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## DENGUE TYPE-2 VIRUS ENVELOPE PROTEIN MADE USING RECOMBINANT BACULOVIRUS PROTECTS MICE AGAINST VIRUS CHALLENGE

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**Abstract.** The gene coding for the envelope (E) glycoprotein of dengue-2 virus was cloned into baculovirus (*Autographa californica* nuclear polyhedrosis virus). The recombinant virus contained the entire E protein gene, preceded by 38 nucleotides from the end of the prematrix glycoprotein gene and followed by the first 83 nucleotides of nonstructural protein 1. When expressed in *Spodoptera frugiperda* (Sf9) cells, approximately 1 mg of recombinant E antigen was made per  $10^9$  cells. This antigen reacted with polyclonal, anti-dengue type-2 antibody and a dengue type-2-specific, neutralizing monoclonal antibody. BALB/c mice immunized with the recombinant antigen produced only non-neutralizing antibody against dengue-2 virus, but were partially protected against morbidity and mortality after intracranial challenge with virulent dengue-2 virus.

Dengue is an acute disease of viral etiology characterized by fever, headache, body ache, and rash. Arguably, the most medically important arthropod-borne flavivirus, millions of dengue virus infections occur each year in tropical and subtropical regions of the world, where four serotypes are circulating.<sup>1,2</sup>

Like other flaviviruses, dengue virus is an enveloped, positive-strand RNA virus approximately 50 nm in diameter. A single open-reading frame of approximately 10.5 kilobases (kb) encodes all viral proteins, which are derived via cotranslational and post-translational proteolytic processing of a precursor polyprotein. The structural capsid (C) protein, the prematrix (prM) glycoprotein, the matrix (M) protein, and the envelope (E) glycoprotein are encoded at the 5' end; these are followed by the nonstructural (NS) proteins, NS1 through NS5.<sup>3</sup>

There are, at present, no safe and effective vaccines against dengue. Efforts to develop subunit vaccines have focused mainly on the E glycoprotein, the major surface antigen of the virion. It binds to cellular receptors and effects virus entry by fusion at acidic pH with host cell membrane.<sup>4,5</sup> The E antigen contains both T and B cell epitopes that appear to play an important role in immunity,<sup>6-9</sup> and the isolated, native protein has been demonstrated to subserve protection in the mouse model.<sup>10</sup> Therefore E antigen may serve as a subunit vaccine.

Positive results have been obtained in the mouse protection model with several recombinant flavivirus antigens, especially those made in vaccinia and baculovirus.<sup>11</sup> The baculovirus expression system appears especially promising for producing subunit protein vaccines because this insect virus is not pathogenic for humans and has the capability to correctly process most eukaryotic proteins. We have previously demonstrated that mice are protected against Japanese encephalitis virus (JEV) and against dengue 1 virus with recombinant E antigens made in baculovirus expression systems.<sup>12,13</sup>

The aim of the present study was to evaluate in mice a baculovirus-recombinant dengue-2 E antigen. We found that the recombinant immunogen conferred partial protection to mice against morbidity and mortality from virulent dengue-2 virus.

### MATERIALS AND METHODS

#### *Construction of a baculovirus-recombinant dengue 2 E glycoprotein gene and expression in Spodoptera frugiperda (Sf9) cells*

The RNA genome of dengue 2 virus (PR159 strain) was cloned and sequenced prior to this study, and a plasmid containing the E gene was a gift from Dr. James Strauss (California Institute of Technology, Pasadena, CA).<sup>14</sup> A DNA restriction fragment containing the complete E gene was obtained by cutting the plasmid with

\* Deceased.

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restriction endonucleases *Ple* I (cuts at nucleotide 899) and *Eco* RI (cuts at nucleotide 2504). This restriction fragment was treated with bacteriophage T4 DNA polymerase and all four deoxynucleoside triphosphates to make blunt ends and cloned into the unique *Sma* I site of a baculovirus recombination vector, pAcMGS, which has been described previously.<sup>12,13</sup> Recombinant baculovirus was obtained by cotransfecting Sf9 cells with the plasmid and wild-type baculovirus DNA and then screening for polyhedrin-negative plaques. A stock of the recombinant virus was used to infect Sf9 cells in suspension culture at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. Infected cells were harvested by centrifugation at  $1,000 \times g$  for 5 min, lysed by Dounce homogenization, and membrane fractions enriched for E protein were prepared from cell lysates.<sup>13</sup>

