Synergistic Neutralizing Antibody Response to a Dengue Virus Type 2 DNA Vaccine by Incorporation of Lysosome-Associated Membrane Protein Sequences and Use of Plasmid Expressing GM-CSF

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We have previously shown that a dengue virus type 1 DNA vaccine expressing premembrane (prM) and envelope (E) genes was immunogenic in mice and monkeys and that rhesus monkeys vaccinated with this construct were completely protected from virus challenge. In order to improve the immunogenicity of dengue DNA vaccines, we have evaluated the effect of lysosome targeting of antigens and coimmunization with a plasmid expressing GM-CSF on antibody responses. A dengue virus type 2 candidate vaccine containing prM and E genes was constructed in which the transmembrane and cytoplasmic regions of E were replaced by those of the lysosome-associated membrane protein (LAMP). The modified vaccine construct expressed antigen that was colocalized with endogenous LAMP in lysosomal vesicles of transfected cells, whereas the antigen expressed from the unmodified construct was not. It was hypothesized that targeting of antigen to the lysosomal compartment will increase antigen presentation by MHC class II, leading to stronger CD4-mediated immune responses. Mice immunized with the modified construct responded with significantly higher levels of virus neutralizing antibodies compared to those immunized with the unmodified construct. Coimmunization of mice with a plasmid expressing murine GM-CSF enhanced the antibody response obtained with either the unmodified or the modified construct alone. The highest antibody responses were noted when the modified construct was coinjected with plasmid expressing the GM-CSF gene. These results could form the basis for an effective tetravalent dengue virus DNA vaccine.

Key Words: dengue; DNA vaccine; LAMP; GM-CSF; neutralizing antibody; lysosome targeting.

INTRODUCTION

Dengue is one of the most important arboviral diseases of humans. The four antigenically distinct serotypes of dengue virus (Genus Flavivirus, Family Flaviviridae) cause an estimated 100 million infections resulting in widespread dengue fever (DF) and at least 250,000 cases of dengue hemorrhagic fever (DHF) each year (Halstead, 1988). These viruses are transmitted primarily by the mosquito Aedes aegypti and are endemic throughout the tropical and subtropical regions of the world. A major expansion of dengue transmission has occurred throughout the tropical Americas accompanied by a large increase in the number of DHF cases. Despite the public health importance of DF and DHF, no effective control measure is available other than vector reduction, which has proven difficult and costly to sustain over time (Gubler and Trent, 1994). Although vaccines are available for two related flaviviruses, yellow fever (YF) virus and Japanese encephalitis (JE) virus (Barrett, 1997a,b), decades of effort have not produced a successful dengue virus vaccine. Live attenuated dengue viruses, while immunogenic, have been associated with high reactogenicity and clinical complications (Edelman et al., 1994; McKee et al., 1987). Inactivated dengue viruses have been reported to be less immunogenic (Schlesinger, 1977), and recombinant dengue proteins have failed to protect nonhuman primates from homologous dengue virus challenge (Deubel et al., 1988; Eckels et al., 1994).

We have been exploring DNA vaccine technology in which plasmids expressing appropriate viral antigens are used for immunization. This approach has been shown to induce an immune response in animal models against a number of different viruses, including several flaviviruses (Colombage et al., 1998; Konishi et al., 1998; Phillpotts et al., 1996; Schmaljohn et al., 1997). We have previously shown that a dengue-2 DNA vaccine containing the genes for premembrane (prM) and 92% of envelope (E) protein induced neutralizing antibody response in mice (Kochel et al., 1997) and the vaccinated mice had a higher survival rate following dengue-2 virus challenge (Porter et al., 1998). When four different dengue-1 DNA
vaccine candidates expressing truncated or full-length E with or without prM were compared, we observed that the candidate expressing prM and full-length E produced virus-like particles and elicited long-lasting neutralizing antibodies in mice (Raviprakash et al., 2000a). This dengue-1 vaccine was further shown to be immunogenic in nonhuman primates and afforded complete to partial protection from challenge (Kochel et al., 2000; Raviprakash et al., 2000b). In all these studies, the virus neutralizing antibody levels were low. We are now attempting to modify the dengue DNA vaccine to achieve enhanced antibody response.

