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Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge

Ruhe Men[a], Linda Wyatt[b], Issei Tokimatsu[a], Sakae Arakaki[a], Golam Shameem[a], Randy Elkins[a], Robert Chanock[a], Bernard Moss[b], Ching-Juh Lai[a,*]

[a]*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA*
[b]*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA*

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Abstract

Dengue epidemics increasingly pose a public health problem in most countries of the tropical and subtropical areas. Despite decades of research, development of a safe and effective live dengue virus vaccine is still at the experimental stage. To explore an alternative vaccine strategy, we employed the highly attenuated, replication-deficient modified vaccinia Ankara (MVA) as a vector to construct recombinants for expression of the major envelope glycoprotein of one or more dengue virus serotypes. MVA recombinants expressing the highly immunogenic C-terminally truncated dengue type 2 virus (DEN2) or dengue type 4 virus (DEN4) envelope protein (E), approx. 80% of the full-length, were evaluated for their protective immunity in animal models. Each of these recombinants elicited an elevated antibody response to DEN2 or DEN4 E in mice following the booster inoculation, as detected by radio-immunoprecipitation. Recombinant MVA-DEN2 80%E, but not MVA-DEN4 80%E, induced a neutralizing antibody response. The MVA-DEN2 80%E recombinant was chosen to further evaluate its ability to induce resistance to wild type DEN2 challenge in monkeys. Monkeys immunized twice with recombinant MVA-DEN2 80%E developed a low to moderate antibody response and were partially protected against DEN2 challenge, as determined by the viremia pattern. Importantly, the subsequent study showed that all four monkeys immunized with the recombinant in a three dose schedule developed an increased level of antibodies and were completely protected against DEN2 challenge. The potential efficacy of recombinant MVA-DEN2 80%E to protect primates against dengue infection suggests that construction and evaluation of MVA recombinants expressing other serotypes of dengue virus E for use in a tetravalent vaccine strategy might be warranted. Published by Elsevier Science Ltd.

Keywords: Modified vaccinia virus Ankara; Dengue virus; Envelope protein; Protective immunity

1. Introduction

Dengue epidemics increasingly pose a public health problem in most tropical and subtropical regions of Southeast Asia, the Caribbean, the Central and South Americas. According to estimates, as many as 100 million dengue infections occur every year world wide [1,2]. Most dengue infections produce a self-limited, acute febrile disease. Less often, a severe form of dengue, characterized by dengue hemorrhagic fever (DHF) or dengue hemorrhagic fever with shock syndrome (DHF/DSS), may occur especially in young children or infants. There are four dengue viruses, each of which is transmitted principally by mosquitos

* Corresponding author. Tel.: +1-301-496-5262; fax: +1-301-402-6413.
E-mail address: clai@niaid.nih.gov (C.-J. Lai).

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of the genus *Aedes*. The research efforts to develop an inactivated or live attenuated dengue vaccine have not yet produced a satisfactory result [3–8]. A vaccinia vectored recombinant vaccine strategy that delivers the highly immunogenic dengue virus subunit envelope protein (E) may prove to be an acceptable alternative.

Previously, we constructed recombinants of the vaccinia virus WR strain to express the DEN4 structural proteins E and preM, and nonstructural protein NS1, singly or in combination expressed from the polyprotein [9–11]. Immunization of mice with each of these recombinants induced resistance to fatal encephalitis caused by dengue virus challenge, indicating that each of these dengue virus proteins represents an independent protective antigen. Those studies also showed that though mice were completely protected, only a low level of E specific antibody response was detected by radio-immunoprecipitation or by a plaque reduction neutralization assay [12,13]. A similar low level of E antibodies was also detected in monkeys immunized with the vaccinia recombinant expressing the DEN4 C-PreM-E-NS1 polyprotein or the subunit E protein alone produced by baculovirus recombinants [14]. The poor immunogenicity of E could also explain the observation that immunized monkeys were only partially protected against DEN4 challenge [14]. In an effort to improve immunogenicity, we investigated the antigenic structure of DEN4 E by systematic carboxy-terminal deletion and expression of the resulting truncated E product in a vaccinia virus recombinant [15]. Unlike the full-length E, truncated E of approx. 80% in length was detected in high concentration on the cell surface and secreted extracellularly. Significantly, recombinant vaccinia virus expressing the DEN4 80%E elicited a higher level of antibody response than did the recombinant expressing the full-length E in mice. The C-terminal truncation strategy was also successfully applied to increase the immunogenicity of DEN2 E as well as Japanese encephalitis virus E [15,16]. These results were encouraging in our effort to develop an alternative vaccine strategy based on the use of cloned dengue cDNA for synthesis of dengue protective antigens.