#### *Cell cultures, viruses, and antisera*

The Sf9 cells were propagated and infected with baculovirus as previously described.<sup>12,13</sup> *Aedes albopictis* cells (clone C6/36)<sup>15</sup> were propagated at 28°C in Eagle's minimum essential medium containing Earle's salts (EMEM; Gibco, Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS, heat inactivated at 56°C for 30 min), penicillin (100 units/ml), streptomycin (100 µg/ml), 2 mM glutamine, and 0.1 mM nonessential amino acids. For preparation of dengue-2 virus, C6/36 cells were grown to 95% confluence in 150-cm<sup>2</sup> tissue culture flasks. Cells were infected with dengue-2 virus at an MOI of 0.1 PFU per cell and maintained in EMEM with 0.6% bovine plasma albumin replacing FBS. After six to eight days of incubation, the media was harvested, clarified by centrifugation at  $16,000 \times g$  for 10 min, and viral antigen in the supernate was concentrated approximately 100-fold by centrifugation at  $100,000 \times g$  for 3 hr at 4°C. Viral antigen, composed primarily of virion-associated proteins and NS1, was resuspended in a small volume of phosphate-buffered saline, pH 7.4 (PBS) and stored at -80°C until use.

Polyclonal anti-dengue type-2 hyperimmune mouse ascitic fluid (HMAF), and monoclonal antibodies (MAbs) 3H5, 4G2, and 2H3 (E antigen-specific) have been characterized previously.<sup>6,16</sup>

#### *Polyacrylamide gel electrophoresis*

Proteins were solubilized in 66 mM Tris, pH 6.8, 1% sodium dodecyl sulfate (SDS), 1% glycerol, and 0.66% bromphenol blue at room temperature (not boiled), and electrophoresed on 15% polyacrylamide gels<sup>17</sup> modified by the inclusion of SDS in the running buffer but not in the gel. Molecular weights were determined by coelectrophoresis of prestained protein standards (Bethesda Research Laboratories, Rockville, MD).

#### *Western blot assay*

Proteins were analyzed by a nonisotopic modification of the Western blot procedure,<sup>18</sup> using an enzyme-linked second antibody. Briefly, proteins were separated on polyacrylamide gels and transferred electrophoretically to nitrocellulose paper. The paper was blocked with 5% casein in PBS, cut into 0.5 cm-wide strips, and incubated overnight at room temperature with either HMAF or sera obtained from recombinant-immunized mice. After the strip was washed with PBS, biotin-conjugated, goat anti-mouse serum (Sigma, St. Louis, MO) was added and incubated for 2 hr. The strips were washed and avidin-biotin-alkaline phosphatase conjugate (1:250 in 5% casein) was added for 45 min. The conjugate was removed, and after the strip was washed, substrate (5-bromo-4-chloro-3-indolyl phosphate with nitroblue tetrazolium in dimethylformamide; Sigma) was added. The reaction was stopped by washing the blots with water after maximum color development had occurred.

#### *Radioimmunoassay (RIA)*

The RIA was performed using antigens concentrated from dengue-2 virus-infected or uninfected C6/36 cell supernates; these were reacted with either dengue-2-specific HMAF (positive control), ascitic fluid from nonimmunized mice (negative control), or sera from mice immunized with recombinant protein. Antigens were spotted on nitrocellulose papers and the papers were blocked with 5% nonfat dry milk containing 0.001% sodium azide for 20 min. Sera or ascitic fluids, diluted 1:500 in PBS, were added to the papers and incubated overnight at room temperature. Bound antibody was detected using <sup>125</sup>I-labeled, goat anti-mouse IgG. After the pa-