Although DNA vaccines give rise to antigen-specific CTL responses in vaccinated animals, they have performed poorly with regard to stimulation of antibody production. This is probably because the majority of the endogenously produced antigen is sequestered into the major histocompatibility complex (MHC) class I pathway of antigen presentation. For dengue viruses, it is believed that antibody can afford protection against infection and that neutralizing antibody is an important component of this protection. It is therefore desirable that a dengue vaccine produce high titers of neutralizing antibody. For successful immune response and antibody production, it is critical that the antigen be presented to CD4+ T helper cells by the class II MHC molecules. The MHC class II antigen processing and presentation pathway that conventionally operates in professional antigen-presenting cells (APC) is accessed by endocytosis or phagocytosis of extracellular antigen. This pathway, therefore, may not operate efficiently in cells that take up the vaccine DNA and synthesize the antigen intracellularly. It may thus be beneficial to target the antigen expressed by DNA vaccines to the lysosomal compartment for degradation and successful loading onto MHC class II molecules, consequently leading to enhanced CD4+ helper T cell response.

The lysosome-associated membrane protein (LAMP) is a major protein of mouse embryo 3T3 cells and is specifically associated with the lysosomal membrane. A tetrapeptide Tyr-X-X-hydrophobic residue sequence in the carboxyl-terminal sequence of LAMP has been identified as the sequence responsible for its targeting to the lysosomal membrane (Guarnieri et al., 1993). Viral proteins to which the carboxyl-terminal sequence of LAMP has been fused have been shown to accumulate in the lysosomes of transfected cells (Rowell et al., 1995). We have constructed dengue-2 DNA vaccine candidates that express prM and E (D2ME) or prM and E-LAMP (D2MEL) chimeric protein. Here we report that the protein expressed by D2MEL and not D2ME is colocalized with endogenous LAMP. When injected into mice, D2MEL elicited an enhanced antibody response, which was further augmented by coimmunization with a plasmid expressing mouse granulocyte-monocyte colony stimulating factor (GM-CSF).

**RESULTS**

Results discussed in this section are representative of three independent experiments utilizing separate batches of purified DNA. The mice were vaccinated with three doses of DNA in quick succession on days 1, 11, and 21. This immunization schedule was borrowed from our earlier studies (Porter et al., 1998) where the intent was to challenge mice when they are still young. However, similar antibody responses were observed when mice were given two doses on days 1 and 28.

**D2MEL vaccine is more immunogenic than D2ME**

The results of an experiment in which 1040D2ME (dengue-2 prM and E genes cloned into vector pVR1040) was compared with 1040D2MEL (1040D2ME–LAMP chimera) for immunogenicity in mice are summarized in Fig. 1. Groups of mice (n = 5) were immunized on days 1, 11, and 21 with 50 μg of VR1040, 1040D2ME, or 1040D2MEL DNA with or without VR1701 (plasmid expressing murine GM-CSF). No anti-dengue antibody was detected in mice vaccinated with control DNAs VR1040 and VR1701 (not shown). None of the 5 mice immunized with 1040D2ME seroconverted on day 30, and only 2 had seroconverted on day 120 (Fig. 1A). In contrast, all the 5 mice immunized with 1040D2MEL had seroconverted on day 30 and their antibody levels remained stable through day 120 (Fig. 1B).

**Codelivery of a plasmid expressing murine GM-CSF augments antibody response**

We evaluated the effect of coadministration of DNA expressing the murine GM-CSF gene by vaccinating groups of mice (n = 5) with 50 μg 1040D2ME or 1040D2MEL, plus 10 μg VR1701 plasmid. It was clear that the addition of VR1701 had a marked effect on the antibody response. All 5 mice seroconverted on day 30 after immunizing with D2ME + VR1701 compared to no seroconversion when injected with D2ME alone (Figs. 1A and 1C). Further, the antibody levels increased dramatically by day 120. In mice that were vaccinated with D2MEL + VR1701, the highest antibody levels were seen on day 30 and were sustained through day 120 (Fig. 1D).

