophenylene diamine (OPD, 0.5 mg/mL in phosphate-citrate buffer containing 0.052M citric acid, 0.102M disodium hydrogen orthophosphate (pH 5.0), and 0.1% hydrogen peroxide) at 37°C for 10 minutes. The reaction was stopped by adding 50 µL of 5N H_2SO_4. Absorbance was determined at 492 nm on a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, California, USA). A positive control using ATCC anti-JEV antibody (Sigma-Aldrich, St. Louis, USA) and a negative control using all reagents except the primary antibody were taken in each ELISA plate.

Assay for JEV Neutralizing Antibody

Serum samples from individual mice obtained one week after the second booster dose were assayed for JEV neutralizing activity by the plaque reduction neutralization test with a 50% end-point ($PRNT_{50}$), as described elsewhere.[9] Briefly, two-fold serial dilutions of mice sera were heat inactivated at 56°C for 30 minutes. These were then incubated with ~100 plaque-forming units (pfu) of a Vellore strain of JEV at 37°C for 1-hour before being added to a well of the 6-well tissue culture plate containing ~70% confluent monolayer of PS cells. The inoculum was removed after 1-hour and the cell monolayer overlayed with tissue culture medium containing 1% agarose. The plate was incubated for 72 hours at 37°C/5% CO_2 for plaque development. The percent neutralization was calculated from the difference in the number of plaques obtained in the absence and in the presence of immune sera. The reciprocal of the highest serum dilution giving at least 50% neutralization was regarded as the JEV neutralization titer.

Statistical Analysis

The statistical significance of different findings between mouse groups was determined by the student's t test or Fisher's exact test; $p \leq 0.05$ was considered to be significant.

RESULTS

Anti-JEV Antibody Titers

Groups of 4-week-old Balb/c mice were immunized with plasmids pMEa/pcMEa or vaccine by direct intramuscular (IM) injection using a 26G needle and syringe. Figure 1 shows that anti-JEV antibodies were detectable in all immunization groups after the primary immunization. The antibody titers increased following the booster doses. However, there were no significant differences in the titers between the individual plasmid construct groups. There was, however, a significant ($p \leq 0.05$) increase in the antibody titer of the vaccine group (Group F) when compared to majority of the plasmid construct groups, except against group C (100 pcMEa). Hence, a dose of 100 µg of pcMEa may be considered to be equivalent to the mouse brain-grown inactivated JEV vaccine, when compared on the basis of antibody inductive potency.

JEV Neutralizing Antibody Titers

Serum samples from individual mice obtained one week after the second booster dose were assayed for JEV neutralizing activity (Figure 2). The JEV neutralizing antibody titers appear to be much more revealing than the anti-JEV antibody titers. Since the neutralizing activity is considered as the gold standard for an effective vaccine, the JEV neutralizing antibody titers in the present case should be given greater weightage than the anti-JEV antibody titers.

The comparison of the two plasmid constructs, on the basis of equal weight (µg of plasmid DNA administered per dose) in the higher dose range, showed that the pCDNA construct was superior to the pCEP4 construct. A 100 µg of pcMEa (Group C) induced ~1.8-folds higher neutralizing antibody titer than 100 µg of pMEa (Group A) (p=0.107). In the lower dose range, 58 µg of pcMEa (Group D) induced ~2.2 folds higher neutralizing antibody titer than 58 µg of pMEa (Group B) (p=0.051). On an equi-molecular basis in the higher dose range, the pCDNA construct (Group D) induced ~1.8 fold higher neutralizing antibody than the pCEP4 construct (Group A) (p=0.107).
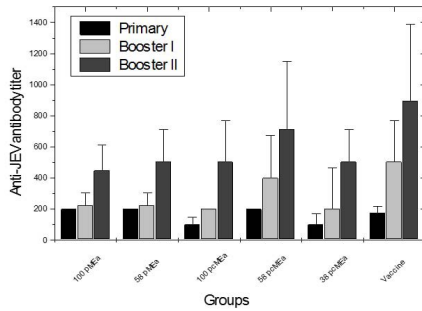
Figure 1: Anti-JEV antibody assay. Mice were immunized with plasmid constructs pMEa or pcMEa by IM needle injection. These mice received booster doses 3 and 5 weeks later. Mice were bled one day before each booster dose and challenged (6th week). Two-fold serial dilutions of sera (starting at 1:25) were assayed by ELISA for the end-point anti-JEV antibody titers. Shown above are geometrical mean titers (as bars) and standard deviation (SD) calculated from the arithmetic mean titers. The black bars represent the pre-booster titers, the light gray bars represent the titers after the first booster, and the dark gray colored bars represent the titers after the second booster dose.
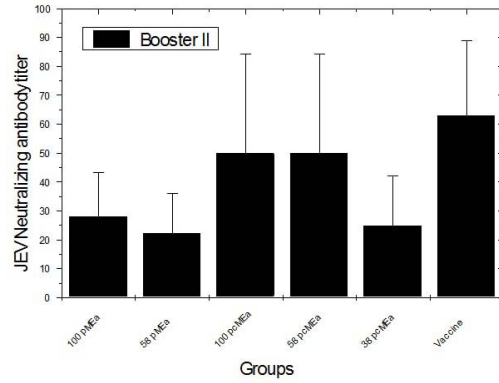

Figure 2: JEV neutralizing antibody assay. Mice were immunized with plasmid constructs pMEa or pcMEa by IM needle injection. These mice received booster doses 3 and 5 weeks later. Mice were bled one week after the second booster and two-fold serial dilutions of sera (starting at 1:25) were assayed for the JEV neutralizing antibody titers. The black bars show the geometrical mean titers along with standard deviations (SD) calculated from the arithmetic mean titers.