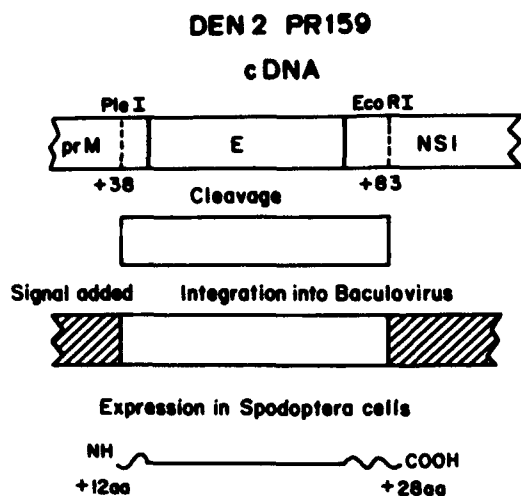


FIGURE 1. The envelope (E) E glycoprotein gene of dengue-2 (DEN 2) virus expressed in recombinant baculovirus. Shown (top to bottom) are the restriction sites used to generate a cleavage fragment that contains 38 nucleotides from the end of prematrix (prM) glycoprotein encoding a 12-amino acid (aa) hydrophobic signal sequence, the entire E protein-coding region, and 83 nucleotides (27 amino acids) from the beginning of nonstructural protein 1 (NS1). This restriction fragment was cloned into a plasmid transfer vector (pAcMGS) and integrated into baculovirus by homologous recombination. For expression, *Spodoptera frugiperda* (Sf9) cells were infected with the recombinant baculovirus.

pers were washed, they were counted in an LKB gamma counter (LKB-Wallac, Wallac, Inc., Turku, Finland).

#### Virus plaque reduction neutralization 50% (PRNT<sub>50</sub>) assay

Mouse sera were assayed for dengue-2 virus neutralizing antibody by PRNT assay with a 50% neutralization endpoint.<sup>13</sup>

#### Immunization of mice and protection assay

Mice (three-week-old, female, BALB/c) were immunized by intramuscular (im) and intraperitoneal injection with 0.1 ml of baculovirus-infected Sf9 cell microsomal membrane protein preparation (100,000 × g/hr lysate pellet) enriched for recombinant dengue-2 E antigen, or an equivalent amount of membrane protein preparation without E antigen (negative control). Intraperitoneal injections were given without ad-

TABLE 1

Reaction of recombinant and authentic dengue-2 envelope (E) antigens with monoclonal and polyclonal antibodies

| Antibodies* | Antigens       |              |
|-------------|----------------|--------------|
|             | Recombinant E† | Authentic E‡ |
| 4G2         | 1.3 (–)        | +            |
| 2H3         | 0.6 (–)        | +            |
| 3H5         | 3.2 (+)        | +            |
| HMAF        | 3.3 (+)        | +            |
| NMAF        | 0.9 (–)        | –            |

\* Monoclonal antibody ascitic fluids 4G2, 2H3, and 3H5 (E antigen-specific) were used at a 1:250 dilution. Polyclonal anti-dengue-2 hyperimmune mouse ascitic fluid (HMAF) and normal mouse ascitic fluid (NMAF) were diluted 1:500.

† A radioimmunoassay was performed using antigen prepared from recombinant baculovirus-infected Sf9 cell lysate or control antigen prepared from wild-type baculovirus-infected Sf9 cell lysate containing an equivalent amount of protein. A test was reported as positive (+) if the counts per min (cpm) obtained with recombinant antigen divided by the cpm obtained with control antigen was greater than 2.0.

‡ A Western blot was performed as described in the Materials and Methods with dengue-2 virus from C6/36 cells. A positive reaction indicates specific staining of the E protein band.

juvant, and im injections were given with an equal volume of Freund's complete adjuvant for the first inoculation (day 0), and with Freund's incomplete adjuvant for two boosters (day 7 and day 14). Sera were obtained one day prior to dengue virus challenge from blood collected aseptically from the retro-orbital plexus with a sterile glass capillary pipette. Mice were challenged on day 21 (at six weeks of age) by intracranial (ic) injection with 10<sup>5</sup> PFU (approximately 100 50% lethal doses) of live, mouse-adapted dengue-2 virus (NGC strain). Mice were observed for up to 24 days for morbidity and mortality and categorized as 1) healthy (no apparent signs of infection), 2) ruffled (the fur had a ruffled look compared with normal mice), or 3) paralyzed (slowed gait, partial or complete inhibition of hind limb mobility). Data were tested for statistical significance using the chi-square or Fisher's exact tests, as appropriate.

#### RESULTS

##### Analysis of the recombinant dengue-2 E protein

For expression of the dengue-2 E gene in Sf9 cells, a recombinant baculovirus was constructed as shown in Figure 1. Cell lysates from recombinant baculovirus-infected Sf9 cells and concentrated culture media from dengue-2 virus-infected and uninfected C6/36 cells were compared by Western blotting with dengue-2 vi-

