Acta Tropica 171 (2017) 233–238

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Construct and expression of recombinant domains I/II of dengue virus- 2 and its efficacy to evaluate immune response in endemic area: Possible use in prognosis

Alfredo Eduardo Montes-Gómez[a], Hector Vivanco-Cid[b], José Bustos-Arriaga[c], Mussaret Bano Zaidi[d,e], Jazmin Garcia-Machorro[f], Benito Gutierrez-Castañeda[g], Leticia Cedillo-Barron[a,*]

[a] Departamento de Biomedicina Molecular, CINVESTAV IPN, Av. IPN # 2508, Col. San Pedro Zacatenco, 07360 México City, Mexico
[b] Laboratorio Multidisciplinario en Ciencias Biomédicas, Instituto de Investigaciones Médico-Biológicas, Universidad Veracruzana, Veracruz, Mexico
[c] Molecular Biology and Immunology of Arbovirus Laboratory 17, Biomedicine Unit (UBIMED), Mexico
[d] Infectious Diseases Research Unit, Hospital General O'Horan, Merida, Mexico
[e] Department of Epidemiology and Biostatistics, Michigan State University, Lansing, USA
[f] Laboratorios de Modelado Molecular y Diseño de Fármacos, Bioquímica, Medicina de Conservación, Fisiología, Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina, Instituto Politécnico Nacional Plan de San Luis y Díaz Mirón s/n, 11340, Mexico
[g] Immunology Department (UMF) Facultad de Estudios Superiores-Iztacala, Universidad Nacional Autonoma de México, Av. de los Barrios 1, Los Reyes Iztacala, Tlalnepantla, Edo. de México, 54090, Mexico

ARTICLE INFO

Keywords:
Antibody response
Dengue virus
Specific antibodies

ABSTRACT

The envelope (E) protein from DENV, contain three functional and structural domains (DI, DII and DIII). Some studies suggest that neutralizing antibodies during natural DENV infection are predominantly against DI and DII, in contrast, low proportion of the antibodies were against DIII. Thus it is necessary to establish the proportion of human antibodies against DENV E protein that bind to DI and DII during the normal course of infection; as an indicator of the quality of the antibody response and to further design new vaccine candidates for DENV. The aim of this study was to express recombinant proteins harboring a 240-aminoacid fragment of the E protein from DI and DII of DENV serotypes 2 and 3 in a eukaryotic S2 system. Further, we evaluate the antibodies against these antigens in samples from patients in acute phase of DF or DHF and compare it with the response of samples from healthy individuals from the same endemic areas and samples from healthy individuals from a non-endemic area (EA and NEA, respectively). These results suggest that the presence of antibodies against rEDI/DII might be used to identify patients at risk for severe disease.

1. Introduction

Dengue virus (DENV) infection is the main mosquito-borne disease worldwide (Murray et al., 2013). The four known DENV serotypes co-circulate and cause a self-limiting febrile illness known as dengue fever (DF) or a more severe clinical illness known as dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (Martina et al., 2009).

DENV belongs to the *Flaviviridae* family, whose members have an RNA genome. Mature virions are composed of 90 antiparallel E-protein dimers. Each E protein is 493–495 amino acids long and is organized as three functional and structural domains (DI, DII, and DIII). DI has eight β-helix chains, whereas DII has 12 β chains, two α helices, and a fusion peptide and is the main site of dimerization (Rodenhuis-Zybert et al., 2010; Modis et al., 2005). DIII has 10 β chains that form an immunoglobulin-like structure; the sequence is associated with binding of the virus to its cellular receptor (Modis et al., 2004). It also contains a hinge connecting DI and DII that allows conformational changes required to expose the fusion peptide (Modis et al., 2005).

The E protein is the most exposed and immunogenic antigen on the surface of the DENV particle; as such, it is recognized by protective neutralizing antibodies (Roehrig et al., 1998). During the acute phase of primary DENV infection, neutralizing antibodies confer heterologous short term protection to serotypes 1–4 (Sabin, 1952); in contrast, serotype-specific protection can last decades and shows low affinity to heterologous serotypes (Zompi et al., 2012; Toh et al., 2014).

Previous studies characterizing the immune response against E

* Corresponding author.
E-mail address: lcedillo@cinvestav.mx (L. Cedillo-Barron).

http://dx.doi.org/10.1016/j.actatropica.2017.04.006
Received 20 December 2016; Received in revised form 10 April 2017; Accepted 14 April 2017
Available online 17 April 2017
0001-706X/

protein in a mouse model found that DIII is the main antigen that elicits protection (Gromowski et al., 2008; Sukupolvi-Petty et al., 2010; Poggianella et al., 2015; Coconi-Linares et al., 2013). However, broadly neutralizing antibodies are induced during natural DENV infection and specific B cell clones are predominantly directed against epitopes in DI and DII (Wahala and Kraus, 2009; Sukupolvi-Petty et al., 2010; Williams et al., 2012) and against E dimers of DENV E (i.e., envelope dimer epitopes) in the four serotypes (America et al., 2014; Rouvinski et al., 2015). Specific antibodies against DIII, which comprise a low proportion of the total polyclonal response were detected in human sera from patients with primary and secondary DENV infection; however, antibodies recognizing an epitope close to or within the fusion loop constituted up to 54% of the total antibodies present in these patients (Crill et al., 2009). Therefore, it is necessary to establish the proportion of human antibodies against DENV E protein that bind to DI and DII during the normal course of infection; this information could be used as an indicator of the quality of the antibody response to provide an effective and economic suitable screening method for evaluation of the immunological status against DENV, thereby allowing the rational design of a candidate vaccine for DENV.