**Virus neutralization**

The ability of antibody produced in response to vaccines to neutralize dengue virus in vitro was measured by the plaque reduction neutralization test (PRNT). The neutralization of virus at each dilution of the serum is shown in Fig. 2. Some virus neutralizing activity (<50%) was present in the pooled sera from the group vaccinated with the control plasmids VR1040 and VR1701. This activity, which was observed only on day 30, disappeared in later bleeds. Sera from the group vaccinated with 1040D2MEL also showed low neutralizing activity that persisted through day 60. In contrast, sera from mice
immunized with 1040D2MEL had 50% neutralization titers (PRNT50) of 48–270 from day 30 to 120 (Fig. 2 and Table 1). Coimmunization with pVR1701 markedly increased the PRNT50 titers for both 1040D2MEL and 1040D2ME immunized mice. Mice vaccinated with 1040D2ME + pVR1701 had PRNT50 titers of 40–120 from day 60 to 120. The highest titers of 340–1250 from day 30 to 120 were seen in mice vaccinated with 1040D2MEL + pVR1701. These titers persisted for more than a year (not shown).

Antibody avidity

In order to determine whether there was a qualitative difference in the antibody produced by different immunities, we evaluated the avidity of antibody to bind antigen. Each serum sample was diluted so as to obtain an absorbance of 0.8–1.5 by ELISA. The ability of antibody to bind antigen in the presence or absence of 6 M urea was measured as described under Materials and Methods, and an avidity index (AI) for each serum sample was calculated. There was no significant difference in the avidity of antibody from mice vaccinated with D2ME + VR1021, D2MEL, or D2MEL + VR1021 (Fig. 3). The mean AI for these groups was 44.4, 51.7, and 45, respectively.

Antigen expressed from D2MEL chimeric vaccine construct is colocalized with endogenous LAMP

NIH3T3 cells were transfected with 1040D2ME or 1040D2MEL and the expression and cellular steady-state localization of the dengue virus antigen were compared to those of the endogenous LAMP proteins of the cell by dual fluorescence staining and confocal microscopy. Endogenous LAMP showed the typical localization in lysosomal bodies in the cells transfected with either the wild type (1040D2ME) or the modified (1040D2MEL) construct (Figs. 4A and 4E). When cells were stained using the dengue-2 E-specific monoclonal antibody 3H5, those transfected with the wild-type 1040D2ME showed the expected membrane reticular distribution associated with viral envelope protein (Fig. 4B). In contrast, the chimeric protein in 1040D2MEL transfected cells was distributed in a pattern similar to that of the endogenous LAMP (Fig. 4F). When Figs. 4E and 4F are superimposed, extensive colocalization of the chimeric D2MEL protein with endogenous LAMP protein was observed (Figs. 4G and 4H). No such colocalization of the unmodified D2ME was evident when Figs. 4A and 4B were superimposed (Figs. 4C and 4D).

**DISCUSSION**

We have shown that a modified dengue virus E protein in which the ectodomain of E is fused with the transmembrane and cytoplasmic domains of LAMP-1 is effectively targeted to the lysosomes of NIH3T3 cells where it is colocalized with endogenous LAMP. Further, we have shown that the vaccine construct expressing this chimeric antigen elicited markedly better neutralizing antibody responses than its wild-type counterpart and that the antibody responses could be further augmented by coimmunization with a plasmid expressing the mouse GM-CSF gene.
Previously, we have shown that a dengue virus type 1 DNA vaccine expressing prM and E genes was immunogenic in mice (Raviprakash et al., 2000a). In rhesus macaques, as well as in the New World Aotus monkeys, this dengue-1 vaccine elicited immune responses that afforded complete to partial protection from viremia upon virus challenge (Kochel et al., 2000; Raviprakash et al., 2000b). Neutralizing antibodies are thought to be of paramount importance for protection against dengue disease (Kliks et al., 1988). Because DNA vaccines cause intracellular synthesis of foreign antigens, the antigens more efficiently enter the MHC class I pathway of antigen presentation, leading to better cellular immune responses than humoral immune responses. To increase the antibody responses, it is important that the antigen be efficiently presented by class II MHC to CD4+ helper T cells. Therefore, it may be beneficial to target the antigen to intracellular compartments that are rich in class II MHC. Several reports have shown that LAMP and MHC II are colocalized in specialized cellular vesicles.