The WR strain-vectored recombinants are not suitable for clinical investigation, mainly because of the concern about unrestricted replication of the WR virus in immune-compromised individuals. Recently, several highly attenuated vaccinia virus vectors, such as the naturally occurring canary poxvirus (ALVAC) [17] and host range-restricted modified vaccinia Ankara (MVA) [18–21] have been developed for construction of recombinants. Among these vectors the highly attenuated modified MVA is particularly attractive because its safety for humans has been clinically tested in a large number of individuals. Recombinants derived from MVA also proved to retain their attenuation phenotype in vitro, i.e. the capacity to replicate efficiently in chicken embryo fibroblast cells, but not in human or most other mammalian cells [18]. Studies have shown that MVA recombinants expressing the major protective antigens of influenza virus and human parainfluenza type 3 virus [19,21,22] induced solid protective immunity against these viruses in mice. MVA was therefore chosen as a vector for expression of the C-terminally truncated E of DEN4 and DEN2 for analysis of their protective immunity. The result of this study show that monkeys responded to multiple immunizations with an MVA recombinant expressing DEN2 80%E and that immunized monkeys were fully protected against homotypic dengue virus challenge.

2. Materials and methods

2.1. Viruses and cells

Primary chicken embryo fibroblast cells (CEF) prepared from 9-day-old embryos were routinely grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). MVA and MVA recombinants expressing the DEN2 or DEN4 80%E were grown in CEF cells in MEM supplemented with 2% FBS. MVA and MVA recombinants were titered on CEF monolayers. Prototype DEN2 New Guinea C (NGC) that was used for challenge of rhesus monkeys was propagated in LLC-MK_2 cells grown in medium 199 supplement with 2% FBS as described earlier [23]. DEN2 NGC was titered on simian LLC-MK_2 cells by plaque assay.

2.2. Plasmid construction

DEN2 NGC DNA fragment coding for the C-terminally truncated E spanning amino acids 1–405, or approx. 80% of the full-length E, was prepared by polymerase chain reaction (PCR) using DEN2 C-preM-E DNA of p5′-2 (*Xho*I) [24] as the template and the appropriate primer pair. The PCR DNA product was cleaved with *Bgl*II and cloned into the *Bam*HI site of MVA intermediate vector pLW-9 [19]. In this manner, the DEN2 E DNA was placed under the control of an early/late modified vaccinia H5 promoter. The DEN2 E DNA insert in the recombinant plasmid was sequenced to confirm the inclusion of a translation start codon at the 5′ end and a stop codon at the 3′ end. Similarly, the DEN4 DNA fragment coding for 80%E (amino acids 1–405) was also prepared by PCR using full-length DEN4 cDNA [25] as the template and the appropriate primer pair. Cloning of the DEN4 E DNA insert into the unique *Bam*HI site of the pLW-9 vector and screening for the recombinant plasmid were performed as described above. The entire

length of the DEN4 80%E DNA insert in the recombinant plasmid was sequenced to determine its authenticity.

2.3. Generation of MVA recombinants

The recombinant plasmid that contained the DEN2 80%E or DEN4 80%E DNA insert was analyzed for its ability to produce the DEN2 or DEN4 80%E product in transfected CEF cells. Briefly, CEF cells in a 6-well tissue culture plate were infected with MVA at a multiplicity of infection (MOI) of 10 for 90 min and then transfected with 5 μg of plasmid DNA in the presence of DOTAP (Boehringer, Mannheim, Germany). Production of the DEN2 or DEN4 80%E by the CEF cells was identified by immuno-staining using DEN2 or DEN4 hyperimmune mouse ascitic fluid (HMAF) and peroxidase conjugated anti-mouse immunoglobulin (Amersham, Arlington Heights, IL, USA). *O*-dianisidine was used as the substrate for coloration (Sigma, St Louis, MO, USA). To generate MVA recombinants, CEF cells in a 6-well tissue culture plate were infected with MVA at MOI of 0.05 for 90 min and then transfected with 5 μg of the plasmid containing the DEN2 or DEN4 80%E insert. The recombinant progeny of MVA-DEN2 80%E or DEN4 80%E were purified through five successive rounds of plaque purification and immunostaining as described above. Each recombinant was amplified by propagation in CEF cells starting from a single plaque.

2.4. Radio-labeling of recombinant virus infected cells and protein analysis

CEF cells in a T_{25} flask were infected with either MVA recombinant at MOI of 10 for 2 h. The infected cells were then labeled with [^{35}S]-L-methionine at 300 μCi (sp. act. 3000 Ci/mmol) in 3 ml of methionine-free MEM containing 2% FBS. At the end of a 15-h labeling period, the medium fraction and the cell fraction were separately prepared in RIPA buffer (0.1% sodium dodecyl sulfate, 1% NP40, 1% sodium deoxycholate, 0.1 M Tris, pH 7.5, 0.15 M NaCl) and followed by centrifugation to remove cell debris [9]. Immunoprecipitation of the DEN2 or DEN4 80%E protein present in the medium or the cell lysate fraction was performed using DEN2 or DEN4 specific HMAF at a dilution of 1:20. The immunoprecipitates were collected with Pansorbin beads (Calbiochem-Boehringer, CA, USA), washed with the RIPA buffer and analyzed by electrophoresis on 12% SDS–polyacrylamide gel. Radio-labeled protein bands were visualized by fluorography.

