

## PROTECTION AGAINST SCRUB TYPHUS BY A PLASMID VACCINE ENCODING THE 56-KD OUTER MEMBRANE PROTEIN ANTIGEN GENE

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**Abstract.** The 56-kD outer membrane protein of *Orientia tsutsugamushi* has previously been shown to be the immunodominant antigen in scrub typhus infections. Its gene was cloned into the DNA vaccine vector pVR1012 as a vaccine candidate (pKarp56). The *in vitro* expression of this 56-kD antigen by pKarp56 was confirmed in tissue culture by an indirect fluorescence assay and Western blot analysis. The initial antibody responses of mice immunized with varied doses of the pKarp56 were barely detected, but increases were observed after each of three subsequent booster immunizations. Although no protection was observed with a single immunization of pKarp56, after four immunizations, 60% of the mice survived a 1,000 × 50% lethal dose (LD<sub>50</sub>) challenge. These results specifically confirm the importance of the 56-kD protein antigen in protective immunity against *O. tsutsugamushi* and demonstrate the feasibility of DNA vaccines for the prevention of scrub typhus.

### INTRODUCTION

Scrub typhus is caused by infection with the obligate, intracellular, gram-negative bacterium *Orientia tsutsugamushi*. This disease can account for up to 23% of all febrile episodes in disease-endemic areas of the Asia-Pacific region and can cause up to 35% mortality if left untreated.<sup>1,2</sup> Vaccines offer the potential of long-term prevention from morbidity and mortality caused by scrub typhus. They also obviate the difficulties posed by vector control and preventive chemoprophylaxis. The recent evidence for antibiotic resistance of *O. tsutsugamushi* further emphasizes the need for a scrub typhus vaccine.<sup>3–5</sup> Prior vaccine development efforts using the whole organism have suggested that a scrub typhus vaccine is feasible. Immunization of volunteers with live vaccine in combination with chloramphenicol prophylaxis elicited immunity comparable to that of natural infection.<sup>6,7</sup> A polyvalent gamma irradiated vaccine that elicited protection against heterologous serologic types has also been demonstrated.<sup>8</sup> A polyvalent vaccine is necessary for *Orientia* because numerous antigenic types have been identified and are highly focal in distribution, and cross-protective immunity elicited by a single strain is weaker and persists for a shorter period of time than homologous immunity.<sup>7–9</sup> However, considerable difficulties exist in mass production of purified *O. tsutsugamushi* and in retaining their stability upon storage. Consequently, whole-cell vaccine products are unlikely to be economically feasible or suitable for manufacturing to meet current Good Manufacturing Practices Act standards of purity, potency, and lot-to-lot consistency.<sup>10</sup>

Recent investigations suggest that the major outer membrane 56-kD protein is a protective antigen that can be produced as a suitable recombinant protein for use as a vaccine<sup>11–13</sup> and as a diagnostic reagent.<sup>14–17</sup> Sera from 95–99% of patients with a history of scrub typhus react strongly with this protein, which reflects the abundance of the molecule on the cell surface and its high immunogenicity.<sup>15,16</sup> The 56-kD

protein also appears to play a role in the adhesion and internalization of *O. tsutsugamushi* into host cells because both polyclonal and monoclonal antibodies against this antigen can block the infection of fibroblasts in tissue culture.<sup>13,18</sup> Recombinant 56-kD protein has been shown to elicit specific antibodies and T cell responses that react with *O. tsutsugamushi*.<sup>12,19</sup> All these results suggest that the 56-kD antigen is an ideal candidate for subunit vaccine development.

DNA vaccines have many advantages over other types of vaccines.<sup>20–22</sup> Wolff and others have shown that direct intramuscular inoculation of plasmid or naked DNA encoding several reporter genes could induce protein expression within muscle cells.<sup>23</sup> By cellular uptake and intracellular expression of the target antigen, DNA vaccines can induce strong cellular immune responses that mimic the effects of live attenuated vaccines but eliminate the safety concerns associated with live vaccines.<sup>22</sup> Immunization with naked DNA plasmids containing sequences expressing specific antigens has been found efficacious in protecting animals against microbial pathogens.<sup>24–26</sup> DNA vaccines are easy to construct in various forms and can be produced in large quantities. These characteristics have led to the formulation of multi-component vaccines that provide broader protection against different strains or even different species of pathogens and made them much easier to produce than other vaccines.<sup>20</sup> DNA vaccines are much more stable than attenuated live vaccines or whole-cell antigen vaccines and can be produced at much lower costs. The move towards using naked DNA vaccines based on the *Orientia* 56-kD protein antigen gene has the potential to shorten the time necessary for developing and fielding of an effective polyvalent vaccine against scrub typhus because, unlike purification of antigenic recombinant proteins, the methodology and standardization for each plasmid component of the vaccine is the same. This is especially important because of the extensive antigenic diversity in the major antigen 56-kD protein found among various strains of *O. tsutsugamushi*.<sup>27,28</sup> We cloned the gene encoding the full open reading frame (ORF) of the 56-kD protein antigen from the Karp strain into a VR1012 plasmid (pKarp56) as a DNA vaccine candidate. In this report, we demonstrate that pKarp56 is both immunogenic and protective, and DNA vaccination is a promising approach to the development of vaccines for the prevention of scrub typhus.