In the lower dose range, equi-molecular amounts of the two constructs (Group B vs. Group E) behaved similarly (p=0.651), indicating that the lower threshold limit for the two plasmid constructs had been crossed, which for pMEa is 100 µg and for pcMEa is 58 µg of plasmid DNA.

Mice Challenge Studies

Mice immunized with the plasmid DNAs were challenged by IC inoculation of a highly lethal dose (100 LD_{50}) of JEV. Table 2 corroborates the findings presented in Figures 1 and 2. The higher neutralizing antibody titers that were observed for groups C and D, having 100 and 58 µg of pcMEa, respectively are well reflected in the mouse protection data. A 83% protection observed in these two groups indicates two things. Firstly, the pCDNA construct was superior to the pCEP4 construct, both in terms of weight and the number of molecules of each plasmid administered.

Moreover, the results are significant from the process-economics standpoint, especially as only 58 µg of the pCDNA construct elicited comparable protective antibody titers to 100 µg of the same construct. Secondly, only 58 µg of the pCDNA construct was capable of inducing equivalent amount of protection to that of the conventional JEV vaccine. None of the unimmunized control mice were protected against the virus challenge.

DISCUSSION

Over the past two decades, one of the major thrust areas of DNA vaccine research has been the elucidation of the underlying mechanism by which the antigen encoded by the bacterial plasmid is processed and presented to the immune system *in-vivo*.[10] Given the relatively small amounts of protein synthesized by DNA vaccination, the efficient induction of immune responses must relate to the type of APC transfected and/or the immune-enhancing properties of the DNA itself by way of CpG motifs.

The first and most critical step in the action of a DNA vaccine formulation is the internalization of the antigen-encoding plasmid DNA.[10] It can be presumed, based on the findings, that this step is influenced by the size of the

Table 2: Protection of mice challenged with a lethal dose of JEV

Group	Immunogen	Route and mode of immunization	No. of mice survived/challenged	Protection (%)	p-value
A	100 pMEa	IM, needle	4/6	66	0.030
B	58 pMEa	IM, needle	3/6	50	0.090
C	100 pcMEa	IM, needle	5/6	83	0.0075
D	58 pcMEa	IM, needle	5/6	83	0.0075
E	38 pcMEa	IM, needle	3/6	50	0.090
F	Vaccine	IM, needle	5/6	83	0.0075
G	None	-	0/6	0	-

Mice were immunized with either the pCEP4 construct [pMEa] or the pCDNA construct [pcMEa]. The numbers prefixed before the names of the constructs indicate the dose (in µg) administered to each mouse. Mice were challenged with 100-fold 50% lethal dose (LD_{50}) of Vellore strain JEV administered IC. Mice were observed for mortality for the next 4 weeks. The table above shows the numbers of mice surviving the virus challenge, percent protection and p values as calculated against the control group (G: no immunogen). p ≤ 0.01 is regarded as significant.

plasmid administered. The data indicates that the smaller plasmid construct (pcMEa) was capable of inducing higher JEV neutralizing antibody titers than the larger plasmid construct (pMEa), both when compared on a weight basis (μg of plasmid DNA administered) as well as on the basis of the number of molecules administered.

The second aspect that needs to be addressed while interpreting the results is the immuno-stimulatory properties of the DNA itself, due to the CpG motifs inherently present in the plasmid backbone. A substantial body of evidence indicates that bacterial DNA as well as synthetic oligodeoxynucleotides expressing unmethylated CpG motifs trigger an immunostimulatory cascade that culminates in the maturation, differentiation, and proliferation of multiple immune cells, including B and T lymphocytes, NK cells, monocytes, macrophages, and dendritic cells (DCs).[11-14] Together, these cells secrete cytokines and chemokines that create a pro-inflammatory and T helper 1 (Th1)-biased immune milieu. It has been clearly established that these effects are indeed due to the CpG motifs, as prior treatment of the plasmid with DNAse or Sss I methylase (the latter selectively methylates the cytosine of CpG dinucleotides) uniformly eliminated cytokine production.[15] In order for these events to occur, the plasmid DNA has to be internalized[16] before it can interact with its receptor, thereby setting the cascade into motion. The receptor has since been identified to belong to the class of pattern recognition receptors (PRRs) of the innate immune system that belong to the toll-like receptor (TLR) family, which in case of mice is TLR9. [17,18]

On the basis of the two lines of established evidence discussed above, it appears from the findings that although the larger plasmid construct (pMEa) may have more CpG motifs in its backbone, the efficiency of its endocytosis might be hampered due to its larger size. On the other hand, the smaller pcMEa construct, although having comparatively less CpG motifs, appears to be more efficiently endocytosed, thereby inducing a higher JEV neutralizing antibody titer, and as reflected by the mice protection data following the challenge with JEV. Hence, this study highlights the fact that a smaller plasmid would be a better choice for preparing a DNA vaccine construct.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.