2.5. Immunization of mice

Three-week old female BALB/c mice in groups of six were inoculated intramuscularly (i.m.) with 1×10^8 infectious units (pfu) of MVA, MVA-DEN2 80%E or MVA-DEN4 80%E. Thirty-five days post-inoculation mice were boosted i.m. with the same dose of MVA or one of the MVA recombinants. Sera were obtained 23 days after the primary inoculation and again at 21 days post-booster inoculation for determination of the antibody titer by the plaque reduction-neutralization test.

2.6. Immunization of monkeys with recombinant MVA-DEN2 80%E and challenge with DEN2

Two groups of four juvenile rhesus monkeys that were each sero-negative for dengue viruses and vaccinia virus were used. Each monkey was inoculated i.m. on day 0 and day 28 with 1×10^8 infectious units of MVA or recombinant MVA-DEN2 80%E diluted to a final volume of 1.0 ml in MEM; 0.5 ml was inoculated in the right hip and 0.5 ml in the left hip. Immunized monkeys were challenged by subcutaneous inoculation with 3×10^5 pfu of DEN2 NGC, equally distributed at two sites in the upper shoulder area 28 days later. Sera were collected just prior to booster inoculation or DEN2 challenge. DEN2-challenged monkeys were bled daily for 10 days to collect serum samples for analysis of viremia. In the second experiment four monkeys were given three doses of the MVA-DEN2 80%E recombinant at 4-week interval prior to challenge with 3×10^5 pfu of DEN2 subcutaneously. For viremia analysis by plaque assay, a 50-μl aliquot of serum was diluted in 0.5 ml MEM plus 0.5% human serum albumin (HSA) and added onto mosquito C6/36 cells in T_{75} flask. After incubation at 35°C for 1 h, infected cells were then added with an agarose overlay in 1× Hanks Balance Salt Solution containing 0.5% lactose albumin hydrolysate, 5% unheated FBS, 0.12% sodium bicarbonate and 0.75% SeaKem GTG agarose, allowed to solidify and incubated in a humidified CO_2 incubator at 35°C for 6 days. Cultures were stained using a saline solution containing 0.14 M NaCl, 5.4 mM KCl, 5.6 mM glucose, 0.27 mM $NaHCO_3$ and neutral red (3.3 mg/l) for 3–6 h. After removal of the staining solution, culture flasks were tightly capped and the virus plaques were scored.

2.7. Analysis of sero-response to immunization with MVA recombinants

Dengue virus E specific antibodies in the sera of mice immunized with MVA recombinant were analyzed initially by immunoprecipitation of a [^{35}S]-methionine labeled lysate of DEN2- or DEN4-infected C6/

36 cells prepared in RIPA buffer. Briefly, 100 μl of the labeled lysate was added with 5 μl of undiluted serum or serially diluted HAMF. A 50% focus reduction assay was also performed to determine the titer of dengue virus neutralizing antibodies [26]. For virus neutralization assay 60–70 focus forming units of DEN2 were mixed with serial dilutions of test serum. The reaction mixture (200 μl) was added to the monolayer of BHK-21 cells in a 24-well plate. After adsorption, cells were covered with MEM containing 1% tragacanth gum (Sigma Chemicals, St Louis, MO, USA) and 2% FBS and incubated at 37°C for 4 days. After fixation with methanol, infected cells were immunostained using DEN2 or DEN4 HMAF and peroxidase-labeled anti-mouse immunoglobulins (DAKO Corp., Carpinteria, CA, USA). The presence of dengue virus antigens in the infected cells was visualized by treatment with appropriate substrates. The foci that developed were scored by low magnification microscopy.

3. Results

3.1. Analysis of DEN2 and DEN4 80%E produced in MVA recombinant infected CEF cells

MVA recombinants that expressed the DEN2 or DEN4 80%E on the surface of infected CEF cells were initially identified by immuno-staining. Polyacrylamide gel analysis showed that the DEN4 80%E produced in recombinant infected cells migrated as a single band of 48–50 kDa (Fig. 1). A protein band of size corresponding to the truncated DEN4 E was also detected in the extracellular fraction. Approximately 77% of the labeled DEN4 80%E was detected extracellularly during the 15 h labeling period. Similarly, following infection of CEF cells with the MVA-DEN2 80%E recombinant radio-immunoprecipitation detected a single labeled protein band of predicted size for the truncated DEN2 80%E in the intracellular fraction as well as the secreted fraction (Fig. 1). The amount of DEN2 80%E secreted was approx. 45% of the total DEN2 80%E synthesized.

