Parvovirus B19 Empty Capsids as Antigen Carriers for Presentation of Antigenic Determinants of Dengue 2 Virus

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For the production of dengue-vaccine candidates, empty capsids, or virus-like particles (VLPs), of parvovirus B19 that carry dengue 2–specific epitopes were employed as antigen carriers. Two epitopes (comprising amino acids 352–368 and 386–397) of domain BIII of the envelope glycoprotein were chosen to produce recombinant B19 VLPs for immunization of BALB/c mice. Serum samples from immunized mice revealed that recombinant B19 VLPs elicited strong humoral immune responses. In summary, this B19 VLP–vaccine platform produced high \((\geq 2.0 \times 10^5)\) anti–dengue 2 titers and robust \((\leq 1:120)\) 50%-plaque-reduction neutralization test (PRNT50) titers, which effectively neutralized live dengue 2 virus in PRNT50 assays.

Dengue virus is a member of the Flaviviridae family, which comprises 4 antigenically different serotypes. Dengue-virus infection represents a major cause of mortality and morbidity in the developing world [1]. Infected individuals worldwide suffer from dengue fever \((5–10 \times 10^5\) cases/year), dengue hemorrhagic fever \((2–5 \times 10^5\) cases/year), and, in the most severe cases, dengue shock syndrome, with mortality rates of up to 44% in young children [2]. Strategies for dengue-vaccine development have included the use of live attenuated strains, chimeric or inactivated viruses, subunit vaccines, and DNA vaccines. Although several candidates (live attenuated and recombinant) are in phase 1 and phase 2 stages of clinical evaluation, none has yet emerged as a successful vaccine [3]. In our effort to develop a vaccine against dengue virus, we focused on the envelope glycoprotein (E-glycoprotein), which represents the primary target for induction of neutralizing antibodies. Its fine structure has recently been determined, and 3 structural domains have been identified; of these 3, domain BIII is especially important, because it contains immunoglobulin-like loops with neutralizing epitopes most distal to the capsid surface and receptor-binding motifs.

Parvovirus B19, a nonenveloped single-stranded DNA virus with a 5.6-kb genome, encodes for a major capsid protein (i.e., viral protein 2 [VP2] [58 kDa]) and a minor capsid protein (i.e., viral protein 1 [VP1] [83 kDa]). VP1 and VP2 are derived from overlapping reading frames, and they coassemble to form an icosahedral viral capsid composed of 60 monomers (>95% VP2, <5% VP1). VP1 and VP2 are identical in their sequence except for an extra 228 aa at the VP1 N-terminus, also called the “VP1 unique region.” VP2 will self-assemble, either alone or with VP1, to form virus-like particles (VLPs); VP1 alone does not self-assemble. Studies have shown that the 228-aa VP1 unique region interferes progressively with particle assembly when >70 aa of the unique region are present. The fact that the VP1 unique region is dispensable in capsid formation and that any foreign protein sequence can be substituted for it presents an opportunity to generate recombinant VLPs for antigen presentation. Heterologous proteins, such as antigenic B cell epitopes, can be displayed on the VLP surface. Recombinant B19 VLPs are morphologically identical to native B19 parvovirus and have been observed to elicit neutralizing antibodies in animals and human volunteers [4].

We examined the possibility of generating recombinant B19 VLPs for the presentation of dengue 2 antigens. Dengue 2 epitopes aa 352–368 and aa 386–397 from the immunodominant domain BIII of E-glycoprotein were chosen for the B19 VLP experiment, because previous studies had shown that both epitopes induce anti–dengue 2 antibodies [5, 6]. First, we hypothesized that recombinant B19 VLPs could be generated by replacing the VP1 unique region with heterologous dengue 2 peptides of various lengths (12–100 aa), to create a total of 5 different fusion proteins composed of dengue 2 peptides and full-length (FL) VP2. Expression of these fusion proteins in a baculovirus system led to their self-assembly into 5 distinct recombinant VLPs, which would display dengue 2 epitopes on their capsid surface. Our goal was to determine whether ad-
ministration of recombinant VLPs would induce, in BALB/c mice, specific antibody responses against dengue 2 epitopes aa 352–368 and aa 386–397. Corresponding keyhole limpet hemocyanin (KLH)-conjugated dengue 2 epitopes were also included, to compare immunization results against those observed for this conventional antigen carrier. Finally, our aim was to examine serum samples from immunized mice, to determine their potential to neutralize live dengue 2 virus in 50%-plaque-reduction neutralization test (PRNT50) assays and thus identify potential dengue 2 vaccine candidates.

**Materials and methods.** SF9 cells (Invitrogen), derived from *Spodoptera frugiperda* ovarian cells, were maintained in SF-900 II SFM (Invitrogen) containing antibiotics, in 100% room air at 28°C.

The backbone for VLP production was VP2+10, which included FL VP2 of parvovirus B19 and the last 10 aa (+10) of the C-terminal portion of the VP1 unique region. The FL domain BI11 of dengue 2 E-glycoprotein was amplified by polymerase chain reaction and was ligated into VP2+10. Double-stranded overlapping oligonucleotides encoding for peptides aa 352–368 and aa 386–397 were produced by annealing complementary oligonucleotides and cloning them into VP2+10. DH10Bac cells were transformed as described elsewhere [2].