FIG. 2. Plaque reduction neutralization test. Sera from antibody-positive mice for groups injected with VR1040, VR1040 + VR1701, 1040D2ME, 1040D2ME + VR1701, 1040D2MEL, and 1040D2MEL + VR1701 were pooled and used in PRNT starting at 1:20 dilution. Sera collected on days 30, 60, 90, and 120 were used, and the percentage virus neutralization for each group (mean of duplicate assay) is shown for various serum dilutions, along with the standard error.
cicles, called MIIC of the endosomal/lysosomal vesicular pathway of APC such as dendritic cells and macrophages (Harding and Geuze, 1993; Kleijmeer et al., 1995; Peters et al., 1995; Turley et al., 2000). It has been shown that proteins, when encoded by plasmids engineered to contain a ER translocation signal sequence and the LAMP transmembrane and cytoplasmic domains, are directed to the MHC II compartment (Guarnieri et al., 1997; Wu et al., 1996; Nair et al., 1995; Ruff et al., 1998; Rowell et al., 1995). By applying this LAMP trafficking technology, we have shown here that the dengue-2/LAMP chimeric DNA vaccine encoded antigens, including human papilloma virus E7, HIV gp160, and carcino-embryonic antigen, has been shown to result in a marked increase in both the cellular and the humoral immune responses in vaccinated mice (Lin et al., 1996; Nair et al., 1998; Rowell et al., 1995; Ruff et al., 1997; Wu et al., 1995). We have recently shown that intramuscular injection of rabies virus DNA vaccine expressing the glycoprotein along with GM-CSF plasmid led to increased B and T-helper cell activity to rabies virus (Xiang and Ertl, 1995). It has been recently shown that intramuscular injection of plasmid expressing GM-CSF attracts infiltrates including CD11c⁺ immature dendritic cells and that immune enhancement is associated with the presence of CD11c⁺ cells in the infiltrates (Haddad et al., 2000). We have shown here that when the unmodified dengue-2 DNA vaccine was coinjected with GM-CSF plasmid, significantly higher levels of neutralizing antibody were obtained. This effect was similar to that obtained by dengue-2/LAMP chimeric DNA vaccine in the absence of the adjuvant. Evidently, LAMP targeting and the use of GM-CSF as genetic adjuvant had similar immune enhancements, albeit by different mechanisms. When these two techniques were used together, as in coinjection of D2ME and GM-CSF plasmid, remarkably high neutralizing antibodies were obtained.

Table 1: 50% Virus Neutralization Titers (PRNT<sub>50</sub>)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
<th>Day 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR1040 + VR1701</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1040D2ME</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>1040D2MEL</td>
<td>48</td>
<td>115</td>
<td>94</td>
<td>270</td>
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<tr>
<td>1040D2ME + VR1701</td>
<td>0</td>
<td>120</td>
<td>40</td>
<td>120</td>
</tr>
<tr>
<td>1040D2MEL + VR1701</td>
<td>340</td>
<td>510</td>
<td>1250</td>
<td>1000</td>
</tr>
</tbody>
</table>

Note. For each group, sera from mice that seroconverted were pooled. The pooled sera were used in a plaque reduction neutralization test and PRNT<sub>50</sub> titers were determined by probit analysis.

FIG. 3. Antibody avidity. Antibody avidity for sera from individual mice vaccinated with 1040D2ME + VR1701, 1040D2MEL, or 1040D2MEL + VR1701 was measured by ELISA in the presence and absence of 6 M urea. Avidity index (AI) was calculated as 100 × (OD<sub>urea</sub>/OD<sub>urea, urea</sub>). Mean AI for each group is shown with standard error.
was no significant difference in antibody avidity for the various groups of mice.

It is clear from the results that a significant improvement in the virus neutralizing antibody response against dengue virus type 2 could be obtained by a combination of LAMP-targeting and coimmunization with GM-CSF plasmid. Since tetravalent immunity is key to a successful dengue vaccine, further studies with other dengue serotypes are currently under way. In addition, testing these concepts in nonhuman primates, and possibly in humans, is warranted.

MATERIALS AND METHODS

Cells, virus, and antibodies

Vero cells and NIH3T3 cells were obtained from ATCC (Rockville, MD) and were grown according to the supplier's specifications in MEM (Earl's salts) containing 10% fetal bovine serum (FBS). Dengue virus type 2 New Guinea C strain (ATCC) was propagated in Vero cells to an average titer of $10^6$ plaque-forming units (PFU) per milliliter. The virus was concentrated by precipitation with polyethylene glycol (7%) in the presence of 2.5% NaCl. The virus pellet was washed and the concentrated virions were used to coat ELISA plates for antibody detection. Dengue-2 polyclonal hyperimmune ascitic fluid was obtained from ATCC. Monoclonal antibodies 3H5 (specific for dengue-2 envelope protein) and 1D4B (specific for mouse LAMP protein) were obtained from supernatants of corresponding hybridoma cultures.