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## MATERIALS AND METHODS

**Construction of DNA vaccine vector containing 56-kD gene (pKarp56).** The full ORF of the 56-kD gene was amplified by a polymerase chain reaction (PCR) using the pBR322 plasmid carrying the 56-kD antigen gene (180 ng in a 100- $\mu$ L reaction volume) as the template.<sup>29</sup> The sequence for the forward primer was: 5'-TTA CGC TCT AGA ATG (A/G) AA AAA ATT ATG TTA ATT GCT AGT GCA ATG-3', which corresponds to 45 bases from nucleotide -12 to +33 of the coding sequence (GenBank accession no. M33004). The sequence for the reverse primer was 5'-GCG GAT CCT TCT AGA GAA AAA ACT AGA AGT TAT AGC GAT CAC T-3', which corresponds to 43 bases from 1578 to 1620. Each primer contains an *Xba* I restriction site (underlined). The numbers referred to the sequence published by Stover and others.<sup>29</sup> The PCR was performed for 30 cycles. Each cycle consisted of denaturation at 94°C for one minute, primer annealing at 57°C for two minutes, and extension at 72°C for two minutes. The PCR product was digested with the restriction enzyme *Xba* I and ligated into the expression vector pVR1012 (Vical Inc., San Diego, CA) that contained a human cytomegalovirus promoter and intron A for transcription initiation and bovine growth hormone polyA signal. The sequence of the insert was confirmed with an automated ABI 370 sequencer (Applied Biosystems, Foster City, CA). DH5 $\alpha$  competent cells (Life Technology, Gaithersburg, MD) were transformed with the resultant plasmid and selected for kanamycin (Sigma, St. Louis, MO) resistance. Plasmid control pVR1012 and test vaccine pKarp56 were purified with a Giga kit (Qiagen, Valencia, CA) to be used in experiment 1 or by centrifugation in two successive isopycnic CsCl gradients, followed by extensive dialysis against phosphate-buffered saline (PBS) to be used in experiment 2. Both preparations were highly purified ( $A_{260}/A_{280} > 1.80$ ). It does not seem likely that different plasmid preparations influence protection during mice challenge.

**Confirmation of expression of 56-kDa antigen in human kidney cell line 293.** The human embryonic kidney cell line 293 (CRL-1573; American Type Culture Collection, Manassas, VA) was cultured in Dubecco's modified Eagle medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal calf serum (Mediatech, Inc.) in an atmosphere of 5% CO<sub>2</sub> at 37°C. When the cultured cells reached 25–30% confluence in 60-mm dishes, transfection was carried out with 3–10  $\mu$ g of purified plasmid DNA using the calcium phosphate precipitation method.<sup>30</sup> Cells were harvested 38 hours post-transfection, washed twice in PBS, and centrifuged at 1,000  $\times$  g for 5 minutes.

**Indirect fluorescence assay (IFA).** The cell pellet was resuspended in 1 mL of PBS (Quality Biologic Inc., Gaithersburg, MD). Cells were spotted onto eight-well slides, dried at 37°C for 5 minutes, and fixed with acetone (Sigma) with an additional 1–2 minutes for drying. Monoclonal antibodies Kp56-a, Kp56-b, and Kp56-c, which are specific for Karp 56-kD antigen, were used in the IFA.<sup>31</sup> Monoclonal antibodies diluted in PBS were added to the fixed slide (10  $\mu$ L/well) and incubated for 1 hour at 37°C. The slides were washed twice with PBS (5 minutes/wash). Goat anti-mouse IgG–fluorescein isothiocyanate conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at 1:200 dilution was added to the slide (10  $\mu$ L/well) and incubated for 30 minute. After washing

twice as for the primary antibody, the slides were dried, mounting solution was added, and examined by fluorescence microscopy.