3.2. Immunogenicity of MVA-DEN2 80%E and MVA-DEN4 80%E recombinants in mice

The immunogenicity of MVA-DEN2 80%E, MVA-DEN4 80%E and parental MVA was initially studied by intramuscular inoculation of mice with 10^8 pfu of the recombinant on days 0 and 35. Sera were collected after the primary and the booster inoculations and analyzed for antibodies reactive to vaccinia virus in order to confirm that mice had been successfully immunized. As shown in Table 1, antibodies specific to vaccinia virus were readily detected by an ELISA assay in each of the animals following the primary inoculation. All of the immunized mice developed a booster response following the second inoculation.

The antibody response to DEN2 80%E [Fig. 2(a)]

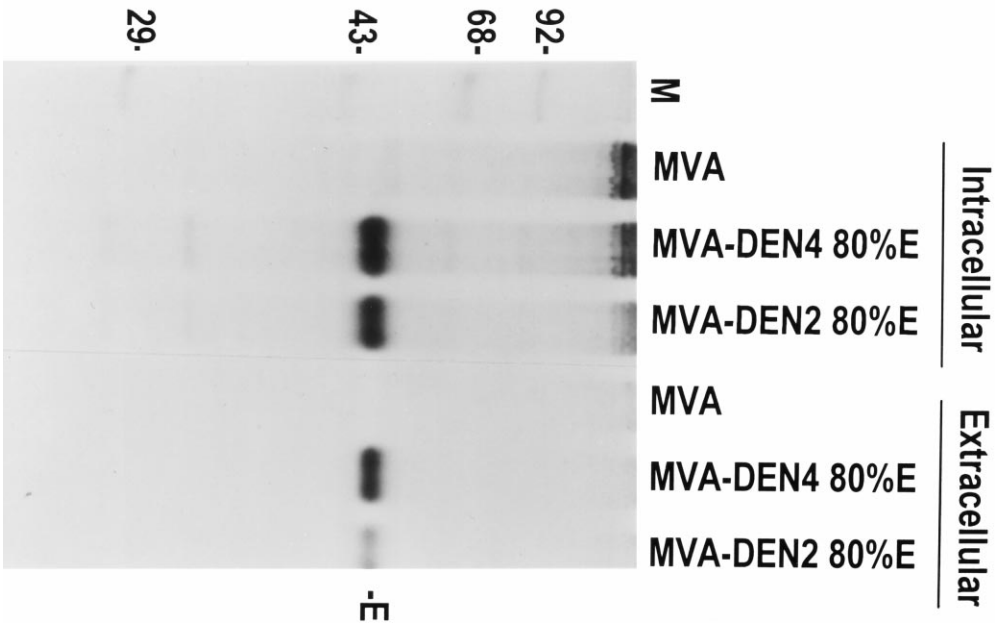

Fig. 1. Analysis of C-terminally truncated DEN2 or DEN4 E protein produced in MVA recombinant infected CEF cells. Primary CEF cells were infected with parental MVA, MVA-DEN4 80%E, or MVA-DEN2 80%E recombinant and labeled with [^{35}S]-L-methionine as described in Section 2. The cell fraction (intracellular) and the medium fraction (extracellular) were separately harvested and prepared in RIPA buffer. The labeled DEN2 or DEN4 E protein was immunoprecipitated using DEN2 or DEN4 HMAF and analyzed by polyacrylamide gel electrophoresis. Lane M represents the molecular size markers in kDa as indicated on the left.

Table 1
Analysis of antibodies specific to vaccinia virus in mice inoculated with MVA recombinants that express 80%E of DEN2 or DEN4[a]

		ELISA antibody titer (log2 of dilution) after:			
		First inoculation		Second inoculation	
Vaccinia virus	Number of mice	Mean	% rise	Mean	% rise
MVA DEN2 80%E	6	13.6	100	16.9	100
MVA DEN4 80%E	6	12.6	100	17.6	100
MVA, control	6	8.6	100	15.3	100

[a] The titer of pre-immune serum in each group was 5.3, as measured by the serum dilution that gave OD reading two fold or more higher than the negative control in the absence of added serum. – % rise indicates the fraction of mice in each group with a serum titer > 5.3.

or DEN4 80%E [Fig. 2(b)] was initially analyzed by radio-immunoprecipitation. As indicated by the labeled E protein band in the immunoprecipitate, DEN2 or DEN4 E specific antibodies were readily detected in mice 5 weeks after primary inoculation. The intensity of the labeled E in the immune precipitate increased noticeably after the second inoculation, indicating that both recombinants induced a booster response. Table 2 shows analysis of DEN2 and DEN4 neutralizing antibodies. By 5 weeks the mice inoculation with the MVA-DEN2 80% E recombinant had developed DEN2 neutralizing antibodies in a titer ranging from 14 to 107 (geometric mean 31). This titer was slightly higher than that observed for mice that were immunized once with a WR vaccinia recombinant virus that expressed DEN2 80% or full-length E (data not shown). The titer of DEN2 neutralizing antibodies increased after the booster inoculation, reaching 620 or greater for each animal with a geometric mean titer of 834 for the group.