Vaccine constructs

Dengue virus type 2 (strain New Guinea C) sequence between nucleotides 367 and 2421 (Irie et al., 1989) was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) of viral RNA using MMuLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) and Taq polymerase (Perkin—Elmer, Palo Alto, CA). These sequences included the carboxy-terminal sequences of the capsid serving as leader sequence, the prM, a second hydrophobic signal sequence, and the E gene. The forward primer contained the Kozak sequences and the reverse primer contained a translational stop codon. The amplified sequences were cloned into vector pVR1040 (Vical, Inc., San Diego, CA), to produce 1040D2ME. This vector is similar to pVR1012 (Hartikka et al., 1996), but has four copies of the mouse immunostimulatory sequence (ISS) containing the CpG motif. 1040D2MEL was constructed by replacing the carboxy-terminal 129 bp of the E gene with sequence encoding the membrane an-
cho and cytoplasmic domains of LAMP-1 (120 bp). A cartoon of the proteins expressed from these constructs is shown in Fig. 5. Plasmid VR1701 expressing the murine GM-CSF gene was from Vical Inc. All vaccine DNAs were purified by alkaline lysis (Maniatis et al., 1984) followed by chromatography on a Sepharose S-1000 (Pharmacia) column (Horn et al., 1995).

Transfection and immunofluorescence

NIH3T3 cells were transfected with plasmids D2ME (encoding dengue-2 prM and E), D2MEL (encoding dengue-2 prM-E-LAMP chimera), or empty vector plasmid pVR1040 using FuGene 6 (Boehringer Mannheim). Cells were grown on coverslips in 6-well tissue culture plates, and 2 µg plasmid with 6 µg FuGene 6 was used per transfection. Forty-eight hours after transfection, the coverslips were transferred to a 24-well plate and washed with PBS (phosphate-buffered saline). The cells were fixed with 2% paraformaldehyde and blocked with 4% normal goat serum and 0.1% saponin in PBS. Dengue envelope was detected with 3H5 monoclonal antibody and FITC-labeled goat anti-mouse IgG. Endogenous LAMP was detected with 1D4B monoclonal antibody and Texas red-labeled goat anti-rat IgG. The coverslips were then mounted onto glass slides using ProLong (Molecular Probe). Fluorescence microscopy and digital image acquisition were carried out with a Nikon TE300 confocal microscope.

Immunization of mice

Unless otherwise indicated, all immunizations were intradermal near the base of the tail with 50 µg for the vaccine or control plasmid and 10 µg of the VR1701 (a plasmid expressing murine GM-CSF gene) where indicated. Six groups (n = 5) of mice were inoculated with three doses on days 1, 11, and 21 as follows: Group A: pVR1040; Group B: pVR1040 + VR1701; Group C: 1040D2ME; Group D: 1040D2MEL + VR1701; Group E: 1040D2MEL; Group F: 1040D2MEL + VR1701. Blood samples were collected on day 30 and monthly thereafter, by the periorbital route. Sera from the samples were stored frozen until used.

Antibody analyses

Measurements of anti-dengue antibody titers in the serum samples were done using twofold serial dilutions of sera in an ELISA according to Ansari et al. (1993) except that polyethylene glycol (PEG) precipitated dengue-2 virions were used as the antigen. End-point titers were defined as the highest dilution of the serum that produced an absorbance that was 3 standard deviations or more higher than the controls.

Antibody avidity measurements were performed by comparing antibody binding to antigen in the presence and absence of 6 M urea in a standard ELISA (Narita et al., 1998). Briefly, sera were diluted so as to obtain a standard ELISA OD value of between 0.8 and 1.5. ELISA was performed in duplicate microtiter plates. After antibody binding, both plates were washed three times with wash buffer. To one plate, 200 µl of wash buffer was added per well and to the other plate, 200 µl of wash buffer containing 6 M urea was added. Both plates were incubated for 5 min at room temperature. The buffers were removed and the plates were washed three more times with wash buffer. Incubations with conjugate and substrate were carried out according to the standard ELISA protocol. The AI was calculated as 100 × (ODurea/ODwater).

Virus neutralization titers were measured by a PRNT as described (Russell et al., 1967). Serial twofold dilutions of the serum samples were used and the PRNT50 titers were determined by probit analysis.

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