**Western blot analysis.** After transfection, the 293 cell pellets were washed twice with PBS and resuspended in Laemmli sodium dodecylsulfate–polyacrylamide gel electrophoresis buffer containing 2-mercaptoethanol, boiled for 5 minutes, and loaded onto a 10% polyacrylamide gel.<sup>32</sup> Renografin density gradient–purified *O. tsutsugamushi* Karp plaque-purified cells, which served as the positive control, were solubilized and loaded onto the gel in a similar way. The protein components were separated on the gel by electrophoresis and electroblotted onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH) overnight at 50 mA in transfer buffer (25 mM sodium phosphate, pH 7.5). After blocking with 5% milk in Tris-buffered saline (0.25 M NaCl in 10 mM Tris HCl, pH 7.5), the membrane was incubated with monoclonal antibody Kp56-b against the 56-kD antigen for 1 hour. The bound antibody was detected by anti-mouse IgG (heavy plus light chain) conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) and subsequent staining with the peroxidase substrate tetramethylbenzidine (Kirkegaard and Perry Laboratories).

**Immunization and challenge of mice.** Female Swiss outbred CD-1 mice (Charles River Laboratories, Wilmington, MA) (weight = 18–24 grams) were used throughout the study. Mice were immunized intramuscularly with a 25-gauge, 0.5 inch needle in each thigh (25  $\mu$ L/site) with a 50- $\mu$ L volume containing different amounts of pKarp56 in PBS. Mice were challenged intraperitoneally with 1,000  $\times$  50% lethal doses (LD<sub>50</sub>) of mouse-passaged plaque-purified Karp strain (20% mouse liver/spleen suspension in 0.2 mL of Snyder I buffer) four weeks after a single immunization (experiment 1, 12 mice in each group) or 45 days after the fourth immunization (experiment 2, 8 mice with pKarp56 and 5 mice with pVR1012 alone). The dates of disease onset and death were recorded for each mouse. The morbidity and mortality were monitored at least twice a day for 21 days post-challenge. Mice were bled from the retro-orbital sinus to obtain sera 3–6 days before each immunization boost or challenge and at four weeks after the fourth immunization.<sup>33</sup> These experiments were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

**Monitor antibody responses by enzyme-linked immunosorbent assay (ELISA).** The antibody level in serum from each mouse was determined by using microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) coated with purified, truncated, recombinant 56-kD antigen (Kp r56) as described previously.<sup>14</sup> Briefly, 96-well microtiter plates were coated overnight at 4°C with Kp r56 (0.3  $\mu$ g/well) diluted in PBS, blocked with 2% bovine serum albumin for 1 hour at 37°C, and rinsed with PBS twice. Mouse sera were diluted 1:200 in PBS, 0.1% Tween 20 with 20  $\mu$ g/mL of control protein extracts prepared from *Escherichia coli* BL21 using a procedure identical to that used for purifying Kp r56, pre-absorbed for 1 hour at room temperature, and then added to the ELISA plates. The plates were incubated for 1 hour at room temperature and washed four times with 0.1% Triton X-100 in PBS. Peroxidase-conjugated goat anti-mouse IgG (heavy plus light chain) (Bio-Rad Laboratories) at a 1:1,000 dilution was then added. After

incubation for 1 hour at room temperature, the plates were washed four times with 0.1% Triton X-100 in PBS and then once with PBS before the addition of substrate (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] [ABTS]; Kirkegaard and Perry Laboratories). Optical densities were measured with a Vmax/Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) at 405 nm after incubation for 15 minutes at room temperature.

## RESULTS

The Vical vector pVR1012 is suitable for human use because it contains a human cytomegalovirus promoter, intron A for transcription initiation, an enhancer element, and bovine growth hormone polyA signal. The expression of 56-kD antigen from pKarp56 was confirmed by IFA and Western blotting of extracts from transfected human kidney 293 cells. Three Kp56-specific monoclonal antibodies (Kp56-a, Kp56-b, and Kp56-c) were used in IFA detection of the expressed 56-kDa antigen 38 hours after transfection.<sup>31</sup> Monoclonal antibody Kp56-a exhibited stronger binding than Kp56-b and Kp56-c to transfected 293 cells. Western blotting of transformed cell extracts confirmed that a full-length 56-kD antigen was expressed in tissue culture (Figure 1). The precise reasons for the low reactivity of antigen expressed by the plasmid is unknown, but poor expression or rapid degradation of expressed antigen are two possibilities.