The titer of DEN4 neutralizing antibodies in the sera of MVA-DEN4 80%E immunized mice was analyzed similarly (Table 2). After the first inoculation mice developed a detectable but low titer of DEN4 neutralizing antibodies with a mean titer of 15. Surprisingly, the titer of DEN4 neutralizing antibodies in

Table 2
Homotypic antibody response of mice inoculated with MVA recombinants that express C-terminally truncated dengue virus envelope protein

		Homotypic antibody titer[b] after:			
		First inoculation		Booster inoculation	
Mouse	Recombinant vaccinia virus inoculated[a]	Individual	Geometric mean	Individual	Geometric mean
011	MVA-DEN2 80%E	38	31	620	834
012	MVA-DEN2 80%E	14		900	
013	MVA-DEN2 80%E	25		960	
014	MVA-DEN2 80%E	19		800	
015	MVA-DEN2 80%E	107		710	
016	MVA-DEN2 80%E	33		1110	
023	MVA-DEN4 80%E	< 10	15	15	14
024	MVA-DEN4 80%E	< 10		< 10	
025	MVA-DEN4 80%E	28		< 10	
026	MVA-DEN4 80%E	31		24	
027	MVA-DEN4 80%E	26		37	
028	MVA-DEN4 80%E	17		18	
017	MVA, control	< 10	< 10	< 10	< 10
018	MVA, control	< 10		< 10	
019	MVA, control	< 10		< 10	
020	MVA, control	< 10		< 10	
021	MVA, control	< 10		< 10	
022	MVA, control	< 10		< 10	
Anti-DEN2 HMAF				2400	
Anti-DEN4 HMAF				2560	

[a] Mouse was inoculated intramuscularly with 10^8 pfu of indicated recombinant vaccinia virus and boosted with the same 5 weeks later.
[b] Antibody titer was the reciprocal of antibody dilution that yielded 50% focus reduction. For calculation of geometric mean, titer of < 10 was assigned 5, because two-fold dilution was used.

each of the six mice remained essentially the same after the booster inoculation. The reason that booster inoculation with the MVA-DEN4 80%E recombinant increased only the level of E-reactive antibodies, but failed to induce a similar increase in the DEN4 neutralizing antibody titer in mice is not understood. However, sequence analysis failed to provide an answer because the sequence of the DEN4 80% E cDNA insert in the MVA recombinant was identical to that present in the infectious cDNA clone from which the E cDNA was derived. Because of its poor immune response in mice, the MVA-DEN4 80%E recombinant was not studied further. However, recombinant MVA-DEN2 80%E was evaluated further in monkeys.