SF9 cells were infected with baculovirus stocks at an MOI of 5 pfu/cell and were harvested 72 h after infection. Recombinant capsids were harvested, purified, and prepared for electron microscopy, as described elsewhere [2].

Five BALB/c H-2d mice per construct were immunized intraperitoneally. The initial dose was 20 μg of purified recombinant VLPs suspended in complete Freud’s adjuvant, followed by 3 booster immunizations with 20 μg of recombinant VLPs and incomplete Freud’s adjuvant, at days 14, 35, and 56. Serum samples were collected from VLP-immunized BALB/c mice at days 28, 49, and 71. A mouse anti-parvovirus B19 (human) monoclonal antibody (MAB8293; Chemicon) was used for western blots; this antibody is directed against epitope aa 328–344 of the VP2 capsid.

Postimmunization serum samples were first diluted 50-fold

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**Figure 1.** A, Schematic representation of recombinant dengue 2 virus-like particles (VLPs). Dengue 2 antigens on the B19 capsid surface were expressed as single (SE), tandem (TD), or full length (FL). E-glycoprotein antigenic determinants (E) and glycine-spacers (g) were used in SE and TD epitopes. B, Electron-microscopic view of recombinant VLP 352–368 SE. C, Immunoblot analysis of recombinant dengue 2 VLPs. Recombinant VLPs and capsids composed of viral protein 2 (VP2) plus the last 10 aa of the C-terminal portion of the unique region of viral protein 1 were subjected to 8% SDS PAGE (0.2–1.0 μg of capsid/well). Immunoblot analysis was performed with an anti-B19 monoclonal antibody (MAB8293; Chemicon) that recognizes the empty capsid VP2.

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and then further in 2-fold serial dilutions. Detection was accomplished by use of a horseradish peroxidase–conjugated secondary antibody (Abcam) and ABTS substrate (Invitrogen).

Wild-type dengue 2 virus preincubated with postimmunization serum was used to infect nearly-confluent LLC-MK2 (monkey kidney) cells. Cells were overlaid with agarose and incubated in CO₂ for 6 days at 37°C. For plaque analysis, a second agarose overlay, with 2% neutral red, was added, and cells were incubated in CO₂ overnight at 37°C; viral plaques were counted the next day.

**Results.** The following 3 types of recombinant B19 VLPs carrying dengue 2 epitopes of domain BIII of E-glycoprotein were designed: 1 FL domain BIII; 2 single (SE) epitopes, 352–368 SE and 386–397 SE; and 2 tandem (TD) epitopes, 352–386 TD and 386–397 TD (figure 1A). The TD epitopes contained 3 repeats of the respective dengue 2 epitope, interrupted by glycine spacers at every 6 aa of length. Sf9 cells were infected with recombinant baculoviruses, without being coinfected with VP2. The generation of recombinant dengue 2 VLPs was confirmed, by electron microscopy, in purified VLP samples (figure 1B). Monoclonal antibody against parvovirus B19 was used to verify that the isolated band contained recombinant B19 capsids. Purified VLPs reacted with B19 monoclonal antibody MAB8293 and produced an ∼58-kDa band on the western blot (figure 1C).

A total of 40 BALB/c mice were immunized with 5 recombinant dengue 2 VLPs and with 2 KLH-conjugated samples (KLH 352–368 and KLH 386–397). Purified VP2 capsids (which contained no heterologous protein) were used as negative controls. Groups of 5 mice were immunized intraperitoneally with 20 µg of either purified recombinant B19 VLPs or KLH-conjugated peptides, at days 0, 14, 35, and 56. Mouse serum samples collected at days 28, 49, and 71 were screened for anti-dengue 2 antibody, on ELISA plates coated with the corresponding synthetic dengue 2-specific peptides. For determination of the anti-VP2 titer, plates were coated with 2 µg of VP2 capsid/mL (50 µL/well). ELISA demonstrated that all recombinant B19 VLPs generated robust anti-dengue 2 antibody titers (figure 2). Overall, serum samples showed increased levels of antibody titers in all recombinant B19 VLP samples, at days 28, 49, and 71. Of the 2 KLH-conjugated samples that were