Experiment 1 demonstrated that one immunization with various doses of pKarp56 did not provide protection against live challenge with  $1,000 \times LD_{50}$  Karp (Table 1). Following the challenge, immunized mice died within a similar number of days as those immunized with the control plasmid pVR1012. No specific antibody responses were observed in sera obtained three weeks after the single immunization in any of the groups.

In experiment 2, eight mice were immunized intramuscularly with 100  $\mu$ g of pKarp56 and five mice with pVR1012 alone, four times at approximately four-week intervals (days 1, 27, 51, and 79). Although the average antibody levels to the 56-kD antigen were barely detectable after one dose, they became clear following the second and particularly the third immunization (Figure 2). An increase in endpoint titer with

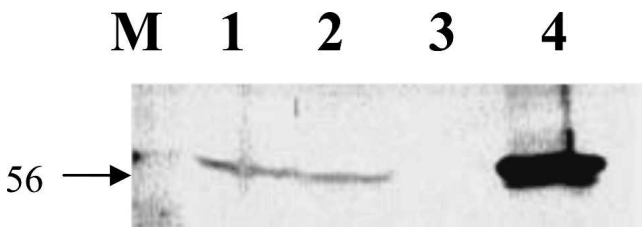


FIGURE 1. Western blot analysis of transiently transfected human kidney 293 cells for the expression of the 56-kD antigen. The cells were collected 38 hours post-transfection. Lanes 1 and 2, lysates of approximately  $10^4$  cells transfected with two individual plasmid preparations carrying the 56-kD gene in pVR1012 (pKarp56); lane 3, lysate from the same number of cells transfected with control pVR1012; lane 4; renografin density-purified *Orientia tsutsugamushi* Karp whole-cell lysate (10  $\mu$ g). Monoclonal antibody Kp56-a was used as the primary monoclonal antibody to detect 56-kD antigen. Lane M, molecular mass marker. The **arrow** indicates the position of the 56-kD protein.

TABLE 1

Protection of mice against lethal Karp challenge by pVR1012 control and pKarp56 vaccine\*

Experiment 1: Single immunization	
Plasmid	Survival
None (PBS)	1/12
pVR1012 (100 $\mu$ g)	1/12
pKarp56 (100 $\mu$ g)	0/12
pKarp56 (25 $\mu$ g)	0/12
pKarp56 (6 $\mu$ g)	0/12
Experiment 2: Four immunizations	
Plasmid	Survival
pVR1012 (100 $\mu$ g)	0/5
pKarp56 (100 $\mu$ g)	5/8

\* PBS = phosphate-buffered saline.

time after vaccination could also be demonstrated. Forty-five days after the fourth immunization, mice from each group were challenged with  $1,000 \times LD_{50}$  of *O. tsutsugamushi* Karp strain, and the morbidity and mortality were monitored twice a day. Five mice (60%) immunized with pKarp56 survived the lethal challenge after four immunizations (Table 1). Multiple immunizations with DNA vaccine also shortened the number of days of illness and increased the survival time among those that eventually died (Figure 3). All control mice became sick at day 7, while immunized mice became sick one day later. The sick mice were inactive, anorexic, and their fur appeared ruffled. Three of five mice in the control group were dead by day 9, one died on day 10, and the last one died on day 13. In the immunized group, only one of eight mice died on day 9, one on day 10, and one on day 15. All survivors in the immunized group became active again on day 12 post-challenge. By day 17, all the mice looked normal again, they were very active, the fur was smooth, and they were eating well. The antibody responses of immunized mice determined by Kp r56 ELISA did not correlate with their ability to survive a lethal challenge (Table 2). For example, although mouse 17 had the same antibody response as that of mouse no. 18 and its response was much higher than those of mice 12, 14, 15, and 16, it died quickly. Similarly, mouse 12 had a low antibody response but survived. We have observed a similar lack of correlation of survival to challenge and antibody responses to r56 in other experiments using recombinant r56 protein as the immunogen (Ching W-M and others, unpublished data).

