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Generation and characterization of a monoclonal antibody that cross-reacts with the envelope protein from the four dengue virus serotypes

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Dengue viruses (DENVs; serotypes 1–4) are members of the flavivirus family. The envelope protein (E) of DENV has been defined as the principal antigenic target in terms of protection and diagnosis. Antibodies that can reliably detect the E surface glycoprotein are necessary for describing and mapping new DENV epitopes as well as for developing more reliable and inexpensive diagnostic assays. In this study, we describe the production and characterization of a monoclonal antibody (mAb) against a recombinant DENV-2 E protein that recognizes a sequential antigen in both native and recombinant form located in domain II of the E protein of all four DENV serotypes. We confirmed that this mAb, C21, recognizes a sequence located in the fusion peptide. In addition, C21 does not have neutralizing activity against DENV-2 in an *in vitro* system. Furthermore, the C21 mAb is an ideal candidate for the development of research reagents for studying DENV biology because it cross-reacts with the four dengue serotypes.

Key words: Dengue virus; monoclonal antibody; dengue viruse-envelope protein.

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The four dengue viruses (DENVs 1–4) belong to the *Flaviviridae* family. The DENV genome encodes a polypeptide precursor that is proteolytically cleaved into the structural proteins, capsid (C), premembrane (prM) and envelope (E) protein, and the non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (1, 2).

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The flavivirus E glycoprotein dimmer is the most exposed antigen of the *Flaviviridae* family and induces protective immunity. This protein is critical for binding to cellular receptors and endosomal membrane fusion. Based on the crystallography studies, the E protein folds itself into three distinct structural and functional domains (3). Domain I (DI) is organized into an eight-stranded, central β-barrel structure. This domain contains both virus-specific and cross-reactive epitopes, which are predominately non-neutralizing. In

contrast, domain II (DII) contains a conserved 'fusion loop', which actively participates in structural rearrangements that occur at low pH when the virus fuses with an endosomal membrane. In addition, DII has several overlapping immunodominant epitopes that stimulate neutralizing antibodies. These epitopes are clustered at the tip of DII, in the hinge region between DI and DII. Finally, Domain III (DIII) can fold independently into an immunoglobulin-like module. This domain also contains the host cell receptor-recognition site, which elicits the production of virus-specific, highly protective neutralizing antibodies that are conformation-dependent (4–6). Because this protein is involved in the immune response, several groups have generated monoclonal antibodies (mAbs) against DENV. Modis et al. (7) developed a mAb that efficiently binds to pH 5.0-treated virus particles and defined a common sequence of amino acid residues at positions 98–110 that are characteristic of the flavivirus group. This amino acid sequence is the defined DII viral fusion sequence. An additional antibody has been recently reported, and it also has variable neutralizing activity. Den et al. (2011) described a monoclonal antibody, 2A10G6, directed at the conserved fusion loop, which also possesses neutralizing activity (8). Strong evidence indicates that antibodies directed to DII of the E protein can affect fusion of the virus and the endosomal compartment by generating changes in structural integrity (9). Moreover, cumulative evidence has shown that a natural infection with DENV or any other flavivirus induces cross- reactive antibodies directed toward the fusion peptide in DII of the E protein. These antibodies have either weak or no neutralizing activity (10–12). Furthermore, an analysis of memory B cells from DENV-infected individuals identified potently neutralizing, serotype-specific IgGs that bind to unknown epitopes on either DI or DII in the loop or adjacent to it (13).

The identification of antibodies with neutralizing or non-neutralizing activity is crucial for the study of virus-host cell interactions, protection, and pathogenesis of hemorrhagic dengue fever. Monoclonal antibodies directed against fusion peptide regions have been characterized, but currently, there is still not enough information about this region. Thus, it is necessary to continue characterizing the epitopes located in this region. In this study, we characterized a mouse mAb, C21, which was developed against the DENV-2 E recombinant protein. Previous mAbs were generated against conformational fusion loop and non-fusion residues in the adjacent region; in this work, we describe a mAb that recognizes a highly conserved linear sequence close to the fusion peptide located in DII. No neutralizing activity was observed *in vitro*, and no significant protection against the homologous virus was observed *in vivo*. Due to its cross-reactivity with the four serotypes, C21 is an ideal candidate for the development of research reagents for investigating DENV biology. Furthermore, dengue-specific mAbs that recognize antigenically different serotypes are particularly important for reliable dengue antigen-capture enzyme-linked immunosorbent assays (ELISAs) for clinical diagnosis.