2. Materials and methods

2.1. Ethics statement

The study protocol, was approved by institutional review boards of the Veracruz University's Institute for Biomedical Research Ethics Committee (Protocol number 18/2010).

A single blood sample was collected from patients 6 to 60 years of age, 5–7 days after the onset of symptoms. Based on clinical and laboratory criteria of the World Health Organization 1997, patients were classified as having DF or DHF. All DHF patients had laboratory evidence of thrombocytopenia ($< 100{,}000$ counts/mm^3) and a positive tourniquet test.

Patients ranged from 6 to 60 years old, as shown in Table 1. Hereafter, samples from these patients will be referred to as acute-phase samples. A single blood sample was also collected from 20 healthy individuals between 20 and 25 years of age from the same endemic area (EA) of Veracruz, to compare the sero-prevalence against the EDI/DII recombinant proteins. Finally, 20 samples from healthy individuals from non-endemic DENV areas (NEA) for dengue virus were collected as negative controls.

2.2. Vector construction

DI and DII from E protein sequences were cloned into the pMTBvector. The EDI/DII sequences were amplified by reverse transcription (RT)-PCR from DENV-2- or −3-infected *Aedes albopictus* C6/36 cells using appropriate oligonucleotides: pMTBFDV-2 (5'-TCAGAATTCCACCATG ACATTGGATTTT-3') and pMTBRDV-2 (5'-GACAAGTGTCCTGTATAGAGCTCTCA-3') for serotype 2 and pMTBFDV-3 (5'-TCAGAATTCAACCATGACGCTGGATATA-3') and pMTBFDV-3 (5'-ACTCTCGAGATAATACTTGTGCCTCC-3') for serotype 3. The forward and reverse oligonucleotides contained restriction sites for *Eco*RI and *Xho*I, respectively. PCR products were digested with the appropriate restriction enzymes and inserted into the pMT-BiP/V5-His expression plasmid of *Drosophila melanogaster* (Invitrogen, Carlsbad, CA, USA), which is under the control of the metallothionein promoter and it's inducible by $CuSO_4$ at a concentration of 500 mM.

Table 1
Characteristics of the patients analyzed.

n	Diagnosis	Stage of infection
20	Dengue fever	Acute phase
20	Dengue hemorrhagic fever	Acute phase
20	Healthy (endemic)	–
20	Healthy (non endemic)	–

2.3. Transient expression of the recombinant proteins

The plasmids pMTEDI/DII serotype 2 and 3 were transiently and then stably transfected into *Drosophila* S2 cells (supplemented with 10% FBS) as described else were. After induction, S2 cells were subjected to immunofluorescence analysis as described previously (Mellado-Sánchez et al., 2005). The S2 cell monolayers then were stained for 60 min with a monoclonal antibody against DENV E protein (León-Juárez et al., 2013). Irrelevant isotype-matched monoclonal antibodies were used as negative controls.

2.4. Stable transfectant cells

The plasmids pMTEDI/DII-DENV2 and pMTEDI/DII-DV3 were stably co-transfected into *Drosophila* S2 cells with a plasmid with blasticidin resistance gene (pCoBlast Invitrogene) using calcium phosphate according to the manufacturer's instructions. The S2 cells were incubated at 28 °C, at selection with blasticidin at a final concentration of 25 μg/ml every four days during three weeks until stable colonies appeared.

Protein concentration was quantified using the Bradford protocol (Bradford, 1976) and 50 μg of each sample were resolved on a SDS-PAGE gel and a Western blot assay was performed using an anti-E monoclonal antibody (León-Juárez et al., 2013)

The secreted recombinant proteins were concentrated on 10 kDa columns (Millipore) to a final volume of 15 mL and resolved on a 15% SDS-PAGE slab gel in order to purify the proteins. (Mellado-Sánchez et al., 2005)

2.5. Statistical analysis

Prism v.6 software was used for statistical analyses (GraphPad Inc., La Jolla, CA, USA). Student's *t*-test; $P \leq 0.05$ was considered significant and cut-off points were the standard error of healthy individuals from NEAs.

3. Results

3.1. Cloning of rEDI/DII recombinant genes of DENV serotypes 2 and 3

We used the *Drosophila melanogaster* S2 cell expression system with a leader sequence to ensure efficient secretion of 6His-tagged soluble recombinant DENV proteins (rEDI/DII). rEDI/DII sequences were amplified by RT-PCR from C6/36 cells infected with DENV serotype 2 or 3. The constructs (pMTEDI/DII-DENV2 and pMTEDI/DII-DENV3) are illustrated in Fig. 1A. The amplified sequences included amino acids 40–280 from the E protein of DENV-2 and 40–278 DENV-3; restriction analysis of the recombinant plasmids yielded the corresponding 756 and 750 bp cassette (Fig. 1B). Protein conformations were modelled using USCF-Chimera NIH (Pettersen et al., 2004).

3.2. Expression and purification of rEDI/DII-DV2 and DV3 in S2 cells

The expression of recombinant proteins driven by the *metallothionein* promoter was evaluated. S2 cells transfected with pMTBEDI/II-DENV2 and -DENV3 were treated with brefeldin A and evaluated for protein expression by immunofluorescence (Fig. 2A). Positive immunoreactivity was detected using a specific anti-E protein antibody (León-Juárez et al., 2013), suggesting that the recombinant and native proteins had a similar structure. This monoclonal anti-E antibody was shown to bind the complete virus in previous studies conducted by our group (León-Juárez et al., 2013). The supernatant was analysed by