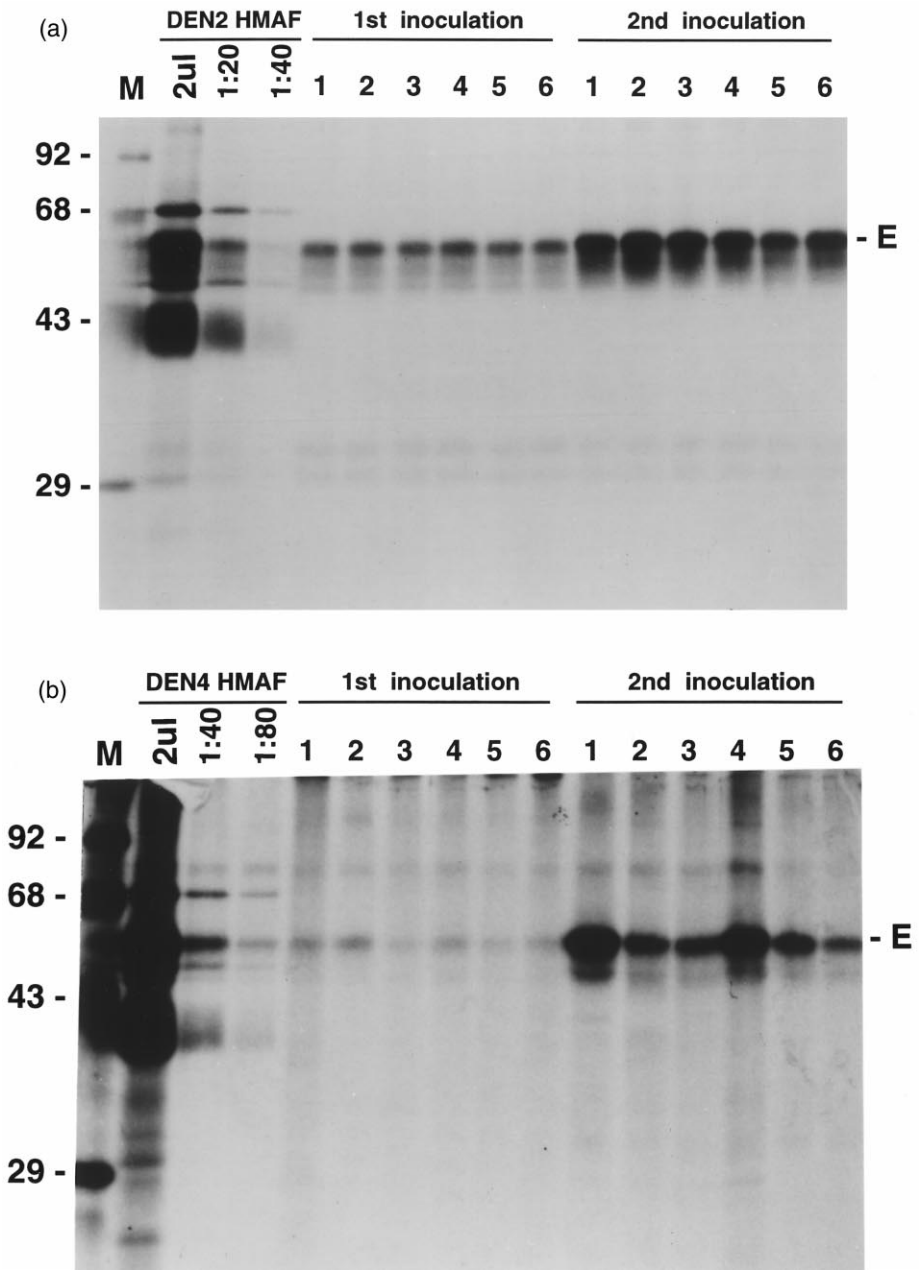

Fig. 2. Analysis of sero-response in mice following primary and booster inoculation with MVA recombinant expressing DEN2 or DEN4 80%E by radio-immunoprecipitation. Three-week old BALB/c mice in groups of six were inoculated intramuscularly in the hind leg with 10^8 infectious units of: (a) MVA-DEN2 80%E or (b) MVA-DEN4 80%E recombinant and again with the same 35 days later. Immunized animals were bled one day prior to and 21 days after the booster inoculation. Serum samples were analyzed by radio-immunoprecipitation. Radio-immunoprecipitation was performed for semi-quantitative analysis using 5 μl of serum in parallel with 5 μl of serially diluted (or 2 μl of undiluted) DEN2 or DEN4 HMAF.

3.3. Immunogenicity and protective efficacy of MVA-DEN2 80%E in twice-immunized rhesus monkeys

Mice are not a natural host for dengue virus infection and only certain mouse-adapted dengue virus strains replicate in mice only when inoculated intracerebrally [3]. Mice inoculated intracerebrally with such a dengue virus strain develop encephalitis and eventually succumb to infection. In contrast, certain Old and New World primates can be infected with dengue virus following inoculation by a peripheral route. The response of monkeys to dengue virus infection is similar to that of humans in that there is a viremia lasting several days [27]. However, dengue virus infected monkeys do not develop symptoms of dengue disease. Recombinant MVA-DEN2 80%E was evaluated in monkeys for immunogenicity and for protective efficacy against DEN2 challenge. In the first experiment, four monkeys were inoculated i.m. with 10^8 pfu of recombinant MVA-DEN2 80%E or MVA on days 0 and 28. Four weeks after the primary inoculation, the titer of DEN2 neutralizing antibodies in the sera of the MVA-DEN2 80%E infected monkeys was less than 10, essentially same as in the sera of the control monkeys. Following the booster inoculation, the DEN2 neutralizing antibody titer was detected in the low to moderate range, i.e. 32–78 (Table 3).

The monkeys inoculated twice with recombinant MVA-DEN2 80%E was tested for evidence of protective immunity to DEN2 challenge. Monkeys in the control group developed viremia lasting 3 days following DEN2 challenge (Table 3). Viremia was not detected in the two immunized monkeys that developed a neutralizing antibody titer of 70 or higher. The other two monkeys whose neutralizing antibody titer was 38 or lower developed viremia lasting 2–3 days. Although there were only four monkeys in the immunized group, there appeared to be a correlation between the level of neutralizing antibodies and protective immunity against DEN2 challenge.

3.4. Protection against DEN2 challenge following three inoculations of recombinant MVA-DEN2 80%E

Previous experiment showed that immunization twice with recombinant MVA-DEN2 80%E induced booster antibody response in mice and in monkeys. Because monkeys inoculated twice with MVA-DEN2 80%E were only partially protected against dengue virus challenge, it was possible that additional immunization might elicit further increase of antibody response and achieve complete protection against dengue challenge. For this reason, the effect of a third immunization with MVA-DEN2 80%E was investigated (Table 3, Exp. 2). Three of the four monkeys that received three doses of MVA-DEN2 80%E recombinant developed a neutralizing antibody titer higher than that attained after the second inoculation. The antibody titer increase detected in each of these animals was only up to two fold that was within one dilution range. The small increase of antibody titers suggests that immunity to recombinant MVA vector infection may limit response to the third inoculation. Significantly, all four monkeys were completely protected against DEN2 challenge, as shown by the absence of viremia.