MATERIALS AND METHODS

Preparation of viral stocks and cells

All four DENV serotypes were used in this study. The DENV-2 clinical isolate was described previously (14); DENV-1 (Hawaii M340), DENV-3 (M93110), and DENV-4 (H241) were also used in this study. Mosquito C6/36 cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) at 34 °C. Baby hamster kidney (BHK-21) cells were cultured at 37 °C in the presence of 5% CO_2 in MEM supplemented with 10% FBS, 1 IU/mL penicillin, 1 μg/mL streptomycin and 2.4 ng/mL amphotericin B. Each virus stock was prepared by infecting the C6/36 cell monolayer when it reached 75–85% confluence in 75 cm^2 tissue culture flasks. The infected monolayers were homogenized and diluted in 40% polyethylene glycol solution in 2 M NaCl (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 4 °C overnight. The suspension was centrifuged at 20 000 g for 1 h. The viruses were resuspended in 1/15 of the total volume in a glycine buffer (50 mM tris, 200 mM glycine, 100 mM NaCl and 1 mM EDTA) and 1/30 of the total volume in FBS. The viruses were homogenized, aliquoted, and frozen at −70 °C until use. The viral stocks were titrated using a standard plaque-forming assay technique with BHK-21 cells, as described previously (15).

Preparation of DENV-2 E proteins

Cultures of *Escherichia coli* strain BL21 were transformed with the recombinant plasmid, pGEX/E, containing the envelope protein sequence from DENV-2, described previously by Mellado-Sanchez et al. (2010). Protein expression was induced by the addition of 1 mM isopropyl thiogalactoside (IPTG) to the transformed bacteria. The inclusion bodies were prepared, and the GST-E fusion protein was then purified from a 10% preparative SDS-PAGE gel, as described previously (16). In addition, the following two DENV-2 recombinant proteins (García Machorro J, López González M, Barrios Rojas O, Fernández Pomares , Sandoval Montes C, Santos Argumedo L., Gutiérrez Castañeda B, Cedillo Barrón L., Submitted paper) were constructed: (i) prMEII*, a protein containing aa 1–55 from DENV-2 prM and aa L^{65} to G^{112} from Ell* DII; and (ii) EII*EIII, a protein containing the EII* described above fused to aa 295–394 located in DIII. The DENV-2 (GenBank accession: AF038403) constructs were used as templates (17). The primers coding for prMEII* and EII*EIII are shown in Table 1. The primers were used to obtain PCR products that were cloned in a shuttle vector containing a metallothionein-inducible expression cassette for Drosophila cells. The PCR products were digested and then inserted into the *Bgl*II and *Not*I sites of the pMT/BiP/V5-HisA plasmid (Invitrogen, Carlsbad, CA, USA). The DENV-E sequences were placed in-frame with a BiP secretory sequence. In the expression vector, the prMEII* and EII*EIII sequences are followed by the V5 epitope and six histidine residues at their C-termini. *Drosophila* S2 cells (Invitrogen) were stably transfected, and $CuSO_4$ (Sigma-Aldrich) was added at a final concentration of 750 μM to induce synthesis and secretion of recombinant soluble DENV-prMEII* and DENV-EII*EIII proteins. The cell culture supernatants were passed through 0.2 μm filters. The protein samples were concentrated using 10 000-MWCO Millipore columns (Viva sciences, Hannover, Germany) and were then dialyzed in PBS.

Table 1. Primers used in this study

Primer	Sequence
prMF	5′-GGGGTACCT ACCATGTTCCATTTAACCACA
prMR	5′-ATCACGTACMGIGTCCTTTTGA ATTCCCG
EII*EIIIF	5′-CCAGGTACCAAACCATGGCAA AAMCAAA
EII*EIIIR	5′-GCTATGT TCACATGCGGGAA TTCC

Production of mAbs specific for E protein

Four 6–8-week-old BALB/c mice ($H-2^d$) were obtained from the breeding facilities at the CINVESTAV (Centro de Investigation y de Estudios Avanzados Del Instituto Politecnico Nacional). All animals were housed and handled in accordance with institutional guidelines. The mice were immunized with four doses of 50 μg of recombinant GST-E protein. These immunizations were administered subcutaneously with incomplete Freund's adjuvant. A final protein boost was administered intravenously 3 days before the splenocytes were harvested. The splenocytes were fused at a ratio of 10:1 with the mouse plasmacytoma cell line, Ag8, using polyethylene glycol 1500 (Sigma-Aldrich), as described by Kohler (18). The fused cells were resuspended in 40 mL of selection medium consisting of RPMI 1640 medium with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μM hypoxanthine, 16 μM thymidine and 400 nM aminopterin. The cell suspension (100 μL) was dispensed into 96-well plates and incubated at 37 °C in a 5% CO_2 atmosphere. After 12 h, an additional 100 μL of selection medium was added to each well. After 24 h, half the medium from each well was removed, and fresh selection medium was added. Every 2 or 3 days, the medium was replaced with fresh selection medium. After 10 days, aminopterin was omitted from the medium. Between the 12th and 14th days, the supernatants from the 96-well plates were screened by ELISA.