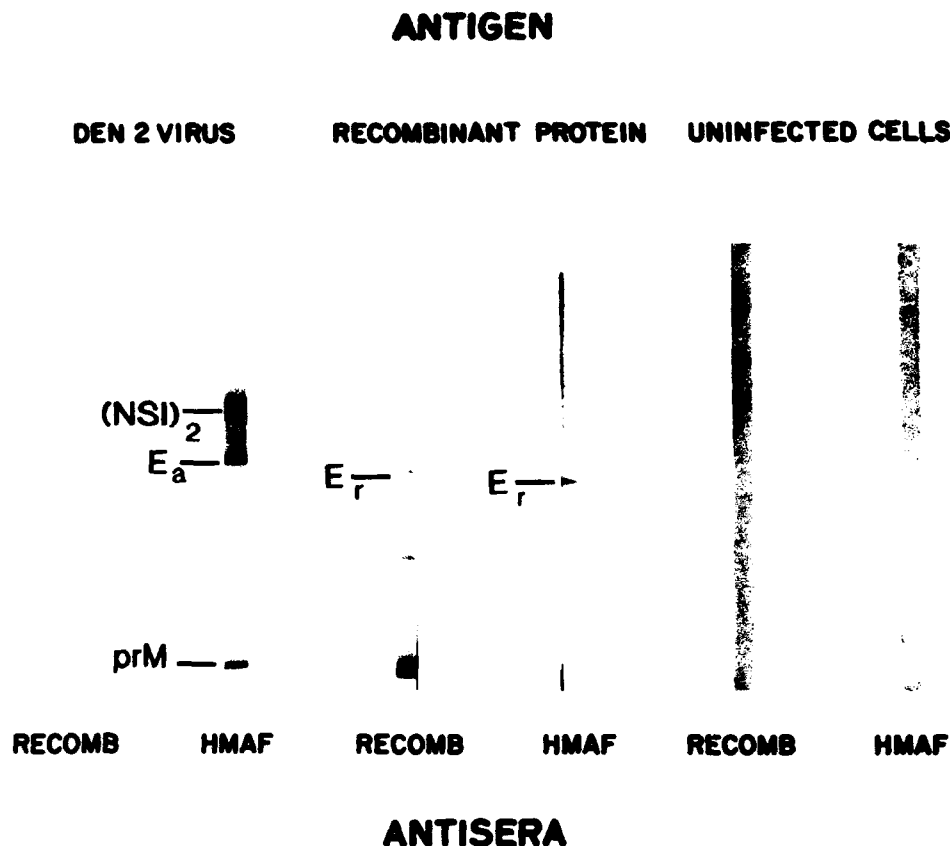


FIGURE 2. Western blot analysis of the baculovirus-recombinant (RECOMB) dengue-2 (DEN 2) envelope (E) protein. Authentic dengue-2 viral antigens from C6/36 cells (two left lanes), recombinant dengue-2 E antigen from baculovirus-infected Sf9 cells (two center lanes), and uninfected C6/36 cells (two right lanes) were subjected to polyacrylamide gel electrophoresis and Western blotted with anti-dengue-2 hyperimmune mouse ascitic fluid (HMAF) or antisera from mice immunized with the recombinant dengue-2 E antigen. Apparent molecular weights were determined using prestained protein standards that were electrophoresed on adjacent lanes of the same gel (not shown). Bands indicated are nonstructural protein 1 (NS1)<sub>2</sub> (NS1 dimer), authentic E protein (E<sub>a</sub>), recombinant E protein (E<sub>r</sub>), protein), and prematrix (prM) glycoprotein.

rus-specific, HMAF, or sera from mice immunized with the recombinant lysate (Figure 2). The HMAF reacted with a single protein in the recombinant baculovirus-infected cell lysate that had approximately the same electrophoretic mobility as authentic E protein. Sera from mice immunized with the recombinant lysate also reacted with the same protein and with additional protein bands presumed to be Sf9 cell antigens. The HMAF, but not sera from recombinant baculovirus-immunized mice, reacted with authentic dengue-2 virus proteins, NS1 dimer (~70 kD), E (~60 kD), and prM (~20 kD).

To verify the antigenic identity of the recombinant E protein, Mabs with demonstrated spec-

ificity to authentic E antigen<sup>6</sup> were reacted in an RIA with recombinant E antigen. A positive reaction was seen with one MAb, 3H5, while no reaction was seen with two others, 4G2 or 2H3 (Table 1).

The recombinant E antigen made in baculovirus-infected Sf9 cells was detected by RIA in cell lysates but not in extracellular medium, and, therefore, appeared to be cell-associated. The yield of recombinant E antigen was estimated to be approximately 1 mg per 10<sup>9</sup> cells by comparing the intensity of bands on a Western blot assay of recombinant-infected cell lysate and highly-purified E antigen of known concentration.