Table 3
Antibody response of monkeys inoculated with MVA-DEN2 80%E recombinant and viremia response to subsequent DEN2 challenge

			Neutralizing antibody titer[a] after:			
Exp.	Virus	Monkey	1st inoc.[b]	2nd inoc.	3rd inoc.	Total viremia days after challenge (virus titer, pfu/ml)
1	MVA, control	H-381	< 10	< 10		3 (180, 220, 220)
		H-385	< 10	< 10		3 (60, 60, 60)
		9627	< 10	< 10		3 (960, 300, 40)
		9652	< 10	< 10		3 (460, 240, 80)
	MVA-DEN2 80%E	9654	< 10	32		3 (100, 200, 220)
		9657	< 10	38		2 (940, 80)
		9658	< 10	70		0
		9667	< 10	78		0
2	MVA-DEN2 80%E	H-357	< 10	100	180	0
		H-383	20	200	280	0
		H-405	< 10	60	120	0
		6049	< 10	180	160	0

[a] Reciprocal of antibody dilution that neutralized DEN2 by 50%.
[b] Monkeys were inoculated with 10^8 pfu of MVA or MVA-DEN2 80%E recombinant at each time indicated and subsequently challenged with 10^5 pfu of DEN2 NGC.

4. Discussion

MVA is a highly attenuated deletion mutant of vaccinia virus, derived from the parent virus by multiple serial passage in chicken embryo fibroblast cells. In these cells MVA replicates efficiently and grows to a high titer. In human and most other mammalian cells, MVA produces abundant early and late viral proteins, but is deficient for replication, apparently resulting from a block in virion assembly [18]. Previous studies demonstrated that MVA recombinants that express foreign genes under the control of an early/late promoter efficiently also retain the host range restriction phenotype [19]. Importantly, various recombinants of MVA have been shown to elicit protective immunity in animal models against a variety of infectious viral agents, such as influenza, and parainfluenza viruses, comparable to that of the recombinants derived from the replication-competent WR strain [19,21,22]. These studies suggested that MVA vectored recombinants might merit further evaluation as vaccine candidates for humans, because MVA has proven to be safe in a clinical trial involving more than a hundred thousand humans.

Recent efforts to develop a safe and effective vaccine against dengue have focused largely on live virus strains attenuated by serial passage of the wild type virus in cell culture or by genetic modification of the viral genome [6,8,23]. In addition, progress has been made to produce an inactivated whole dengue virus vaccine [28]. Nevertheless, studies of promising candidate dengue vaccines still remain in the experimental stage. MVA vectored recombinants expressing the C-terminally truncated E of one or more of the four dengue virus serotypes represent an alternative dengue vaccine strategy. In the current study, two MVA recombinants that expressed the C-terminally truncated DEN2 or DEN4 80%E were constructed. Each of these recombinants induced a significant antibody response in mice, reaching a level comparable to $>1/20$ dilution of DEN2 HMAF or $>1/40$ dilution of DEN4 HMAF, when assayed by radio-immunoprecipitation. We found that intramuscular immunization with the MVA recombinants in mice induced an antibody response comparable to that observed earlier following intraperitoneal inoculation with the WR vaccinia virus-derived DEN4 or DEN2 E recombinants. In the earlier study, the level of antibody response to a WR recombinant expressing DEN2 80%E was sufficient to solidly protect mice against dengue fatal encephalitis caused by dengue virus challenge, whereas the WR recombinant expressing full-length DEN2 E induced an antibody response that conferred less than 50% protection [15].

Because neutralizing antibodies most likely play a pivotal role in the protection from virus infection, determination of the titer of dengue virus neutralizing antibodies in the mouse sera should provide a direct assessment of the protective immunity of these recombinants. Recombinant MVA-DEN2 80%E induced a high level of DEN2 neutralizing antibodies in mice. Surprisingly, the titer of neutralizing antibodies in mice immunized twice with recombinant MVA-DEN4 80%E remained low and could not be boosted. The reason that recombinant MVA DEN4 80%E failed to induce an elevated level of neutralizing antibodies in mice in a manner similar to that was observed with recombinant MVA-DEN2 80% following the booster inoculation is not immediately clear. It is possible that other mouse strains may respond to MVA DEN4 80%E differently from the Balb/c strain that was used in the current evaluation of immunogenicity. Importantly, the nucleotide sequence of the truncated DEN4 E in the MVA recombinant was identical to that present in the infectious DEN4 cDNA. Thus, a mutation or deletion in the DEN4 80%E sequence that might account for the loss of one or more of the antigenic determinants can be ruled out. DEN4 derived from cDNA was previously shown to induce high titer of neutralizing antibodies in monkeys [23]. It appears that DEN4, similar to DEN2, reacted readily with its homotypic neutralizing antibodies.