## DISCUSSION

In this report, we demonstrated that multiple immunizations of pKarp56 without any adjuvant protected 60% of outbred CD1 mice against a lethal dose of live *O. tsutsugamushi* Karp strain. Preliminary data showed that highly purified whole-cell antigen, a truncated recombinant 56-kD antigen, or irradiated *O. tsutsugamushi* could provide full homologous protection after one immunization in a mouse model (Ching WM, Dasch, GA, unpublished data). Previous studies by Jerrells and others demonstrated that immunity can be elicited in mice and established by administration of viable *O. tsutsugamushi* or  $\gamma$ -irradiated *O. tsutsugamushi*.<sup>34,35</sup> The development of immunity was associated with the presence of cell-

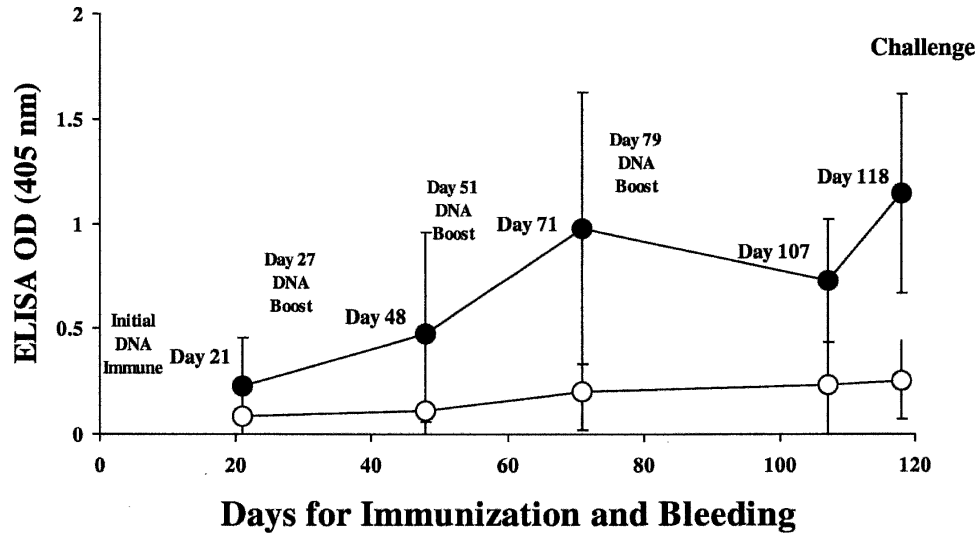


FIGURE 2. Enzyme-linked immunosorbent assay (ELISA) antibody responses of mice to the 56-kD protein antigen following DNA vaccination. Mice were immunized on days 0, 27, 51, and 79 and challenged on day 124. Sera were collected at days 21, 48, 71, 107, and 118. Mean and range of antibody responses (15 mice per point) at a 1:200 dilution are indicated. OD = optical density. ● = mice immunized with pKarp56; ○ = control mice immunized with pVR1012.

mediated immunity as shown by delayed-type hypersensitivity, antigen-induced lymphocyte proliferation, and cytokine production. The lack of correlation between antibody level against 56-kD antigen and protection against live challenge

with the homologous strain is consistent with the notion that cellular immunity is important. One of the advantages of DNA vaccines is that they can stimulate memory cells.<sup>36</sup> Another advantage is that additional antigens can be added to increase the breadth of protection.<sup>21</sup> Therefore, we studied the feasibility of DNA vaccine approach with our first-generation DNA vaccine candidate pKarp56. The construction of a second-generation DNA vaccine candidate that includes the 56-kD antigen gene from other strains in addition to Karp has been initiated. The results presented in this study demonstrated the efficacy of a DNA vaccine for the prevention of scrub typhus. These results specifically confirmed the importance of the 56-kD protein antigen in protective immunity to *O. tsutsugamushi*. Although no protection was observed following a single dose of pKarp56, after four immunizations 60% of the mice survived a 1,000 × LD<sub>50</sub> lethal challenge. In a separate experiment, inbred C3HeB/FeJ mice were also protected by immunization with a recombinant 56-kD protein antigen (Ching W-M, Dasch GA, unpublished data). The protective efficacy in outbred mice is very significant because human populations to be protected will have various genetic backgrounds. Protective efficacy of DNA vac-