TABLE 2  
Antibody response of mice immunized with baculovirus-recombinant dengue-2 envelope (E) antigen\*

| Total antigen dose ( $\mu$ g) | RIA (P/N)† | PRNT <sub>50</sub> ‡ |
|-------------------------------|------------|----------------------|
| 1.5                           | 7.4        | <10                  |
| 9.0                           | 13.5       | <10                  |
| 19.5                          | 164.0      | <10                  |

\* Reaction by radioimmunoassay (RIA) and dengue virus plaque-reduction neutralization (PRNT) assay of sera from mice immunized with different doses of recombinant E antigen.

† Positive/negative (P/N) = counts per min (cpm) obtained with antigen prepared from dengue-2 virus-infected C6/36 cell supernates, divided by cpm obtained with uninfected C6/36 cell supernates used at an approximately equal total protein concentration. Mouse sera were used at a 1:500 dilution. P/N values >2.0 were considered positive.

‡ Virus PRNT assay with a 50% endpoint (PRNT<sub>50</sub>). Values of 10 or greater were considered positive, <10 negative.

#### Immunization of BALB/c mice with recombinant dengue 2 E antigen

To test the recombinant E protein as a protective immunogen, groups of containing 10 mice each were immunized with 1.5, 9, or 19.5  $\mu$ g of

recombinant protein administered in three equal-volume doses. After immunization, the mice were bled and sera were tested for virus-binding and neutralizing antibody against dengue-2 virus. Sera from recombinant-immunized mice showed antibody that bound to dengue virus antigen, with the highest level seen in mice immunized with the highest dose of recombinant antigen. However, none of the sera had measurable titers of anti-dengue-2 virus-neutralizing antibody (Table 2).

Immunized mice were then challenged with live dengue-2 virus and observed for morbidity and mortality (Table 3 and Figure 3). The number of mice that survived challenge was one of 10 in the control group, five of 10 in the group that received 1.5  $\mu$ g of recombinant antigen ( $P = 0.05$ , by Fisher's exact test), nine of 10 in the group which received 9  $\mu$ g ( $P = 0.0005$ ), and six of 10 in the group that received 19.5  $\mu$ g ( $P = 0.03$ ). Although the protection rate decreased