Monoclonal antibody screening and isotype determination

Specific mAb-secreting hybridomas were initially identified by ELISA using 96-well polyvinyl plates (Nunc, Roskilde, Denmark) previously coated with either purified recombinant GST-E (6 μg/mL) or GST protein only as a control. The wells were blocked with 0.05% Tween-20 and 3% skim milk in PBS. The supernatants were then incubated at 4 °C overnight. The plates were washed three times with PBS containing 0.1% Tween-20 (PBS-T) and were incubated with HRP-conjugated anti- mouse IgG (Zymed Laboratories Inc., San Francisco, CA, USA). The plates were washed again and then incubated with the peroxidase substrate, *o*-phenylenediamine dihydrochloride in citrate buffer, pH 5.6. The reaction was stopped with 2 N H_2SO_4, and the plates were read at 490 nm using a microplate reader (Sunrise Tecan, Salzburg, Austria). The selected clones were subcloned using the limiting dilution technique. The mAb isotypes were determined using a Monoclonal Antibody Isotyping Kit II [Mouse MonoAB ID kit (HRP); Invitrogene] according to the manufacturer's instructions.

Western blot analyses of Vero cells infected with DENV (1–4)

Vero cells were infected with DENV-1, DENV-2, DENV-3 or DENV-4 or were mock-infected. After 72 h, the cells were washed and lysed with RIPA buffer (100 mM Tris–HCl pH 8.3, 2% Triton X-100, 150 mM NaCl, 0.6 M KCl, 5 mM EDTA, 1% aprotinin, 3 mM PMSF, 1 μg/mL leupeptin, and 5 μg/mL soybean trypsin inhibitor). The cell lysates were analyzed by SDS-PAGE and transferred to nitrocellulose membranes; the membranes were then incubated with the C21 mAb, followed by HRP conjugate (Invitrogen).

To identify the epitope recognized by C21, additional Western blot analyses were performed with purified proteins, including prMEII*, EII*EIII, and GST-E, and DENV-infected cells. For these analyses, the membrane was first blotted with the HRP-V5 antibody (Invitrogen), and then the membrane was stripped (19) and re-blotted with C21. Next, the membrane was washed three times with PBS-T and then incubated with anti-mouse HRP-conjugated antibodies for 1 h. Finally, the HRP substrate was added.

Immunofluorescence staining of infected cells

The mAbs were used to identify the native E protein in DENV-infected cells. The cells were seeded on glass coverslips at 6×10^4 cells/coverslip (Bellco, Vineland, NJ, USA). After 48 h, the culture medium was removed, and monolayers were infected with active DENV-2 or UV-inactivated virus, both at five PFU per cell. The cells were then incubated at 37 °C and were analyzed by immunofluorescence microscopy at different times. Briefly, the cells were fixed with 4% *para*-formaldehyde (Sigma-Aldrich) in PBS for 20 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 in PBS and blocked with 10% normal goat serum. The cell monolayer was incubated for 60 min with C21 (anti-E protein), followed by incubation with a fluorochrome-conjugated secondary antibody, mouse IgG H+L (Caltag; Invitrogen). An irrelevant isotype antibody that matched the monoclonal antibody was used as a negative control. Finally, the nuclei were labeled with 1 μg/mL of 4′6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 10 min, and the slides were mounted with Vectashield (Vector, Burlingame, CA, USA). Images were captured using two different confocal microscopes (a Leica SP2 and an OLYMPUS FVX).

Purification and concentration of monoclonal antibodies

The C21 anti-E mAb was purified from cell supernatants using an ultra-filtration system (Stirred Ultrafiltration Cell model 8010; Millipore, Billerica, MA, USA) and a membrane with a cutoff of 30 kDa (GM30; Millipore), which was first washed with 0.1 M sodium hydroxide for 30 min and then concentrated to 10 mL; next, the sample was affinity purified through a column of protein G with an FPLC system (General Electric, Buckinghamshire, UK). The mAb was concentrated and dialyzed in PBS with the Centricon system with a cutoff of 10 kDa and finally the mAb concentration was determined, and then its activity and specificity were measured by ELISA and Western blot.