### Table 1. 50%-Plaque-reduction neutralization test (PRNT<sub>50</sub>) titers generated by antigenic dengue 2 B cell epitopes.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Protein length, aa</th>
<th>Dengue 2 epitope</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt; mean&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain BIII</td>
<td>100</td>
<td>FL</td>
<td>40</td>
</tr>
<tr>
<td>SE</td>
<td>352–368</td>
<td>17</td>
<td>SE</td>
</tr>
<tr>
<td>386–397</td>
<td>12</td>
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<td>40</td>
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<tr>
<td>386–397</td>
<td>54</td>
<td>TD</td>
<td>80</td>
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<tr>
<td>KLH</td>
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<td>17</td>
<td>SE</td>
</tr>
<tr>
<td>386–397</td>
<td>12</td>
<td>SE</td>
<td>ND</td>
</tr>
<tr>
<td>VP2 (negative control)</td>
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* Expressed as reciprocal PRNT<sub>50</sub> titer against live dengue 2 virus. ND, not determined.
included to evaluate their potential to induce anti-dengue 2 antibodies, only KLH 352–368 generated detectable IgG titers. Serial dilutions of serum from VLP-immunized BALB/c mice were tested for their ability to neutralize dengue 2 virus. PRNT \textsubscript{50} assays were performed with serum samples collected at day 71, because these samples had the highest anti-dengue 2 antibody levels in the ELISA. Monkey LLC-MK\textsubscript{2} cells were incubated with serial dilutions (1:10–1:2560) of mouse serum and were then infected with live New Guinea C dengue 2 virus. Pre-immunization samples, medium without serum, and VP2+10 capsids were used as negative controls. The highest PRNT \textsubscript{50} titers were obtained in serum samples from BALB/c mice that had been immunized with recombinant B19 VLPs 352–368 TD and 386–397 TD, followed, in terms of neutralizing activity, by VLPs displaying SE epitopes and FL domain BIII (table 1). Titors for FL construct DIII were comparable to those for 386–397 SE but were below those for 352–368 TD and 386–397 TD. PRNT \textsubscript{50} titers were not detectable in any serum samples from mice immunized with VP2 capsids. KLH-conjugated serum samples showed low (in the case of KLH 352–368) or no (in the case of KLH 386–397) detectable levels of neutralizing activity in PRNT \textsubscript{50} assays.

**Discussion.** We have successfully produced recombinant B19 VLPs that elicit robust humoral immune responses against dengue 2 epitopes in immunized BALB/c mice. B19 VLPs were used for presentation of dengue 2 antigenic determinants because this approach offers several potential advantages for vaccine development [7]. VLPs mimic the structure of native B19 parvovirus, which results in effective uptake and processing of VLPs by the host immune system. The defined physical and biological characteristics of recombinant B19 VLPs provide the noninfectious B19 vaccine platform with a robust safety profile, compared with that observed for attenuated vaccines [8]. The 3 types of recombinant VLPs that we utilized generated high IgG titers and showed potent neutralization of live dengue 2 virus in functional in vitro assays, with PRNT \textsubscript{50} titers up to 1:120. Previously published data have shown that monoclonal antibodies against DIII of E-glycoprotein efficiently block viral absorption and infectivity in vitro [9]. The quantitative and qualitative immune responses after immunization with recombinant B19 VLPs confirm these findings and illustrate the potential that B19 capsids have as antigen carriers—and, therefore, for vaccine development.

Detailed characterization of immune responses to B19 parvovirus has shown that VLPs composed of VP1 plus VP2 induce antibodies that neutralize viral infectivity, whereas VLPs composed only of VP2 fail to do so [10]. The presence of FL VP1 capsid protein in recombinant VLPs appears to facilitate induction of antibodies against VP2 neutralizing epitopes. Immunization of B19-seropositive individuals with VP2-based VLPs should therefore not present an obstacle to an efficient immune response against recombinant B19 VLPs, because they consist only of VP2 and lack FL VP1 capsid protein.

Our immunization protocol induced robust humoral immune responses in BALB/c mice, but further assessment of recombinant B19 VLPs as antigen carriers will have to be examined in future experiments. Immunization against dengue 1, 3, and 4 will require generation of recombinant B19 VLPs with antigenic determinants of E-glycoprotein and testing by inoculation of animals. Recombinant-VLP dengue-vaccine candidates also must be tested in an appropriate nonhuman-primate model. Factors such as antigen dosage, route of administration, immunization schedule, and choice of adjuvant can affect the outcome of humoral and cellular immune responses in a vaccine and must be optimized [11, 12, 13]. Dengue-vaccine candidates other than live attenuated viruses—candidates such as DNA-based and subunit vaccines—are still in the preclinical stage of development [3]. The majority of recombinant-subunit vaccines employ the expression of either the recombinant E-glycoprotein or nonstructural proteins of dengue virus. Monath et al. recently have reported that, in nonhuman primates, PRNT \textsubscript{50} titers as low as 1:20 can be sufficient to confer protection against challenge with live dengue virus and have suggested that cross-neutralizing antibodies or cytotoxic T lymphocytes provide a second line of defense that can clear viral infection [14]. The immunogenic nonstructural proteins of dengue virus are a dominant source of cytotoxic T cell determinants in mice and humans [3].

No biological data are currently available regarding recombinant B19 VLPs that carry dengue virus CD8\textsuperscript{+} epitopes, but theoretically it would be possible to produce these recombinant B19 VLPs and to evaluate their potential to elicit major-histocompatibility-class-I responses in an animal model. Previous studies have shown that recombinant VLPs of porcine and canine parvovirus elicit strong cytotoxic T lymphocyte and T helper responses in mice [15, 16]. Prime-boost strategies have proven to be very effective at triggering potent T cell responses to control infection. Recombinant B19 VLPs could be used in combination with live-virus or DNA-based vaccines, to extend the arsenal of prime-boost regimens in immunization against dengue.

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**References**