The specific immunogenicity of DEN2 or DEN4 E is likely influenced by the physical structure which these antigens present to the host immune system. Recent structural analysis has revealed that the tick-borne encephalitis virus (TBEV) E assumes a dimer structure with an unusually flat and elongated architecture [29]. The significant sequence conservation among flavivirus E predicts that the native form of DEN2 or DEN4 E shares a global structure similar to that of TBEV E, although certain local domains such as those responsible for antigenic determinants of neutralizing antibodies might vary. In addition, most of the antigenic determinants of neutralizing antibodies of flavivirus E are thought to be highly dependent on conformation. The disparity of the neutralizing antibody response between DEN2 80%E and DEN4 80%E in mice suggests that DEN4 80%E may not assume conformation required for optimal presentation of determinants for these antibodies. On the other hand, analysis by radio-immunoprecipitation indicates that DEN4 80% E retained the antigenic determinants for eliciting nonfunctional antibodies reactive to DEN4 E. One interpretation of the data is that C-terminal truncation of DEN4 E results in protein misfolding leading to selective presentation of the epitopes for nonfunctional antibodies and diminished presentation of the epitopes for neutralizing antibodies. As demonstrated earlier by HMAF binding affinity analysis, the length of truncation could critically affect the conformation of the resulting DEN4 E [15]. The mini-

mum length of 80% including Arg at position 392 was identified among a series of truncated DEN4 E constructs. It is conceivable that the truncated DEN4 80%E may only assume the conformation best for eliciting E protein binding antibodies. Other investigators studying the E protein of TBEV or Japanese encephalitis virus, resorted to co-expression of PreM and E to form aggregates in an attempt to improve the immunogenicity [30,31].

Monkeys are the closest surrogates for human dengue virus infection. The response of monkeys to dengue virus infection is similar to that of humans in that there is a viremia lasting several days, although infected monkeys do not develop dengue disease symptoms. Earlier, we employed this primate model to analyze the immunogenicity and protective efficacy of viable chimeric dengue viruses against dengue virus challenge [23]. By comparison, the titer of DEN2 neutralizing antibodies detected in the sera of monkeys inoculated twice with MVA-DEN2 80%E was lower than that found in the sera of monkeys inoculated singly with the wild type DEN2 or the chimeric virus with DEN2 specificity. The antibody titers attained after immunization with two doses of the MVA recombinant provided only partial protection in monkeys. Other investigators also reported that a low neutralizing antibody titer in immunized monkeys was observed with ALVAC expressing Japanese encephalitis virus E and PreM [31].

In the present study preliminary information was obtained concerning the level of DEN2 neutralizing antibodies induced by recombinant MVA-DEN2 80%E required for protection against DEN2 in monkeys. Monkeys with a neutralizing antibody titer of approx. 70 or greater were protected against DEN2 challenge. This protective antibody titer was within the range of protective and non-protective immunity observed in an earlier study in which monkeys were inoculated with live DEN1/DEN4 and DEN2/DEN4 chimeras with type 1 and type 2 specificity, respectively. In that study monkeys that developed a neutralizing antibody titer of 640 or greater were completely protected against homotypic dengue challenge, whereas one of the monkeys with a titer of 20 was not [23]. To increase the antibody response, recombinant MVA-DEN2 80%E would require three immunizations. Alternatively, the MVA recombinant could be used in combination with purified subunit protein or an over-attenuated vaccine. A MVA recombinant vaccine utilizing such a multi-phase immunization strategy might be perceived as cumbersome or even impractical. For this reason, effort should continue to improve the immunogenicity of recombinant expressed DEN2 E. Importantly, the potential efficacy and the expected safety of such a vaccine are encouraging. Since there are four dengue virus serotypes and heterotypic immunity against other dengue serotypes is brief, the current strategy for immunization against dengue favors the use of a vaccine preparation containing all four dengue serotypes. However, before considering further evaluation of the MVA-DEN2 80%E recombinant in clinical trials, study should be initiated to construct MVA recombinants expressing immunogenic E of other dengue virus serotypes. Unexpectedly, recombinant MVA-DEN4 80%E did not elicit an appreciable increase of neutralizing antibody response even after the booster immunization. To address this problem, additional DEN4 constructs should be re-evaluated to determine the extent of C-terminal truncation which could produce a subunit E protein structure best suited to elicit neutralizing antibodies. Clearly, the expected safety and the potential protective efficacy of such a vaccine consisting of all four dengue virus serotypes may merit construction of MVA-recombinants expressing immunogenic E of the other three dengue serotypes.

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