Competition assay

ELISA plates were coated overnight at 4 °C with 6 μg/mL of the GST-E protein, and the C21 mAb was used at 0.2 μg, as previously described. For the competition experiments, a panel of four overlapping peptides of 15 residues was designed to cover 45 residues from the E region of DII protein P1: NTTTDSRCPTQGEPS (aa N^{66}-S^{80}), P2: QGEPSLNEEQDKRFV (aa Q^{77}-V^{92}), P3: DKRF VCKHSMVDRGW (aaD^{87}-W^{102}), and P4: VDRG WGNGCGLFGKG (aaL^{97}-G^{112}) and two additional control peptides, a scrambled peptide from the P4 sequence named P5 (WLGMCNVGR GDGG) and a peptide that included the region shared between peptide 3 and 4 named P6 (VCKHSMVDRGWG (described in the Results section). Three experiments were performed with the four peptides, a scrambled P4 peptide (P5) and an additional peptide that included the region shared between peptide 3 and 4 (P6). All the peptides were synthesized by GenScript Corporation (Piscataway, NJ, USA). For each peptide, we considered the charge of the peptide to determine whether water or an organic solvent should be added. All the stock peptides were prepared at a 1 mg concentration to minimize the potential for precipitation. For the competition experiments, duplicate dilutions of the synthetic peptides, P1, P2, P3, P4, P5, and P6, at final concentrations of 0.25, 0.5, and 1 μg/mL were used. As controls, the C21 mAb incubated with the GST-E protein or prMEII* were used as negative controls so that C21 was inhibited from binding to the GST-E present on the plate. Next, 100 μL of each solution was added to the wells and incubated at room temperature for 1–3 h. The wells were washed four times with PBS-T. Subsequently, 100 μL of HRP-conjugated anti-mouse IgG in blocking buffer was added to each well, and then the HRP substrate was added. The peroxidase reaction was stopped after 20 min by the addition of 50 μL 0.5 M H_2SO_4. The OD values were measured at 490 nm (20).

Neutralization test

The neutralizing antibodies in mice were titrated as described previously (21). Twofold serial dilutions of test sera were mixed with active DENV-2. Briefly, BHK-21 cells were plated at a density of 1.5×10^5 per well in 24-well plates. Next, 30 or 70 PFUs of DENV-2 were added to each well. The DENV-2 was previously mixed with different amounts of the C21 mAb diluted in 2% FBS in Hanks' medium. The plates were incubated for 2 h at 37 °C with a 5% CO_2 atmosphere, and 500 μL of overlay medium was then added to each well. After further incubation for 4–5 days under the same conditions, the supernatant was discarded, and the cells were washed by immersion in water. The cells were then stained with Naphthol blue black dye. The plates were extensively washed with water and air- dried. Viral titers were estimated using the 50% plaque reduction neutralization test ($PRNT_{50}$). The control for the basal viral titer was established by adding normal mouse serum to the virus mixture, and positive controls for anti-DENV were performed in each experiment. The results are expressed as described elsewhere (22).

RESULTS

Generation and identification of mAbs against the DENV-2 E protein

To generate serotype-specific mAbs, we expressed and purified sufficient quantities of the DENV-2 GST-E protein to immunize the mouse used for the hybridoma fusion, as described previously (21). Figure 1A shows the presence of a 72-kDa protein before and after purification (lines 3 and 4) that corresponds to the predicted molecular mass of the GST-E fusion protein. This protein was used to inoculate four BALB/c mice.

After immunization, the four mice exhibited reactivity to the GST-E protein by indirect ELISA (data not shown). Next, successful fusions were obtained using spleen cells from the best-responding immunized mouse. The resultant supernatants from different wells were screened against GST-E in an ELISA assay. To avoid mAbs specific for GST, the same supernatants were simultaneously tested with wells coated with GST. We selected the hybridoma that showed a strong signal in an indirect ELISA against the homologous DENV- 2 E protein from a panel of eight different mAbs (Table 2). Clone C21 was selected for further analysis because of its strong signal in the ELISA and Western blot assays. Furthermore, we evaluated whether the C21 mAb was able to bind to viral DENV-E protein in Con-A coated wells. The results showed a clear positive signal (Table 2, the last line labeled with*). There was no reactivity with the negative controls, which consisted of GST in the same system In addition, no signal was observed in this system when unrelated mAb supernatants were added to the wells (data not shown).