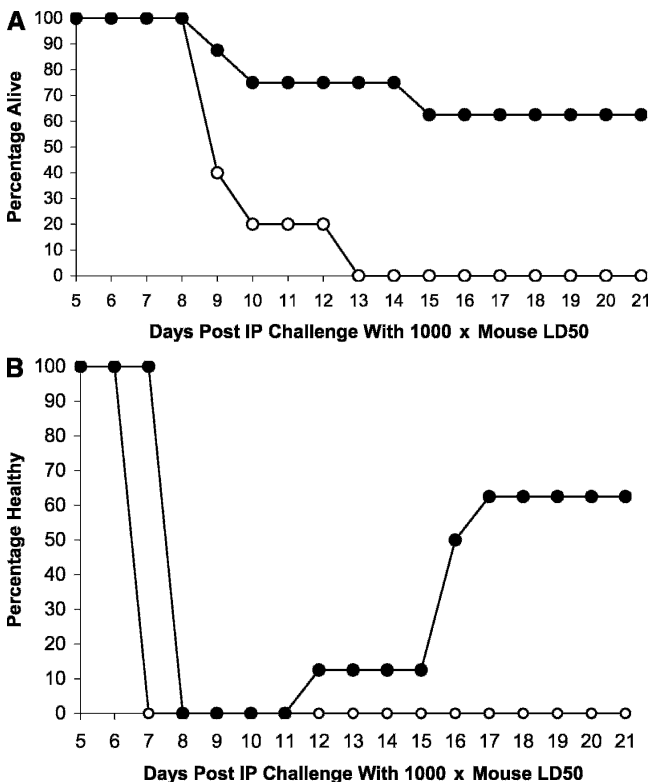


FIGURE 3. Mortality and morbidity of mice immunized four times with the pVR1012 control or pKarp56 and challenged with 1,000 50% lethal doses (LD<sub>50</sub>s) of *Orientia tsutsugamushi* Karp strain. A, Mortality. B, Morbidity. IP = intraperitoneal. ○ = control mice; ● = mice immunized with pKarp56.

TABLE 2

Antibody responses and protection of immunized mice against lethal Karp challenge\*

Mouse	pVR1012		pKarp56		
	ELISA OD	Challenge result	Mouse	ELISA OD	Challenge result
1	0.075	Dead (day 9)	11	0.247	Dead (day 10)
2	0.107	Dead (day 9)	12	0.422	Sick (days 8–15)
3	0.128	Dead (day 10)	13	0.500	Dead (day 15)
4	0.420	Dead (day 13)	14	1.171	Sick (days 8–16)
5	0.420	Dead (day 9)	15	1.272	Sick (days 8–11)
			16	1.498	Sick (days 8–15)
			17	1.690	Dead (day 9)
			18	1.698	Sick (days 8–15)

\* ELISA = enzyme-linked immunosorbent assay; OD = optical density.

cines demonstrated in inbred murine models may not be applicable to outbred species.<sup>37</sup> The protection afforded by the 56-kD antigen observed in both inbred mice and outbred mice suggests that the protective efficacy observed may not be due to hyper-responsiveness to the antigen by a particular host immunotype. Therefore, the 60% protection observed in the CD-1 outbred mice achieved with a naked DNA vaccine is very encouraging.

The 56-kD antigen is an outer membrane protein. Previously, we truncated the membrane anchoring domains at the N-terminus and the C-terminus from the full-length gene and inserted the truncated gene (coding for amino acid 80-456) into an *E. coli* expression vector.<sup>14</sup> The expressed protein easily refolded into a structure similar to the native form, as judged by the reactivity of polyclonal and several monoclonal antibodies. In the construct of our DNA vaccine candidate pKarp56, the intact gene of the 56-kD ORF was inserted into pVR1012. Raviprakash and others have shown that different lengths of expressed dengue antigen exhibited different degrees of degradation.<sup>38</sup> The insertion of the truncated 56-kD gene into pVR1012 may increase the protective efficacy by expressing a soluble protein or by increasing the expression of the antigen. The protective efficacy of this 56-kD antigen-based DNA vaccine may also be improved significantly by several approaches, such as improving the expression level by using different secretory sequences, inclusion of the Kozak consensus translational initiation sequence, co-immunization with immune potentiating adjuvants such as interleukin-12 (IL-12) and granulocyte-macrophage colony-stimulating factor (GM-CSF), cloning the gene into dendritic cell-attracting vectors to enhance the presentation of the 56-kD antigen, or using liposomes for more efficient delivery.<sup>20,37-41</sup> Preliminary experiments using plasmids expressing IL-12 or GM-CSF as adjuvants or DNA encapsulated in liposomes as the delivery system or using DNA vaccine followed by a protein booster appear promising in further enhancing the value of pKarp56 as a DNA vaccine.

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