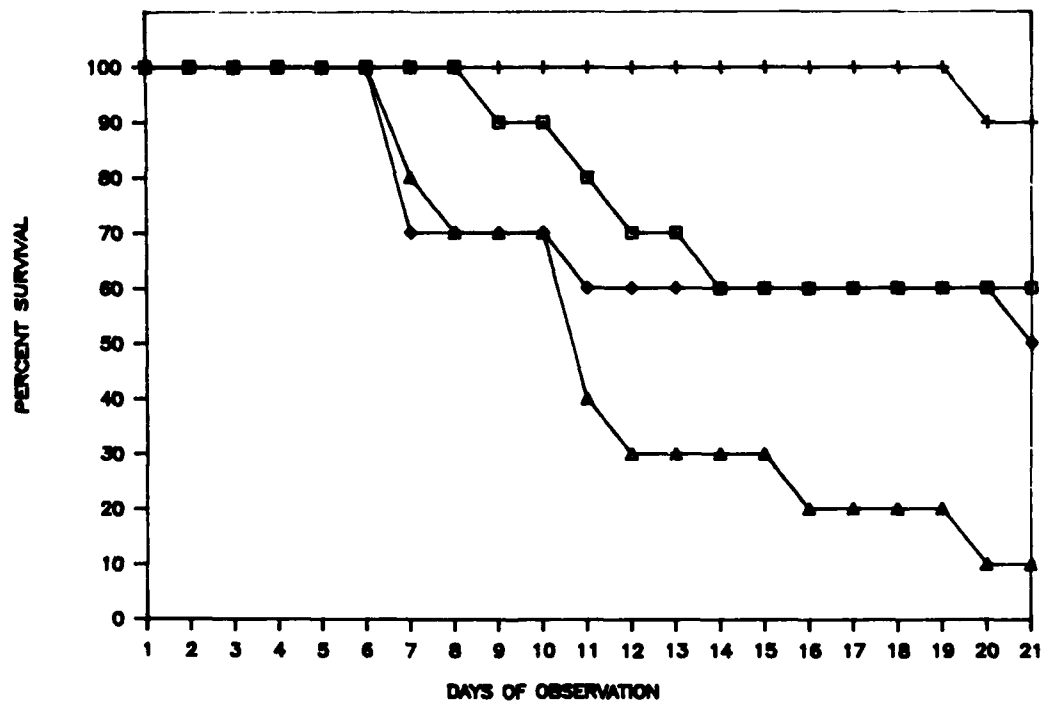


FIGURE 3. Survival of mice immunized with recombinant dengue-2 envelope (E) antigen following challenge with live dengue-2 virus. Groups of 10 mice each were immunized with 1.5  $\mu$ g (◇), 9  $\mu$ g (+), and 19.5  $\mu$ g (□) of baculovirus-recombinant dengue-2 E antigen or with an equivalent amount of protein from baculovirus-infected Sf9 cells that did not contain E antigen (Δ). Following immunization, mice were challenged by intracranial injection with approximately 100 50% lethal doses of dengue-2 virus (NGC strain). The percentage of mice surviving at each day postchallenge is shown.

somewhat in the latter group, this was not statistically significant ( $P = 0.15$ ).

Immunized mice also showed some protection against morbidity that was significant, however, this was observed only in the group that received 9  $\mu$ g of recombinant E antigen ( $P = 0.03$ , by chi-square test). In this group, the day of onset of illness as determined by appearance of ruffled fur or slowed movement was delayed (day 9.6 versus day 5.5 in controls), the length of illness was decreased in those mice that survived challenge (5.4 days versus 14.5 days in controls), and the mean time to death was increased in nonsurvivors (8 days versus 3.5 days in controls).

#### DISCUSSION

A 1.6-kb dengue-2 cDNA restriction fragment encoding a 12-amino acid hydrophobic signal peptide at the end of the prM protein, the entire E protein, and the first 27 amino acids of NS1 protein was cloned and expressed using a recombinant baculovirus. The recombinant protein reacted with anti-dengue-2 HMAF and with a dengue-2-specific, neutralizing MAb (3H5) that recognizes a linear epitope on E protein.<sup>19</sup> However, the recombinant protein failed to react with two other E-specific MAbs (4G2 and 2H3). These MAbs appear to recognize epitopes that are more conformation-dependent<sup>20</sup> (unpublished data), suggesting that significant differences in folding exist between the recombinant and the native protein.

When used to immunize mice, the recombinant E protein stimulated, in a dose-dependent manner, the production of antibody that bound to authentic dengue-2 virus in an RIA. However, this antibody failed to neutralize dengue-2 virus in vitro. Interestingly, although the recombinant E antigen failed to stimulate dengue virus-neutralizing antibody, immunized mice were partially protected (up to 90%) against death from dengue encephalitis.

While several studies, including earlier ones from our laboratory with baculovirus recombinant dengue-1 and JEV E antigens, found a positive correlation between the presence of virus-neutralizing antibody and protection,<sup>12,13</sup> other investigators have shown that mice can be solidly protected against dengue encephalitis in the absence of detectable neutralizing antibody.<sup>21</sup> Results of present study also suggest that factors

TABLE 3

*Effect of vaccination with recombinant envelope antigen on dengue morbidity in mice*

| Dose ( $\mu$ g) | Day of onset of illness* | Length of time (days) from onset of illness to death | Length of illness† | Day of death |
|-----------------|--------------------------|------------------------------------------------------|--------------------|--------------|
| 0               | 5.5                      | 3.5                                                  | 14.5 (1)           | 9.6          |
| 1.5             | 6.5                      | 3.8                                                  | 9 (5)              | 10           |
| 9.0             | 9.6                      | 8                                                    | 5.4 (9)            | 20           |
| 19.5            | 7.7                      | 3                                                    | 9.5 (6)            | 9.5          |

\* Day of onset of illness is the mean determined for all mice in each group.

† Length of illness is the mean number of days of illness determined for those mice that ultimately survived challenge and recovered. The number of survivors is shown in parentheses.

other than antibody play a crucial role in protection. One explanation is that T cell epitopes play a large role. Unlike most B cell epitopes, which seem to depend upon secondary structure,<sup>20</sup> most helper and cytotoxic T cell epitopes appear to be definable by primary structure alone<sup>22</sup>, and therefore may not be so dependent upon antigen conformation. Additional studies need to be done to test this hypothesis and to elucidate other factors that may subserve protection.

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**Disclaimer:** The views presented are those of the authors and should not be construed as official policy positions of the Department of the Army or the DOD.

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