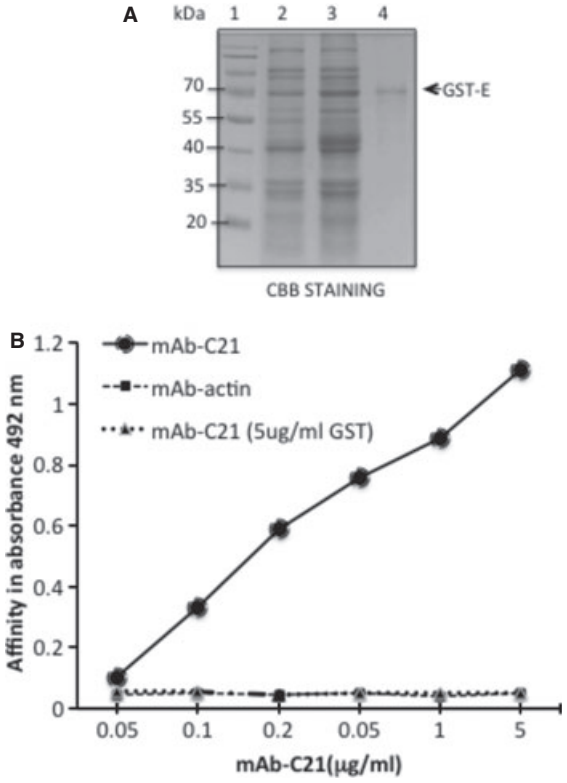

Fig. 1. The steps involved in mAb preparation. (A) Purification of a recombinant dengue virus GST-E fusion protein using 10% SDS-PAGE under denaturing conditions and direct visualization by Coomassie blue staining. Lane 1: MWM, lane 2: IPTG- induced pGEX-E at time 0, lane 3: inclusion bodies of GST-E fusion protein after 16 h IPTG induction in *Escherichia coli* BL21 cells and lane 4: GST-E purified protein. The open arrowhead indicates the GST-E protein with an estimated molecular mass of approximately 72 kDa. (B) Analysis of specific reactivity at different concentrations of the novel C21 mAb against the GST-E protein at a concentration of 5 μg/mL.

Table 2. Screening of different mAbs generated to the DENV-2 virus E protein

Hybridoma mAb-E	GST-E O.D.	GST O.D.
F10-1	0.332	0.114
B10-2	0.328	0.065
E8-2	0.316	0.032
C12-5	0.415	0.093
H5-4	0.302	0.044
C21	1.099	0.035
H6-8	0.363	0.032
F10-8	0.393	0.044
G12-7	0.39	0.041
D11-5	0.316	0.098
C21*	0.567	0.041

The C21 hybridoma was determined to belong to the IgG2b subtype with a kappa light chain. Then, the specific anti-E C21 mAb was concentrated and purified, and titer was determined. ELISA binding assays were performed to quantify the binding affinity of C21. A total of 6 μg/mL of recombinant E protein was used per well for the ELISAs. A positive signal was observed with 0.05 μg of the mAb, and the signal steadily increased until the concentration of the mAb was 1 μg (Fig. 1B). An unrelated monoclonal antibody (anti-actin) incubated with the GST-E antigen was used as a negative control. In addition, a negative control for specificity was performed by coating the plates with GST protein, and we found that the C21 mAb did not recognize GST at any concentration.

Reactivity of anti-E mAb with native or denatured DENV envelope glycoprotein

The capacity of the C21 mAb to recognize the E glycoprotein under denatured conditions might provide information as to whether the epitope recognized by the mAb consists of a consecutive amino acid sequence. To elucidate this, Vero cells were infected for 24 h with each DENV (1–4). The cells were lysed and treated with β-mercaptoethanol for the denatured conditions prior to PAGE and subsequent immunoblotting. We show that the C21 antibody bound to the GST-E recombinant protein (lane 1; 72 kDa in the four panels) and to the four native DENV-E glycoproteins (lane 4; 55 kDa in the four panels) obtained from the infected cells (Fig. 2A). This finding suggests that C21 recognizes a linear or continuous amino acid

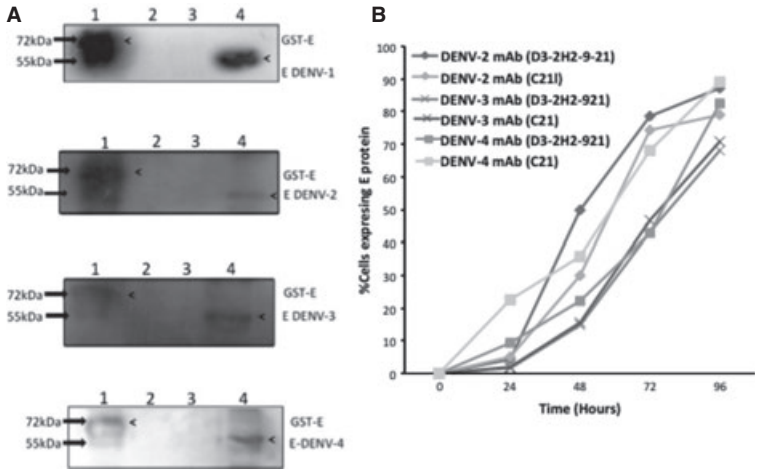

Fig. 2. C21 mAb cross-reactivity: (A) Immunoblot analysis to determine the serotype specificity. Vero cells were infected with 5 m.o.i. of the four serotypes, dengue virus 1 (DENV-1) to DENV4 (all cases, lane 4) and were then lysed and analyzed 24 h post- infection with the C21 mAb. In the four panels, lane 1 shows purified, soluble recombinant GST-E protein from DENV-2, lane 2 shows non-infected cells, lane 3 contains mock-infected cells, and lane 4 shows DENV-infected cells. Protein molecular weights (in kDa) are shown on the left. (B) Specificity of the C21 mAb in Vero cells infected with the DENV serotypes 2–4 and analyzed at different times using immunofluorescence. A commercial antibody against the E protein (D3-2H2-9-21) was used as the positive control simultaneously for each serotype. Five different microscope fields from three independent experiments were analyzed, and 200 cells of each were scored. The results are represented as the percentage of antigen E positive cells.

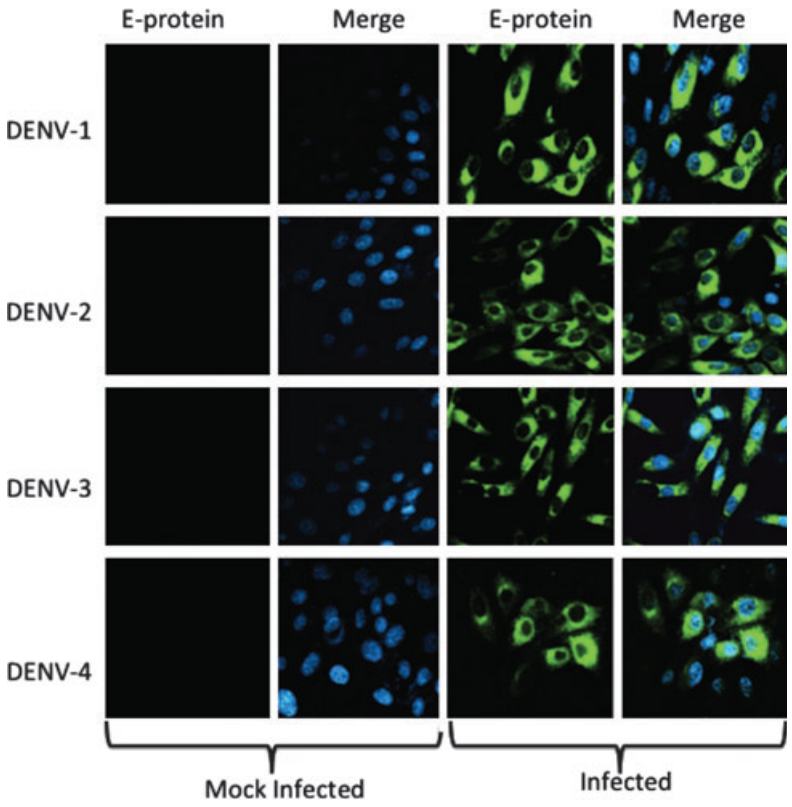

Fig. 3. C21 mAb cross-reactivity in infected cells. Vero cells were either infected or mock-infected with dengue virus (serotypes 1–4) at 5 m.o.i. for 48 h. The immunofluorescent Staining was then performed with the C21 mAb (green signal) to detect E protein. Mock-infected and uninfected cells were used as controls. The nuclei were counterstained with DAPI (blue).

sequence. In contrast, no signal was observed in the uninfected cells or mock-infected cells (lanes 2 and 3 in the four panels).

Furthermore, the specificity of this mAb was evaluated in an experiment on the kinetics of infection with 2 m.o.i. (Multiplicity of infection) of three DENV serotypes (DENV 2, 3, and 4). The results showed that the staining was similar among all of the serotypes tested, and compared with a commercial mAb, no significant differences were observed (Fig. 2B).

In general, specific mAbs have utility as diagnostic tools only when the antibodies recognize the native protein. Therefore, we evaluated the recognition and cross- reactivity of the anti-E mAb with the native E glycoprotein from the four DENV serotypes. This experiment was performed in Vero cells infected with 5 m.o.i. for DENV (1, 2, 3, and 4) particles 48 h after infection. As shown in Fig. 3, the C21 mAb cross-reacted with cells that were infected with all four serotypes of DENV; however, no visible differences in the recognition patterns of the E proteins from the four serotypes were observed. In contrast, there was no evident cross-reactivity with other components of the mock-infected cells, and no response was observed in the negative control (Fig. 3).

The C21 mAb recognizes an epitope present in DII of the E protein

To map the region recognized by the C21 mAb, we used the following two DENV-2 recombinant proteins: (i) prMEII*, which consists of the prM protein (aa 1 to aa 55), and Ell* (aa 65 to aa 112) located in domain II; and (ii) EII*EIII, which is the EII* described above fused to aa 298 to aa 397 located in DIII (Fig. 4A). When the proteins were analyzed by Western blot using an anti-V5 antibody, only the fusion proteins, prMEII*

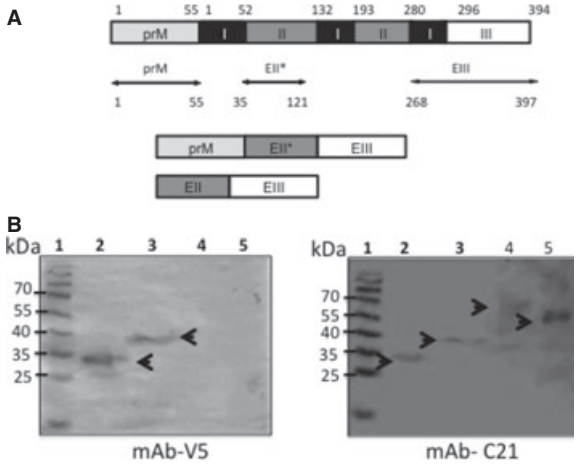

Fig. 4. Schematic representations of the fusion proteins. (A) Diagram of the full-length dengue virus 2 (DENV-2) E sequences and those used to construct the recombinant plasmid prMEII* and EII*EIII. The black arrow represents the EII* region and the gray arrow represents the EIII region. (B) Purified recombinant proteins were analyzed with SDS-PAGE and blotted with the V5 antibody (left panel); then, the membranes were stripped and re-blotted with the C21 mAb (right panel). Lane 1; PMW, lane 2; prMEII*, lane 3; EII*EIII; lane 4; GST-E, lane 5; Vero cells infected with DENV-2 as a positive control.

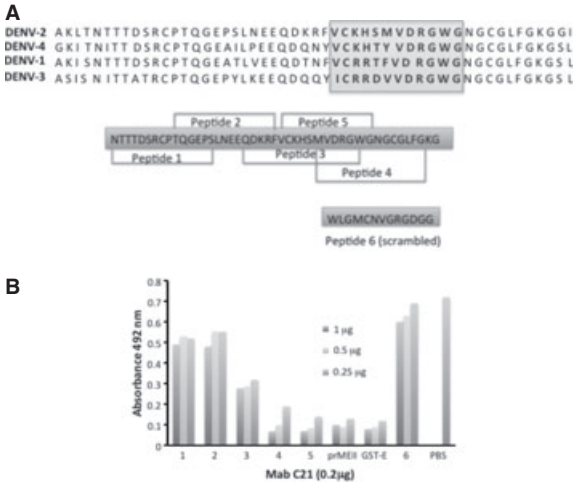

Fig. 5. EII* alignment of the amino acid sequences of the four serotypes and peptide mapping of the C21 mAb. (A) Sequences of the 40 aa surrounding the EII* protein in the four serotypes (N66 to G112) showing the highly conserved fusion sequence (loop). Six peptides were synthesized (P1–P6) The first four peptides were designed with four overlapping aa among them. P5 included P3 and P4, and P6 is a scrambled peptide of P4. The boxes show the residues included in each synthetic peptide. (B) All the peptides were synthesized and tested in competition assays, as described in the Materials and Methods section. ELISA plates were coated with 6 μg/mL of GST-E protein, and different concentrations of the C21 mAb (0.25, 0.5, and 1 μg) were incubated with 0.2 μg of each peptide (1–3, 7–9) and with prMEII, GST-E or PBS as shown on the horizontal axis. These mixtures were then added to the ELISA plates coated with GST-E protein. The ELISAs were performed as described in the Materials and Methods section. The vertical axis denotes the optical density measured at 492 nm.

and EII*EIII (lanes 2 and 3), were detected. GST-E was not detected because no V5 is present in this protein (Fig. 4B). Conversely, when the same membrane was stripped and re-blotted with the C21 mAb, marked binding to DENV-2 infected cell lysates (lane 5), GST-E (full length, lane 4) and the recombinant prM-Ell* and EII*EIII proteins (lanes 2 and 3) were observed. These data strongly suggest that the sequence recognized by the C21 mAb is located in the 40 residues common to the prMEII* and EII*EIII proteins.

Epitope mapping by competition ELISA

As described above, the C21 mAb recognizes an epitope located in DII of the E protein. Using this information coupled with the specificity of the mAb for the four serotypes (Fig. 5A), we identified the 40 residues of DII of the E protein, which were aligned and compared with the corresponding sequences from the four serotypes (Fig. 5A).

To map the epitope, four overlapping peptides of 15 residues each were designed to cover 45 residues from the E region of the prME DII protein. Although this region is relatively well conserved among the four serotypes, the peptides were based on the DENV-2 serotype. The peptides are described in the Materials and Methods as follows: P1: (aa N^{66}-S^{80}), P2: (aa Q^{77}-V^{92}), P3: (aaD^{87}-W^{102}), and P4: (aa L^{97}-G^{112}) (Fig. 5B). Three independent assays were performed to detect the binding of C21 to the four peptides immobilized in wells of an ELISA plate; however, no positive signal was detected (data not shown). Subsequently, a competition binding experiment was then performed. We

evaluated the ability of each peptide to bind to the C21 mAb in solution prior to testing the binding of the C21 mAb to full-length GST-E in ELISA plates. Peptides 3 and 4 blocked the binding of C21 to GST-E- coated ELISA plates (data not shown). Interestingly, P3 and P4 (which compete highly with E protein) contain the fusion peptide sequences. On the basis of these results, we designed two additional control peptides; a scrambled peptide from the P4 sequence named P5 (WLGMCNVGRGDGG) and a peptide that included the region shared between P3 and P4, named P6 (VCKHS MVDRGWG). These peptides were pre-incubated at concentrations of 1, 0.5 or 0.25 μg/mL with the C21 mAb (at 0.2 μg) before being analyzed using ELISA in a plate coated with 6 μg/mL of GST-E protein, resulting in an OD of 0.7 at 495 nm P1 and P2 bound weakly to C21 (only 30%) (Fig. 5B) allowing it to interact with the GST-E protein. The C21 mAb was able to bind to P3 in solution (64% competition), but P4 (VDRGWGNGCGLFGKG) exhibited stronger competition (89.3%), similar to that of P5. In addition, P4 and P5 bound to the C21 mAb and inhibited it from binding to the GST-E protein. The scrambled peptide P6 was not able to inhibit the binding of the C21 mAb to GST-E, and the optical density result was similar to that observed when C21 was incubated with only PBS. This competition result was similar to that obtained when GST-E or prMEII* were incubated with the C21 mAb before the ELISA. These results suggest that P4, in solution, is in a conformation that allows for binding to the C21 mAb. P4 comprises 15 residues of DENV-2 in the region of the fusogenic peptide. It is important to consider that P3 shares 5 aa with P4, suggesting that the epitope recognized by the C21 mAb may consist of aa 97–112, a highly conserved region among the four serotypes.

The C21 mAb does not have neutralizing activity *in vitro*

To determine whether or not the C21 mAb directed against the region close to the fusion peptide of DENV-2 possesses neutralizing activity against DENV-2, 5, 10, 15, and 20 μg of the C21 mAb were tested in a conventional PRNT assay (see Materials and Methods for details). Five days later, the numbers of plaques were recorded. The C21 mAb was not able to reduce the formation of plaques at any concentration (data not shown).

DISCUSSION

The objective of this study is to produce mAbs from mice immunized with the recombinant GST-E protein from DENV-2. We characterized a mAb that was broadly cross-reactive with the four DENV serotypes, as demonstrated by Western blot analysis. The C21 mAb binds to the native DENV 1–4 E proteins under normal or reducing conditions, suggesting that the epitope is linear and not conformational. Evaluating the capacity of any given mAb to bind to fragments of the E protein provides additional information regarding the antigenic map of the DENV-2 E protein (11, 23–25).

Based on our competition assays, the epitope recognized by the C21 mAb was located in DII, close to the fusion peptide C21 and is similar to an antibody raised against DENV that recognizes a region close to DII. Moreover, cross-protection against DENV-2 and DENV-4 infections in mice have also been demonstrated *in vivo* using this and similar antibodies developed by other groups (8, 9, 26). Although most neutralizing antibodies have been directed against conformational epitopes, antibodies should be elicited in response to a continuous string of amino acid sequences, as previously reported in DENV-3-infected patients (27).

Previous work with mAbs directed at the flavivirus fusion loop has suggested that some antibodies, such as E16 and 2A10G6, inhibit infection at the post-attachment step (8, 28). Therefore, a neutralizing assay with the C21 mAb was performed *in vitro*, and no neutralizing activity was observed at high concentrations compared to the control. The epitope recognized by the C21 mAb described in this work recognized a continuous sequence of the E glycoprotein peptide, P4. The sequence of this peptide is VDRGWGNGCGLFGKG (aa $V9^{7}$-G^{111}). The C21 mAb also showed partial inhibition of P3, the sequence of which is DKRFVCKHSMVDRGW (aa D^{87}-V^{101}). These two peptides may be inhibited because

they share aa 99–105. It is important to note that the size of an epitope is 6–7 aa. The residues identified as participating in the flavivirus cross-reactive epitopes are spatially arranged on the DENV-2 E glycoprotein surface in the highly conserved fusion peptide region of DII. In this domain, the residues G104, G106, and L107 are almost completely conserved among the flaviviruses (Fig. 5A). This epitope appears to be dengue-group reactive, reduction-denaturation resistant, and different from previously described epitopes. From the sequence alignment of the four serotypes (Fig. 5A), it was apparent that the conserved region enabled cross- reaction, and similar reaction kinetics were observed for at least three of the four DENV serotypes.

Based on our results, the linear epitopes on the DENV E glycoprotein might be useful in immunologic studies and the diagnosis of dengue infections. It is interesting that although a recombinant DENV antigen was used, a monoclonal antibody raised against DII was identified, as in a natural infection. In general, the novel C21 DENV-specific mAb generated in this study appears to be a very useful tool for the diagnosis of infection and for the study of viral biology in cells because it reacts with the four natural viral antigens of the four DENV serotypes in infected Vero cells in both indirect immunofluorescence and sandwich ELISA. This observation suggests that the recombinant antigen may be useful for obtaining monoclonal antibodies because the GST-E protein shares linear and accessible epitopes with the native viral antigen in DENV-infected cells.

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